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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Gene therapy in pancreatic cancer

Si-Xue Liu, Zhong-Sheng Xia, Ying-Qiang Zhong

Si-Xue Liu, Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China
Zhong-Sheng Xia, Ying-Qiang Zhong, Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China
Author contributions: Liu SX performed the research; Liu SX and Xia ZS analyzed the data; Liu SX and Zhong YQ wrote the review.
Correspondence to: Ying-Qiang Zhong, MD, PhD, Professor, Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Yanjiang West Road 107, Guangdong Province, China. zhongyingqiang@21cn.com
Telephone: +86-20-81332598 Fax: +86-20-81332244
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Abstract

Pancreatic cancer (PC) is a highly lethal disease and notoriously difficult to treat. Only a small proportion of PC patients are eligible for surgical resection, whilst conventional chemoradiotherapy only has a modest effect with substantial toxicity. Gene therapy has become a new widely investigated therapeutic approach for PC. This article reviews the basic rationale, gene delivery methods, therapeutic targets and developments of laboratory research and clinical trials in gene therapy of PC by searching the literature published in English using the PubMed database and analyzing clinical trials registered on the Gene Therapy Clinical Trials Worldwide website (http://www. wiley.co.uk/genmed/ clinical). Viral vectors are main gene delivery tools in gene therapy of cancer, and especially, oncolytic virus shows brighter prospect due to its tumor-targeting property. Efficient therapeutic targets for gene therapy include tumor suppressor gene *p53*, mutant oncogene *K-ras*, anti-angiogenesis gene *VEGFR*, suicide gene *HSK-TK*, cytosine deaminase and cytochrome *p450*, multiple cytokine genes and so on. Combining different targets or combination strategies with traditional chemoradiotherapy may be a more effective approach to improve the efficacy of cancer gene therapy. Cancer gene therapy is not yet applied in clinical practice, but basic and clinical studies have demonstrated its safety and clinical benefits. Gene therapy will be a new and promising field for the treatment of PC.

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Key words: Pancreatic cancer; Gene therapy; Tumor suppressor; Suicide; Anti-angiogenesis; Immunotherapy; Oncogene; Multidrug resistance; Clinical trial

Core tip: This paper tries to present a full picture of gene therapy in pancreatic cancer, providing an unambiguous classification and comprehensive analysis, especially in therapeutic targets and clinical trials worldwide. From our work, you may find the hotspots in related research and the reason why they get there.

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INTRODUCTION

Pancreatic cancer (PC) is an aggressive and highly lethal malignant disease. The incidence of PC is lower than that of many other types of cancer, but it is the fourth most common cause of death from cancer[1]. PC is highly malignant and invasive, owing to nonspecific incipient symptoms and early metastasis. Most patients have local or metastatic spread at the time of presentation, and less than 15% of patients are candidates for surgery. Therefore, the prognosis of the disease remains poor. Recent statistics from the US National Cancer Institute showed that the overall 5-year relative survival rate for 2002-2008 was 5.8%, and nearly 90% of all patients were dead in 1

year from diagnosis, with a median survival less than 6 mo[2,3].

Surgery is still the first line treatment for PC, because it provides the only curable option. Other adjuvant treatments are chemotherapy, radiotherapy, physiotherapy and biotherapy. However, PC is highly resistant to the currently available chemotherapy and radiotherapy, and it is one of the cancers for which survival rate has not been substantially improved during the past 30 years. Therefore, new effective modalities for the treatment of this disease are urgently required. In recent years, with the outstanding progress of modern molecular biology, tumor immunology and gene engineering technology, tumor biotherapy is becoming a perspective and rapidly developing field of modern medicine, which is expected to improve state of or even cure patients who are not curable by classical methods of therapy.

Generally, tumor biotherapy includes immunotherapy and gene therapy, but there is no explicit boundary between them. Gene therapy can be used to transfer genes into tumor cells to render them more highly immunogenic, while cancer immunotherapy utilizes gene engineering technology to produce immunomodulating agents, such as tumor vaccines.

Since the first gene therapy clinical trial was approved by the National Institutes of Health in May 1990, significant progress in gene therapy technology has been achieved. In September 2006, a successful immunogene therapy of two patients with metastatic melanoma was reported. Up to July 2013, we have entries for 1970 trials undertaken in 31 countries, and most of them had been aimed at the treatment of cancer (64.2% of all gene therapy trials)[4]. In 2008, detailed, global, genomic analyses found that PC contained an average of 63 genetic alterations, the majority of which were point mutations. These alterations defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67%-100% of the tumors[5]. PC gene therapy is, therefore, targeting genes involved in these cellular pathways, inducing direct cell apoptosis and/or stimulating host immune defense system against tumor growth and expansion. Highly efficient gene therapy regimen is based on the following key points: efficiency of gene delivery, tumor targeted therapy and selection of efficient targets.

STRATEGIES FOR GENE THERAPY

Gene replacement

This strategy seeks chances of replacing a mutated gene with a normal gene *via in situ* homologous recombination. It is the best way to treat or even cure monogenic diseases, but seldom used for cancer gene therapy because of technical limitations and complex genetic alterations in cancer.

Gene modification

This strategy tries to directly modify the mutated gene and rehabilitate functions of target cells. It is an ideal manner of gene therapy but with great difficulties. Rare research related to this strategy has been reported.

Gene augmentation

Gene augmentation intends to transfer exogenous therapeutic genes into deficient cells and let their expression products make up for the deficiency. This is the most commonly used strategy in gene therapy. Key point of this technology is the selection of therapeutic genes and gene delivery systems. Plenty of efficient delivery systems have been developed to introduce genetic material into eukaryotic cells and get them expressed. The details will be discussed below.

Gene blockade

This strategy seeks to prevent the transcription and translation of certain cancer-associated genes by using short nucleotide sequences that bind in a complementary fashion to specific DNA or RNA, which can block aberrant signal transduction pathway and induce tumor differentiation and apoptosis eventually. It is also known as antisense gene therapy. Common materials used in this strategy include antisense oligonucleotides, ribozymes and small interfering RNAs (siRNAs).

Antisense oligonucleotides: Antisense oligonucleotides are short single-stranded segments of DNA or RNA artificially synthesized *in vitro*, which can selectively inhibit the transcription and translation of the target gene through the Watson-Crick base pairing between the antisense nucleotide and the target RNA or DNA. Since DNA is more easily synthesized and stable in body fluid, antisense oligodeoxyribonucleotides have become the most common material used in practice. Various chemical modifications to its backbone have been used to improve oligonucleotide stability, targeting and transduction efficiency[6].

Ribozymes: Ribozymes are RNA molecules with catalytic activity that are capable of sequence specific cleaving of mRNA molecules. They can selectively bind to target mRNAs through the Watson-Crick base pairing and form a duplex, which includes a highly distorted conformation that is easily hydrolyzed. The hydrolysis of the mRNA can be used for targeted suppression of specific genes[6].

However, a shortcoming of ribozymes is that their RNA backbone makes them easy targets for degradation by the ubiquitous RNAases, so these molecules are biologically unstable *in vivo*. Then researchers found another category of ribozymes, which are called DNAzymes or deoxyribozymes. They are analogs of ribozymes with greater biological stability, employing DNA motifs to replace the RNA backbone. Moreover, these DNAzymes are also easy to modify synthetically, thereby generating even stronger, resilient second-generation analogs, which makes them powerful tools for gene suppression applications[6].

siRNAs: siRNAs are short double-stranded RNA seg-

ments with typically 21- to 23-nucleotide bases that are complementary to the target mRNA sequence. siRNAs can be artificially synthesized *in vitro* and directly transferred into target cells, or be produced in the genetically modified target cells, in which a gene encoding siRNA is introduced *via* appropriate vectors, with the help of endogenous RNAase. When entering into the target cell, siRNAs bind to ribozyme compounds and form RNA-induced silencing complexes (RISCs), which bind to the target mRNA and stimulate mRNA degradation mechanisms, such as nuclease activity, that lead to silencing of the particular gene. Compared with other gene blockade technologies, siRNAs are remarkably superior because of their high degree of specificity to mRNAs, nonimmunogenic nature and high resistance to ribonucleases. Since siRNAs do not integrate into the genome, they offer greater safety than plasmid molecules. Furthermore, siRNAs do not have to transfer through the nuclear membrane and therefore require less sophisticated delivery systems, promising faster development and higher efficiencies[6]. Thanks to these advantages, RNA interfering technique has become one of the hotspots in research of gene therapy.

METHODS FOR GENE DELIVERY

Ex vivo delivery

In this system, the recipient cells which are previously explanted from the target tissue or bone marrow are cultured or proliferated *in vitro* and subsequently reinfused into the patient after therapeutic gene transfer. Obviously, only transplantable cells, such as lymphocytes and medullary cells, are acceptable in this method. In cancer therapy, tumor cells can also be cultured and engineered *in vitro*, but usually they are used to secrete cytokines or act as a vaccine. To improve the therapeutic efficacy, positively transfected cells are screened from the total cells for implantation, which gives *ex vivo* delivery higher transduction efficiency than *in vivo* delivery. However, the shortcomings of *ex vivo* delivery are complex operational process and a low survival rate of reimplanted cells[7,8].

In vivo delivery

In this system, gene vectors carrying therapeutic genes are directly delivered into the target tissues or organs, *via* systemic injection, *in situ* injection, oral agents or spray, of which *in situ* injection into local tumor tissue mediated by imaging methods is the most commonly used and ripest technology. Almost all the clinical trials on *in vivo* cancer gene therapy are based on this method, which includes intratumoral injection mediated by CT or ultrasound, tumor main vascular perfusion and gene-eluting stent implantation.

In vivo delivery is superior for its simple operation, easy preparation, independence on cell culture systems and wide range of application, whereas low efficiency of transduction, short curative effect, poor target cell specificity and immunologic problems are the main problems of this system. *In vivo* delivery might be the most useful strategy in clinical application. If only we overcome the shortcomings of this technique, gene therapy can truly be widespread applied in clinical treatment[9].

VECTOR SYSTEMS FOR GENE DELIVERY

The core problem on whether we choose *in vivo* delivery or *ex vivo* delivery is how to achieve specific gene transfection and highly efficient gene expression in recipient cells. As a consequence, establishing an efficient, safe and specialized delivery system has become the foundation of gene therapy. An ideal gene delivery system should have these characters: (1) non-invasive mode of administration; (2) tumor-specific targeting, including primary lesion and distant metastatic lesion, especially site specific lesion, such as the central nervous system and testis; (3) sustained gene expression; and (4) high insertion capacity, bio-safety, stability and easy preparation.

These vector systems can be divided into two categories: non-viral and viral vector systems. Both of them have been investigated and each of them presents distinct advantages and weaknesses. Viral methods normally offer higher transduction efficiency and long-term gene expression, but it may be associated with toxicity, immunogenicity, mutagenicity, inability to transfer large size genes and high costs. Non-viral methods provide advantages including relative safety, ability to transfer large size genes, less toxicity and easy preparation; they can also be modified with ligands for tissue or cell specific targeting. However, non-viral methods show limitations of low transfection efficiency and poor transgene expression[10,11].

Non-viral vector systems

Non-viral vectors consist of chemical vectors, biological vectors and physical methods of gene transfer to introduce naked DNA (in the form of plasmid DNA), RNA molecules, or oligonucleotides into recipient cells.

Physical delivery

Physical delivery mainly includes microinjection, microparticle bombardment and electroporation.

Microinjection involves the utilization of a micropipette to inject nucleic acid directly into a single living cell at a microscopic level. It is highly efficient since one cell at a time is targeted for DNA transfer. However, this precision is achieved at the expense of time. Microparticle bombardment, also known as ballistic DNA injection, gene gun technology or DNA-coated particle bombardment, is used to transfer plasmid DNA coated with heavy metals, usually gold, tungsten or silver, which are used as payload. These particles can be accelerated by pressurized gas and fire at the target cells or tissues without injuring them. Nevertheless, since direct exposure of target tissues is required, its application is restricted in internal organs. In addition, low efficiency of transfection into the nucleus and plasmid DNA integration into host genome are also problems to be solved. Electroporation

uses high-voltage electrical current to generate transient disruption on the membrane of target cells, which allows the entry of plasmid DNA by diffusion. This technique results in high cell mortality and therefore is not suitable for clinical use.

In conclusion, though significant transfection efficiencies have been achieved using physical techniques, they are extremely difficult to standardize in a clinical setting and are considered laborious, impractical, and invasive[11].

Chemical vectors

Commonly used chemical vectors can be classified into two major types based on the nature of the synthetic material, including cationic lipids and cationic polymers. In recent years, chemical vectors have been widely studied due to their advantages, including safety, large size gene transfer ability, less toxicity, low cost and easiness in preparation.

Cationic lipids (liposomes) are vesicles that consist of an aqueous compartment enclosed in a phospholipid bilayer[6]. DNA is bound by cationic lipids as a result of electrostatic interaction, which allows for fusion of the liposome with the target cell membrane, endocytosis, and delivery of the DNA into the cytoplasm. Cationic lipids mediated gene transfer is the most promising method in non-viral delivery systems. Although this approach has already been applied in clinical trials, some problems still need to be solved for its better application, including the toxicity and lower transfection efficiency *in vivo*[11].

Cationic polymer is an umbrella term of a wide range of chemical compounds, including: (1) natural polymers such as chitosan; (2) dendrimers such as polyamidoamine (PAMAM); (3) polypeptides such as poly-L-lysine (PLL), polyarginine, polyornithine, histones and protamines; and (4) other polymers such as polyethylenimine (PEI) and polyphosphoester[11]. Their transfection activity and toxicity vary dramatically. When mixed with negatively-charged DNA, positive charges of the polymers allow the formation of polymer/DNA complexes (polyplexes) through electrostatic interaction. Polyplexes are nano-sized transfection units that normally have higher stability than lipoplexes. The contributions of cationic polymers are enhancing the DNA uptake *via* endocytosis, protecting DNA from nuclease degradation and facilitating DNA escape from endosomes. Finally, DNA is released into the cytoplasm and migrates into the nucleus in which transgene expression takes place.

Recently, a combination of cationic polymers with liposomes, called polymer/lipid hybrid system, has also been developed and showed some superiority. To prepare this 3-part (lipid/polymer/DNA) system, DNA is precondensed by cationic polymers, followed by the subsequent complexation with liposomes. Using cationic lipids, the polymer/DNA complexes can be further condensed and protected, which can facilitate endocytosis and increase circulating half-life *in vivo*[11].

Furthermore, to improve the tissue or cell specificity of chemical vectors, they are also manipulated with the supplement of ligands or fusogenic peptides, such as transferrin, lectin and epidermal growth factor, which can bind to the receptors on the surface of target cells specifically. This approach is also known as receptor-directed gene transfer.

Biological vectors

Bacteria can be used as gene therapy vectors. When engineered to express the therapeutic transgene, bacteria can introduce both the therapeutic gene and protein product to recipient cells. The types of bacteria used include attenuated strains of *Salmonella*, *Shigella*, *Listeria*, and *Yersinia*, as well as non-pathogenic *Escherichia coli*. For some of these vectors, the mechanism of DNA transfer from the bacteria to the mammalian cell is not yet fully understood, but their potential to deliver therapeutic molecules has been demonstrated *in vitro* and *in vivo* in experimental models[12]. A bacterial cancer vaccine for pancreatic cancer - a live attenuated Listeria strain expressing mesothelin - has entered early-phase clinical trial and demonstrated antitumor effects[13].

In addition, many mammalian cell types can be used as carriers of gene therapy vectors, such as hematological cells and mesenchymal stem cells (MSCs)[14]. MSCs possess natural tropism towards tumors, making them a vehicle for targeted delivery of therapeutic genes into tumors. Many experiments have identified their significant antitumor effects *in vitro* and *in vivo*. However, to effectively use this therapeutic strategy in clinic, we still have to solve a number of technical problems[15].

Viral vector systems

Viral vectors: Viral vectors are the most commonly studied and applied gene delivery systems. More than two-thirds of clinical trials of gene therapy reported are viral therapies. These viruses can use their innate mechanism of infection to enter the cell and transfer DNA molecules into cells without any physical or chemical processing. The therapeutic gene then enters the nucleus, integrates into the host gene pool, and is eventually expressed.

The most common viral vectors in cancer gene therapy are adenovirus (AdV), retrovirus (RV), adeno-associated virus (AAV), lentivirus, herpes simplex virus (HSV), influenza virus, Newcastle disease virus, pox virus, and Epstein-Barr virus (EBV). Both advantages and disadvantages should be considered when selecting a viral vector, including insertion capacity, host range of infection, state of integration into host genome, efficiency of transfection and expression, immunogenicity, bio-safety and difficulty of preparation. The comparison of common viral vectors is shown in Table 1[6,9,16-18].

Oncolytic virus: Referring to viral treatment, there is another related field called oncolytic virotherapy in tumor-targeted gene therapy, which is an emerging treatment modality that uses replication-selective virus (or conditionally replicating virus) to destroy cancers. These natural viruses are genetically modified to be non-pathogenic

Table 1 Comparison of characters of common viral vectors

Virus	Viral genome	Insertion capacity	Host range of infection	State of integration into host genome	Efficiency of gene transfection and expression	Immunogenicity	Titers of preparation *in vitro* (PFU/mL)	Bio-safety
AdV	Double-stranded DNA	38 kb	Broad spectrum (both dividing and non-dividing cells)	No integration	High	High	10^{11}-10^{12}	Safe
AAV	Single-stranded DNA	4.9 kb	Broad spectrum	Site-specific integration on chromosome 19q13.3	High	Low	10^{12}, dependent on helper virus	Safe
RV	Single-stranded RNA	8 kb	Dividing cells only	Integrate randomly	Low	Low	10^{6}-10^{7}	Risk of insertional mutagenesis
Lentivirus	Single-stranded RNA	8 kb	Broad spectrum	Integrate randomly	High	Low	10^{9}-10^{10}	Risk of viral infection and insertional mutagenesis
Pox virus	Souble-stranded DNA	25 kb	Broad spectrum	No integration	High	High, function as immunologic adjuvant	10^{6}-10^{7}	Safe
HSV	Double-stranded DNA	15-30 kb	Nerve cells and epithelial cells, especially neuro- tropic speciality	No integration	High	Moderate	10^{11}-10^{12}	Risk of viral infection

but selectively infectious and cytotoxic to cancer cells. Viruses hijack the host cell's protein factory, disabling its production in favor of viral products, which are intrinsically cytotoxic. The infected host cell eventually lyses, releasing new virions capable of infecting other cells in a "bystander" effect, amplifying and propagating the initial effect of infection. These viruses can also evoke an immune response in the host, but in a tolerable morbidity. In conclusion, two main characteristics of oncolytic viruses are: (1) they replicate selectively in cancer cells and have self-amplification properties; and (2) they have cancer-cell-specific toxicity.

Meanwhile, these oncolytic viruses can also be engineered to carry exogenous genetic materials to produce therapeutic effects such as secreting cytokines and enhancing antitumor immune responses prior to eventual cytolysis[19,20].

Nowadays, oncolytic virotherapy has shown its great potential in cancer therapy, and dozens of clinical trials are under way, some of which have been in phase Ⅲ. The rate of publication of manuscripts on oncolytic virotherapy has now surpassed that on viral cancer gene therapy[20]. In 2005, the Chinese State Food and Drug Administration approved the world's first oncolytic virus for treatment of cancer, an engineered human adenovirus (Oncorine; Shanghai Sunway Biotech, Shanghai, China) for treatment of head and neck carcinoma[21].

Target genes and efficacy

At present, cancer gene therapies are mainly based on two principles: gene augumentation and gene blockade. The former is introducing exogenous genetic materials (therapeutic genes) into cancer cells and let their expression products play a therapeutic role to prevent or reverse the growth of cancer cells, while the latter is inhibiting the excessive expression of intrinsic genes (target genes) in cancer cells. Therefore, the investigated genes of interest can be divided into same categories: therapeutic genes utilized for gene augumentation and target genes for gene blockade (Table 2). Since PC is one of the malignant diseases that have the most complicated genetics and pathogenesis, gene therapy of PC has encompassed almost all these categories mentioned above.

Tumor suppressor genes

$p16^{INK4a}$, $p21^{CIP1/WAF1}$, $p14^{ARF}$, retinoblastoma protein (pRb) and p53: The *pRb* gene is a part of gene family that includes two other members, *p107* and *p130*, which collectively repress genes that regulates the G1 to S checkpoint of the cell cycle. Rb family proteins interact with transcription factor E2Fs, which induce the expression of genes needed for DNA synthesis. When bound at E2F-responsive promoters, Rb family proteins help to repress gene expression[22]. Rb proteins can be phosphorylated by cyclin dependent kinases 2, 4 and 6 (CDK2, 4 and 6), resulting in the release of E2F and gene expression[23].

The $p16^{INK4a}$ gene (*p16*), located on chromosome 9p21, is deleted in 85% of pancreatic adenocarcinomas[24]. It is the first member to be identified in the INK4 family of CDK inhibitors. The *p16* gene product is tight-binding and inhibitory protein for CDK4 to induce G1 arrest of the cell cycle[25], while $p21^{CIP1/WAF1}$ (*p21*) gene product acts as a downstream effector of p53 and mediates G1 cell cycle arrest by inhibiting CDK2. Loss of p21 activity has been observed in approximately 30%-60% of PC specimens[26].

The $p14^{ARF}$ (*p14*) gene, also located on chromosome 9p21, shares an exon with $p16^{INK4a}$ in different reading frames[27]. *Mdm2* (murine double minute) is a *p53*-inducible gene that normally acts to terminate the *p53*

Table 2 Target genes of gene therapy in pancreatic cancer

Strategy	Categories	Examples
Gene augumentation	Tumor suppressor genes	$p16^{INK4a}$, $p21^{CIP1/WAF1}$, $p14^{ARF}$, Retinoblastoma Protein (pRb), *p53*, *p84 /Thoc1*, *p73*, *Smad4/DPC4*
	Drug sensitivity genes/ suicide genes	Herpes Simplex Virus Thymidine Kinase (*HSV-TK*), Cytosine Deaminase (CD), Nitroreductase (NTR), Cytochrome P450 (CYP)
	Anti-angiogenesis genes	Soluble VEGFR, Soluble FGFR, Endostatin, Thrombospondin-1, Angiostatin, Vasostatin, NK4, Matrix metalloproteinases inhibitors (MMIPs/TIMPs), Somatostatin receptors (SSTR)
	Immune related genes	MHC molecules, Co-stimulatory molecules (B7 family, ICAM-1, LFA-3), Inflammatory cytokines (IL-2, IL-12, GM-CSF, IFN-α, IFN-β, IFN-γ, TNF-α), Tumor antigen (CEA, MUC-1, *etc.*)
	Apoptosis related genes	TRAIL
Gene blockade/ antisense therapy	Oncogenes	K-ras, LSM1/CaSm, HER-2/EerB-2
	MDR	MDR1, MRP family, BCRP
	Proliferation related genes	VEGF, hTERT, COX-2

MDR: Multidrug resistance.

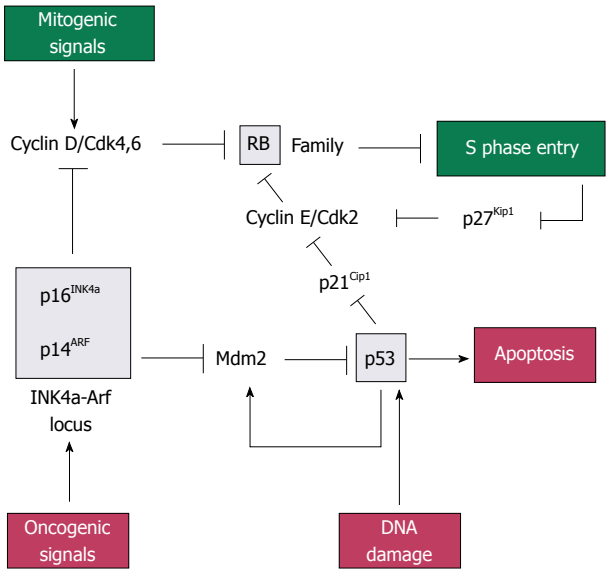

Figure 1 Cell cycle checkpoints. Mitogenic signals activate cyclin D-dependent kinases, which phosphorylate RB and RB family proteins (p107 and p130) to facilitate entry into S phase. The p16INK4a protein inhibits cyclin D/Cdk4, 6 to activate RB and prevent entry into S phase. The p14ARF protein inhibits Mdm2 to induce p53, leading either to p53-dependent apoptosis or to induction of the Cdk2 inhibitor p21Cip1. The p21Cip1 protein inhibits cyclin E/Cdk2 and induces RB-dependent cell cycle arrest.

response. The p14ARF protein inhibits *Mdm2* to induce *p53*, leading to p53-dependent apoptosis[22].

The *p53* gene, located on chromosome 17p, is inactivated by mutation in 70% of pancreatic adenocarcinomas. It can induce apoptosis or G1 cell cycle arrest *via* $p21^{CIP1/WAF1}$. It is normally maintained at a very low level by Mdm2, which targets p53 for ubiquitin-mediated degradation. Stress or mitogenic signals increase the level of $p14^{ARF}$, which in turn inhibits Mdm2 and lead to the stabilisation and activation of p53.

In short, pRb, p53, $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p14^{ARF}$ form part of a signaling network that monitors mitogenic signaling and restrains aberrant growth-promoting signals from driving cell cycle progression inappropriately (Figure 1)[22].

In research of PC therapy, both p16 and p21 have successfully been transduced into pancreatic cancer cell lines by means of adenoviral vectors, which results in growth inhibition and induction of apoptosis *in vitro*[25,28]. One study proved that p16-mediated cytotoxicity is tightly associated with the presence of functional pRb[29]. Liposome-mediated delivery of the $p14^{ARF}$ gene to pancreatic cancer cell lines was capable of resulting in the enhancement of their sensitivity to 5-Fu contrasting with cells devoid of $p14^{ARF}$ expression, which successfully inhibits PC cell proliferation[30].

Of all these tumor suppressor genes, *p53* is the most important and extensively studied one. The first gene therapy for the treatment of cancer in China in 2004 is a replication-defective adenovirus 5 expressing *p53* used for squamous cell carcinoma of the head and neck[21]. Transfer of the *p53* gene using an adenoviral vector also suppressed the growth of human pancreatic cancer cell lines *in vitro*[31]. Reintroduction of *p53* also increased cytotoxicity of gemcitabine *in vitro* and *in vivo*[32], and that of temozolomide *in vitro*[33]. In nude mouse model, intraperitoneal administration of the retroviral *p53* vector resulted in significant inhibition of the growth of primary pancreatic tumor and peritoneal deposits compared to controls[34]. What is more, the inhibitors of Mdm2 are also investigated as new agents for PC treatment, since they induced the growth inhibition through reactivation of the p53 pathway[35].

p84 /Thoc1: The *p84/Thoc1* gene encodes proteins that have similar death domains with other proteins involved in the regulation of apoptosis, which can bind to an amino terminal domain of the Rb1 protein, regulating transcriptional elongation and RNA processing. Its overexpression induces apoptosis of cancer cells. A recent study found that infection of pancreatic adenocarcinoma with adenovirus encoding *p53* and *p84/Thoc1* inhibited growth of cancer cells both *in vitro* and *in vivo* to a greater extent than treatment with either one alone[36].

p73: The *p73* gene, located on chromosome 1p36, is identified as a *p53* family member observed in 45.6% of pancreatic adenocarcinomas. It can induce cell cycle arrest and apoptosis in a p53 manner by binding to *p53* DNA target sites, and transactivates p53-responsive

genes[37]. One research found that an adenoviral vector encoding *p73* was capable of effectively killing several pancreatic cancer cell lines, including those that were completely resistant to p53-mediated apoptosis[38].

Smad4/DPC4: The *Smad4* gene, designated as tumor suppressor gene DPC4, is located on chromosome 18q21.1 and deleted in about 50% of pancreatic adenocarcinomas but only in about 10% or less of other cancers, which suggests that Smad4/ DPC4 may have a specific role in pancreatic tumorigenesis[39]. Smad4 is a member of the Smad family of transcription factors, which potentiates tumor growth, angiogenesis and invasion and is associated with poor prognosis[23]. Restoration of the *Smad4* gene using an adenoviral vector showed inhibition of pancreatic tumor growth in mice[40], while the same effect was seen *in vitro* through a retroviral vector pLXSN containing DPC4[41] .

Drug sensitivity genes/suicide genes

Drug sensitivity gene therapy, also known as gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy, attempts to selectively transduce tumor cells with a gene which, when express an enzyme, will convert a systemically administered nontoxic prodrug into a toxic metabolite.

A large number of enzyme-prodrug systems have been developed for suicide gene therapy in recent years. Examples of enzymes include viral thymidine kinase (TK), bacterial cytosine deaminase (CD), bacterial carboxypeptidase G2 (CPG2), purine nucleotide phosphorylase (PNP), thymidine phosphorylase (TP), nitroreductase (NR), D-amino-acid oxidase (DAAO), xanthine-guanine phosphoribosyl transferase (XGPRT), penicillin-G amidase (PGA), β-lactamase (β-L), multiple-drug activation enzyme (MDAE), β-galactosidase (β-Gal), horseradish peroxidase (HRP), deoxyribonucleotide kinase (DRNK), deoxycytidine kinase (dCK), carboxypeptidase A (CPA), β-glucuronidase (β-Glu), and cytochrome P450 (CYP)[42]. However, among these dazzling choices, the most classic paradigm in PC therapy is herpes simplex virus thymidine kinase (HSV-TK).

HSV-TK/ganciclovir

The *HSV-TK* gene codes for an enzyme that converts the nontoxic prodrug ganciclovir into monophosphorylated ganciclovir, which is subsequently further converted by cellular guanylate kinases to the triphosphorylated forms, blocking DNA synthesis and inducing cell death[43]. The therapeutic effect can also be amplified by a "bystander effect", which means *HSV-TK* transduced tumor cells are toxic to neighbouring unmodified tumor cells. The reason may be related to the uptake of toxic metabolites *via* intercellular communication paths such as gap junctions[44]. It is, in some extent, compensable for the low efficacy of gene transfer.

The HSK-TK delivered by retrovirus and adenovirus has been proved efficient in killing PC cells *in vitro* and *in vivo*[45,46]. The combination of adenovirus- and retrovirus-mediated delivery of HSV-TK appeared to be more effective in tumor reduction compared to either one alone *in vivo*[47]. Liposome mediated transfer of HSV-TK was able to cause regression of tumors in nude mice with peritoneal dissemination of PC[48]. However, there were some studies showing that retrovirally transduced HSV-TK had limited efficacy in PC cell lines both *in vitro* and *in vivo*[49,50]. The main reason of this controversial fact may be related to a poor efficiency of gene transfection *in vivo* and a limited bystander cell killing effect, so further study is required for its clinical application.

CD/5-fluorocytosine (5-FC)

CD is a bacterial enzyme that converts the prodrug 5-FC into the cytotoxic and radiosensitising agent 5-FU, which inhibits DNA replication and protein synthesis. Several studies have proved that the adenovirus carrying CD gene is efficient in inhibiting the growth of murine PC cell lines *in vitro* and *in vivo* when associated with 5-FC[51-53]. Furthermore, when combined with radiation, the adenoviral vector carrying a mutant bacterial *CD* gene (AdbCD-D314A) plus 5-FC significantly increased frequency of tumor regression and the persistence of tumor growth inhibition compared with either radiation or AdbCD-D314A/5-FC therapy alone[54].

There is another gene called *FUR1*, which encodes uracil phosphoribosyltransferase (UPRT), playing a role in CD/5-FC suicide therapy. Since the CD gene, also called *FCY1* gene, often demonstrates resistant to 5-FU, UPRT has an additional advantage as it catalyses the conversion of 5-FU into the toxic metabolite 5-fluorouridine-5'-monophosphate[55]. Combined treatment with 5-FU and E1B-55kDa-deleted adenovirus carrying the *UPRT* gene (AxE1AdB-*UPRT*) dramatically reduced the disseminated tumor burden in mice with peritoneal dissemination of AsPC-1 without causing toxicity in normal tissues[56]. One study showed that the *FCY1* gene alone was ineffective in the treatment of PC *in vitro* and plasmid vectors expressing chimera CD-UPRT (pRSV-CD-*UPRT*) only increased 5-FC sensitivity to some PC cell lines[57]. Another study demonstrated that adenoviral vectors carrying the CD: uracil phosphoribosyltransferase fusion gene (Ad-CD: *UPRT*) resulted in increased 5-FC-mediated cell killing, compared with Ad-CD[58]. Moreover, Ad-CD: UPRT/5-FC combined with monoclonal antibody TRA-8 produces an additive cytotoxic effect in cancer cells both *in vitro* and *in vivo*[58].

Nitroreductase/CB1954

The *Escherichia coli* enzyme nitroreductase (NTR) is able to convert the prodrug CB1954 (5-[aziridin-1-yl]- 2,4-dinitrobenzamide) to 2- and 4-hydroxylamino derivatives, which react with cellular thioesters to generate a potent alkylating agent capable of cross-linking DNA, inducing cell apoptosis eventually[59]. Retrovirus-mediated *NTR* gene delivery showed increased sensitivity up to 500-fold to CB1954 in PC cell lines *in vitro*, through associated by-

Table 3 Active and negative regulatory factors of angiogenesis

Stimulators	Inhibitors
Hypoxia	Angiostatin
Oncogenic proteins such as Ras	TSP-1
Inflammatory cytokines such as IL-8 and IL-6	Endostatin
FGF	Arrestin
TGF-β	Canstatin
HGF	MMPIs
PDGF	Somatostatin
G-CSF	Tumstatin
Angiogenin	VEGI
Leptin	Decoy receptors such as soluble VEGFR
Proliferin	Inflammatory cytokines such as IL-12

G-CSF: Granulocyte colony-stimulating factor; PDGF: Platelet-derived growth factor; HGF: Hepatocyte growth factor; TGF-β: Transforming growth factor-β; FGF: Fibroblast growth factor; TSP-1: Thrombospondin-1; MMPIs: Matrix metalloproteinases inhibitors; VEGI: Vascular endothelial growth inhibitor.

stander effect[60]. In a nude mouse model with subcutaneous PC xenografts, retrovirus vectors expressing the *NTR* gene with administration of CB1954 resulted in tumor regression, growth delay and significantly increased median survival[61]. Another research of nude mouse xenograft model for disseminated peritoneal carcinomatosis with ascites (PC cell line SUIT2) showed that combination of replication-defective adenovirus vectors carrying the *NTR* gene (Ad-CMV-NTR) and *CB1954* almost doubled the median survival from 14 to 26 d[62]. The *NTR*/*CB1954* treatment has been tested in clinical trials of gastrointestinal and liver malignancies, but none for PC.

Cytochrome P450/cyclophosphamide

Cytochrome P450 enzyme converts the chemotherapeutic prodrugs cyclophosphamide (CPA) or iphosphamide (IPA) to toxic metabolites phosphoramide mustard, which is an alkylating agent able to form DNA cross-links in a cell cycle-independent manner. Since cytochrome P450 is predominantly produced in the liver and toxicity of the metabolite is rather systemic and not tumor-specific in human body, the transduction of the CYP gene into the tumor tissue shows its advantages to enhance tumor-specific toxicity.

In vitro study demonstrated that expression of CYP 2B1 enzymes (retrovirus-mediated transduction) led to an up to 13-fold increase in susceptibility to IPA in a range of PC cell lines (BxPC-3, MIA PaCa-2, Hs-766T, PaCa-44 and PANC-1)[63]. *In vivo*, retroviral CYP 2B1 transfer with CPA treatment highly sensitized PC cells NP-9, NP-18, and NP-31, and led to significant differences in tumor volume at the end of the treatment when compared with CPA alone[64]. Furthermore, in tumor-bearing mice model, intratumoral injection of encapsulated cells, which were genetically modified to express the CYP, was able to cause significant tumor reduction[65,66]. In addition, these encapsulated cells have also been used in phase Ⅰ/Ⅱ trials in patients with PC and have shown remarkable early success, with median survival doubled and 1-year survival improved by 3-fold[67].

ANTI-ANGIOGENESIS GENES

Tumor growth is dependent on angiogenesis, in which vascular endothelia growth factor (VEGF) plays a leading role. VEGF is a glycoprotein that has a huge impact on endothelial cell survival, mitogenesis, migration, differentiation, and vascular permeability. It is overexpressed in over 90% of PC and is associated with increased microvessel density, tumor progression and poor prognosis[68]. The VEGF receptor (VEGFR), which is a transmembrane receptor tyrosine kinase of the ErbB family, including VEGFR-1 (FMS-like tyrosine kinase-1, flt-1) and VEGFR-2 [fetal liver kinase-1 (flk-1) or kinase insert domain receptor (KDR)], is also overexpressed in the vasculature of tumors that express VEGF[69,70].

Mechanisms that lead to inappropriate activation of the VEGF pathway include receptor overexpression, activating mutations, overexpression of receptor ligands, and loss of their negative regulatory pathways. Both VEGF and VEGFR are, therefore, appealing targets for anti-angiogenesis therapy. Many molecular targeted agents and monoclonal antibody interfering with VEGF signal system have been developed for cancer therapy, such as Bevacizumab (a humanized antibody against VEGF), Sorafenib (a multi-targeted kinase inhibitor), Erlotinib (an inhibitor of EGFR, the only molecular targeted drug approved by the FDA in 2005 for PC), Axitinib (an inhibitor of both VEGFR and related tyrosine kinase receptors), and Aflibercept (a recombinant fusion protein that functions as a soluble decoy receptor and inhibits VEGF). However, these agents seem unlikely to confer sufficient benefit in the PC clinical trials and their cost-effectiveness has been questioned[71].

Compared to classic non-gene therapy, gene therapy represents a powerful tool for therapeutic intervention to angiogenesis in terms of specific targeting, cost-effectiveness and safety. In this new approach, the VEGF/VEGFR pathway is still the main hotspot. Stimulators and inhibitors that up-regulate and down-regulate VEGF signal pathways are all possible therapeutic targets in cancer treatment (Table 3)[72,73]. Therefore, strategies for anti-angiogenesis gene therapy can be divided into two categories: (1) delivery of genes encoding endogenous angiogenesis inhibitors or their receptors; and (2) blockage of the excessive angiogenesis genes encoding growth factors or growth factor receptors. The former is based on transfer of exogenous genes whereas the latter seeks to block excessive genes in tumor. Materials involved in these two are totally different.

Soluble VEGFR

Soluble forms of VEGFR-1 and VEGFR-2 are a kind of decoy receptor, which can inhibit VEGF dependent tumor angiogenesis, by binding to VEGF and acting as a dominant negative receptor[74]. Recombinant adenovirus-

es encoding soluble VEGFR-2 (Ad Flk1-Fc) and soluble VEGFR-1 (Ad sflt1) showed significant tumor inhibition when injected intravenously and directly into the tumors, respectively[75,76]. Crosslinked polyplex micelles modified by RGD (Arg-Gly-Asp) peptide ligands, a non-viral vector, carrying plasmid DNA expressing a soluble form of VEGFR-1 (sFlt-1), demonstrated significant inhibition of tumor growth *via* anti-angiogenic effect when systemically injected into pancreatic adenocarcinoma bearing mice[77]. A truncated dominant negative mutant of VEGFR-2, which binds to VEGF and decreases the angiogenic stimulus of VEGF, when delivered by replication-defective retroviruses, could also lead to inhibition of tumor growth in each of three human PC cell lines *in vivo*[78]. Similar results were also found in an *in vitro* study with herpes simplex virus (HSV) amplicon mediated delivery of a hypoxia-inducible soluble VEGFR-2 (sFlk-1)[79]. In an comparative research of the antitumor activity of anti-angiogenic proteins, recombinant adenoviruses encoding angiostatin, endostatin, neuropilin, and soluble forms of VEGFR (Flk1, Flt1) all resulted in inhibition of tumor growth through intravenous injection in murine models involving lung cancer, fibrosarcoma and PC, but soluble forms of VEGFR were significantly more effective (approximately 80% inhibition of preexisting tumor growth) than the others[80].

Soluble fibroblast growth-factor receptors (FGFRs)

FGFRs, encoded by four genes (*FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*), are involved in the regulation of organ development, cell proliferation and migration, angiogenesis and other processes. Recent studies have shown that FGFR-activating mutations and overexpression are closely associated with the development and progression of tumors in human[81]. Several monoclonal antibodies and small-molecule FGFR inhibitors have been developed, some of which have already entered early clinical development, such as AZD4547 (AstraZeneca), BGJ398 (Novartis), LY2874455 (Eli Lilly), GP369 (Aveo) and HuGAL-FR21 (Galaxy). Soluble forms of FGFR (sFGFR) had similar mechanism of action with sVEGFR. One study found that replication-defective adenoviral vectors carrying *sFGFR1* gene could effectively suppress tumor angiogenesis and enhance apoptosis among lung cancer cells and pancreatic cancer cells both *in vitro* and *in vivo*, especially in sVEGFR-resistant cancers. Furthermore, the combined usage of sVEGFR plus sFGFR1 produced an enhanced inhibitory effect compared to their individual effects[82].

Endostatin (ES), thrombospondin-1 (TSP-1), angiostatin (AS) and vasostatin

ES, AS, vasostatin and TSP-1 are the most important endogenous angiogenesis inhibitors that have been studied extensively. ES is a cleavage product from the C-terminal portion of collagen XVIII, which has been shown to inhibit endothelial cell migration and proliferation and induce apoptosis. The apoptotic effect of ES is associated with down-regulation of anti-apoptotic proteins, such as Bcl-2 and Bcl-xl, while inhibition of endothelial cell proliferation and migration is *via* interacting with several pathways, such as binding to integrin α5, associating with heparan sulfate proteoglycans on cell surface, and inhibiting VEGFR-2, PDGF, cyclin-D1 and metalloproteinases[83,84]. Recombinant human ES has been developed for cancer treatment and entered in clinical trials[85,86]. TSP-1 is a multifunctional extracellular matrix protein with pivotal roles in the regulation of vascular development and angiogenesis[87], while AS is a potent inhibitor of angiogenesis, selectively inhibiting endothelial cell proliferation and migration through binding a cell surface ATP synthase or inhibiting extracellular matrix (ECM)-stimulated plasminogen[72]. Vasostatin, the N-terminal domain of calreticulin, was also reported to have tumor suppressor and anti-angiogenic function *in vitro* and *in vivo*.

A double-regulated duplicative adenovirus expressing human ES (AdTPHre-hEndo) limited PC growth both *in vitro* and *in vivo*, and the inhibition was significantly higher than non-duplicative adenovirus vectors carrying the ES gene[88]. A eukaryotic expression vector pRC/CMV carrying the AS gene, when delivered into human PC cell line mediated by liposome *in vitro*, also notedly reduced the volume of tumors[89]. A replication deficient recombinant adenovirus encoding vasostatin (Ad-vasostatin) showed less neovascularisation and inhibited tumor growth *in vivo* and *in vitro*[90]. Same results were also discovered in a study with intratumoral delivery of recombinant AAV expressing vasostatin (rAAV-VAS)[91]. The Lister vaccine strain of vaccinia virus armed with the ES-AS fusion gene (*VVhEA*), which displayed high selectivity for cancer cells with effective infection of tumors after both intravenous and intratumoral administrations, showed a significant antitumor effect with evidence of inhibition of angiogenesis both *in vitro* and *in vivo*[92]. A recombinant AAV-mediated gene delivery of 3TSR (the antiangiogenic domain of TSP-1) or ES (rAAV-ES), *via* intratumoral injection, intramuscular injection or intrasplenic injection, also inhibited tumor growth by anti-angiogenic effect[87].

NK4

NK4, composed of the N-terminal hairpin and subsequent 4-kringle fragment of hepatocyte growth factor (HGF), is an HGF antagonist that acts as an angiogenesis inhibitor. NK4 strongly inhibits the infiltration, metastasis, and tumor growth of PC[93]. HGF is overexpressed in 61%-87% of PC cases[94] but frequently expressed in tumor-associated fibroblasts rather than PC cells. HGF promotes growth and enhances cell motility and extracellular matrix breakdown, leading to invasion and metastasis of cancer cells[95].

Strong antiangiogenic activity of an NK4-expressing adenvirus vector (Ad-NK) has been tested on PC cells in both *in vitro* and *in vivo* studies of subcutaneous and orthotopic transplantation, peritoneal dissemination, and liver metastasis[96]. Ad-NK potently inhibited the growth

and invasion of cancer cells in response to HGF[97]. Intrasplenic injection of Ad-NK4 suppressed the number and growth of hepatic metastases[98]. When injected into the peritumoral region combined with gemcitabine, Ad-NK4 completely suppressed peritoneal dissemination and liver metastases, leading to significantly increased survival, compared to Ad-NK or gemcitabine alone[96]. Intraperitoneal injection of Ad-NK4 could also suppress the development of peritoneal dissemination of PC in nude mice[99].

Matrix metalloproteinase (MMP) inhibitors

MMPs are a family of zinc-dependent proteolytic enzymes that degrade the extracellular matrix and play an important role in tumor progression, angiogenesis, immune evasion, invasion and metastasis[100]. Natural tissue inhibitors of metalloproteinases (TIMPs) regulate physiologic MMP activity and are implicated in malignancy. The imbalance between MMPs and TIMPs, which denotes overexpression of MMPs and reduced expression of TIMPs, is frequently found in PC[101]. Some synthetic MMPIs have been exploited for antitumor therapy, such as BAY 12-9566 and Marimastat, but showed limited survival benefit in clinical trials in spite of their effectiveness in animal models[102-104]. When undergoing gene transfection to overexpress *TIMP-1 in vitro*, human PC cells were less likely to implant, grow and migrate in nude mice and showed increased apoptosis, and decreased angiogenesis[105]. Adenoviral vectors encoding human TIMP1 or TIMP2 limited development of PC and led to prolonged survival *in vivo*[106].

Somatostatin (SST) receptors (SSTRs)

SST is a natural peptide hormone secreted in various parts of the human body, and participates in a wide variety of biological processes including neurotransmission and negative control of exocrine and endocrine secretions. Meanwhile, SST exerts a strong antiproliferative effect in normal as well as tumor cells by interacting with SSTRs, including apoptotic effects, growth factor inhibition, antiangiogenic and immuno-modulating activities[107,108]. SSTRs consist of five different G-protein coupled receptor subtypes (SSTR1-5), which are differently expressed in the various types of tumor. The antineoplastic activity of SST and its analogues depends on the receptor subtypes they are bound to. Among these receptor subtypes, SSTR-1 and SSTR-2 play a predominant role in mediating the anti-proliferative effect[109]. However, only *SSTR2* expression is significantly inactivated in 90% of PC cases and SSTR-2, therefore, occupies the majority of gene therapy studies of SSTRs[110,111].

When introduced into PC cell line PC-3 with lipofectamine, SSTR2 showed inhibition of *VEGF* and *MMP-2* expression *in vitro*[112]. A targeting adenoviral vector driven by MUC1-promoter expressing SSTR2 gene showed significant cell proliferation inhibition *in vitro*, though there was no AdMUC1-SSTR2-induced apoptosis[113]. Intratumoral transfer of SSTR2 using the synthetic vector linear polymers of ethylenimine (PEI), strongly inhibited tumor progression of pancreatic adenocarcinoma *in vivo*, while depleting SST by RNA interference completely reversed SSTR2's antitumoral effect on *VEGF* expression and tumor angiogenesis[107]. Another study demonstrated that SSTR2 restoration mediated by oncolytic adenovirus (ZD55-hSSTR2) alone had a minor antitumor effect, but antitumor efficacy can be enhanced with the combination of ZD55-TRAIL (TNF-related apoptosis-inducing ligand) *in vitro* and *in vivo*[114]. Gene transfer using SSTR1 also displayed growth inhibition of PC by inducing cell cycle arrest *in vitro* and *in vivo*[115]. What is more, cotransfection of *SSTR1* and *SSTR2* showed a synergistic inhibitory effect on tumor proliferation and rendered Panc-1 cells more responsive to an SST analogue[116].

IMMUNE RELATED GENES

Tumor cells generally have a low immunogenicity and are able to escape surveillance by the host. Cancer immunotherapy has been developed to overcome this immune tolerance, including active and passive immunity. Passive approach encompasses administration of cytokines, activated effector cells or specific monoclonal antibodies targeting tumor cells. Active immunotherapy involves stimulation of immune response to tumor-associated antigens (TAAs), *via* employing cancer vaccines. Meanwhile, gene therapy is helpful by transferring genes into tumor cells or immune cells to render them more immunogenic and more effective, respectively. This combination is also known as immunogene therapy (Figure 2)[117].

In pathway A, allogeneic or autologous tumor cell vaccines that are genetically modified to secrete cytokines or co-stimulatory molecules are designed to elicit systemic immune responses to attack tumor tissue. Though autologous tumor cell vaccines showed promising results in clinical trials, a number of technical problems were uncovered, including the requirement of labor-intensive procedures for production of an individualized vaccine and the difficulty of expanding primary human tumor cells to the high numbers required for vaccination[118]. Fortunately, allogeneic whole tumor cell vaccines had been also proved to successfully induce systemic tumor-specific immune responses without the need to be HLA compatible with the host, and became the major way in study of tumor vaccines[119]. In pathway C, immune cells, especially dendritic cells (DCs), are *ex vitro* cultured autologous cells that are genetically modified, stimulated by specific antigens or activated by multiple cytokines to bypass the dysfunction of endogenous immune cells, restore immune surveillance, induce cancer regression or stabilization or delay and prevent its recurrence.

Host immune responses against a tumor antigen include cellular and/or humoral immune responses, starting with the processing of tumor antigens by antigen presenting cells (APCs) and recognition by T lymphocytes. T-cell activation requires not only the interaction between

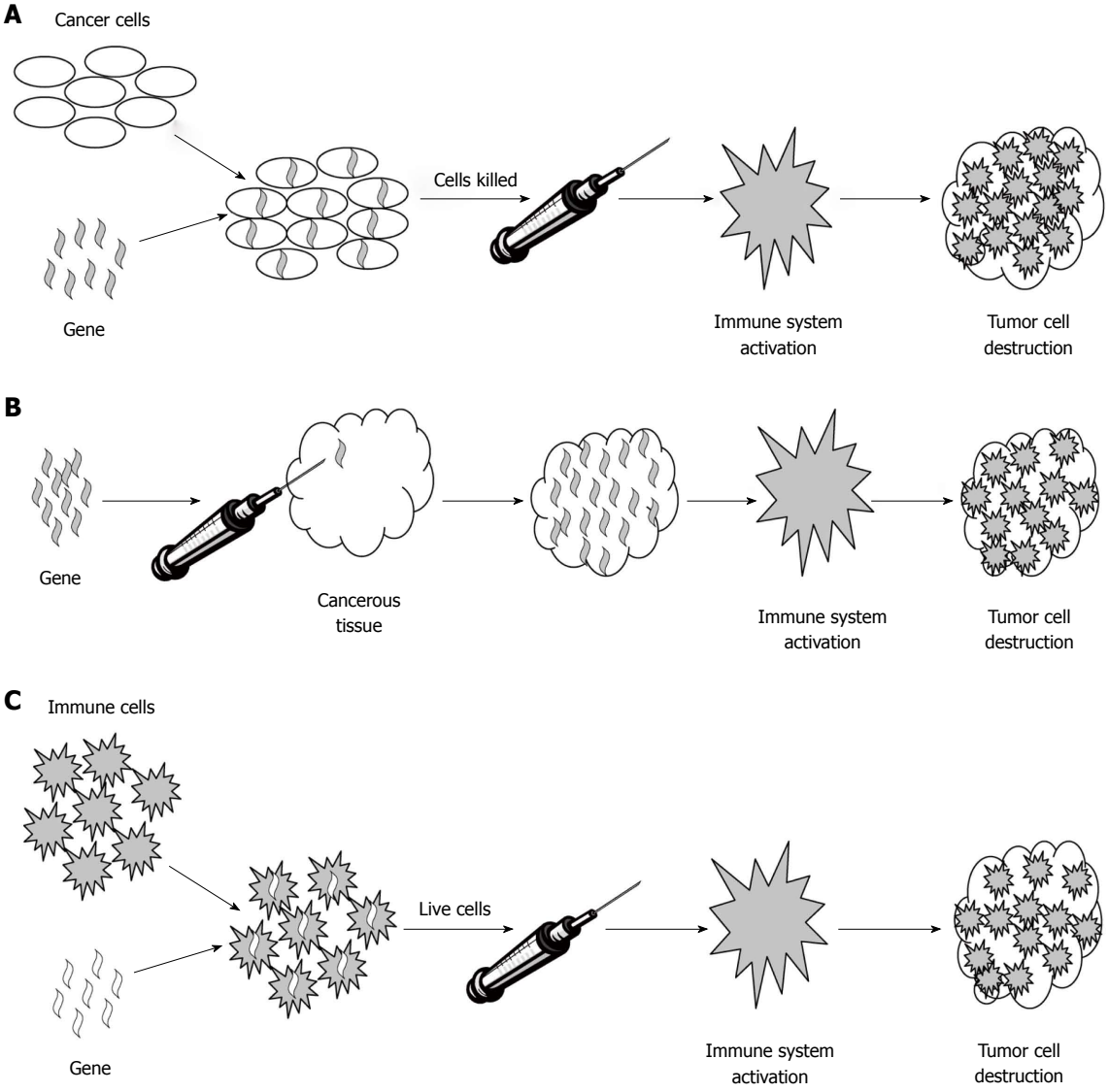

Figure 2 Schematic diagram of immunogene therapy. A: Immunogene therapy with altered cancer cells, which are harvested from patients (autologous cells) or from established cancer cell lines (allogeneic) and then cultured *in vitro* and inactivated to yield a vaccine; B: Immunogene therapy with *in vivo* gene transfer; C: Immunogene therapy using altered immune cells.

major histocompatibility complex (MHC) antigens bearing a specific peptide and the T-cell receptors, but also non-antigen-specific co-stimulatory activation by interaction of molecules expressed on the T-cells and APCs. Mechanisms of tumor immune tolerance include: underexpression of MHC antigens, loss of co-stimulatory molecule expression, alteration of tumor antigens, and secretion of immunosuppressive factors. Aiming at these mechanisms, at least four kinds of genes can be utilized in immunogene therapy: (1) major histocompatibility complex (MHC) antigens/human leukocyte antigens (HLAs), (2) co-stimulatory molecule genes, (3) tumor antigen genes; and (4) inflammatory cytokine genes.

MHC molecules and co-stimulatory molecules

Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the co-stimulatory signals necessary for full activation of T cells. Therefore, one approach to induce tumor-specific immune responses is to genetically modify tumor cells to express MHC molecules and co-stimulatory molecules on their cell surface. This kind of modification makes tumor cells to function as professional APCs and enhance their ability to directly stimulate T cells. In addition, this way is also appropriate for manipulation of DC cancer vaccines[120].

Loss or reduced expression of MHC-class-I molecules in many cancer cells have been found in mice and humans and have been identified as an important form of immune evasion. Despite that a large number of potential cancer antigens were discovered, clinical trials of immunization were disappointing due to the loss of *MHC-I* expression in tumors[121]. In animal models, *in vivo* gene transfer of foreign MHC-class-I *H-2K* gene into tumors successfully induced a cytotoxic T-cell response and attenuated tumor growth and caused complete tumor regression in murine models[122,123]. Transfection of

tumor cells with syngeneic MHC-class-Ⅱ or allogeneic MHC-class- Ⅰ genes improved tumor-specific immunity in the autologous host[124]. Therefore, combining MHC molecules with other tumor antigens could emerge as an attractive approach in cancer immunotherapy. No correlative research in PC has been reported yet.

In studies of costimulatory molecules, B7 family is one of the most important members and is constitutively expressed by most APCs, as the ligand for two receptors expressed on T cells, CD28 and CTLA-4. In animal studies, transfection of B7.1 into some tumors resulted in tumor rejection and generated systemic immunity against wild-type tumor challenges *via* stimulating CD8+ T cells[125]. Cotransfection of B7 with MHC-Ⅱ molecules was capable of inducing potent systemic immunity *via* both CD4+ and CD8+ T cells pathways[126]. A combinatory vaccine regimen (PANVAC-VF) composed of vaccinia virus and fowlpox virus expressing tumor-associated antigens (CEA and MUC1) and costimulatory molecules [B7.1, intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-3 (LFA-3)], when administered by subcutaneous injection with adjuvant GM-CSF, showed a significantly great therapeutic effect both in animal models and in clinical trials with advanced PC patients[127,128].

Inflammatory cytokines

Numerous cytokines have been studied in *in vivo* gene transfer. Human PC cells that underwent retrovirus-mediated gene transfer of IL-2, IL-4, IL-6, IL-27 and granulocyte macrophage-colony stimulating factor (GM-CSF) showed significant retardation and even regression when inoculated into BALB/c nude mice[129,130]. *In vivo* gene delivery of IL-1β, IL-24, IFN-α, IFN-β and IFN-γ by different viral vectors have been proved to lead to significant tumor growth inhibition in many PC models[131-135]. In addition, combined application of cytokine transfection and traditional chemotherapy, and combining immune genes with either tumor suppressor genes or suicide genes, are also common strategies in cancer immunogen therapy. For instance, combination of intratumoral human TNF-α gene delivery with gemcitabine produced marked delays in the growth of human pancreatic xenograft tumors relative to either agent alone *in vivo*[136,137]. Combination of IFN-α gene delivery with 5-FU had similar results as well[137]. An *in vitro* study of combining INF-β gene tranfection with gemcitabine also showed tumor growth inhibition[138]. In a phase Ⅰ / Ⅱ trial, intratumoral gene delivery of replication-deficient adenovirus encoding TNF-α, combined with standard chemoradiation, showed promising clinical outcome with dose-limiting effect and toxicity[139].

In studies of tumor cell based vaccines, cytokine genes have been also introduced into tumor cells to render them tumorigenicity and immunogenicity. One study that directly compared and contrasted effects of different cytokines in murine tumor models demonstrated that the tumors transduced with GM-CSF produced the greatest degree of systemic immunity relative to irradiated non-transduced tumor cells[140]. In a phase Ⅰ trial of allogeneic GM-CSF-secreting cancer vaccines in 14 patients with pancreatic adenocarcinoma, no local or systemic dose-limiting toxicities were observed and three subjects who received the highest two dose levels showed increased disease-free survival time (more than 2 years)[141]. In a comparative clinical trial of GM-CSF-secreting cancer vaccines alone and combined with cyclophosphamide in patients with metastatic PC, minimal treatment-related toxicity was found and cyclophosphamide cohort exhibited longer progression-free survival and overall survival[142]. Another clinical trial of ipilimumab (anti-CTLA-4) in combination with allogeneic PC cells transfected with a *GM-CSF* gene also showed prolonged disease stabilization in previously treated advanced PC[143].

Tumor antigens

Clinical studies of immunotherapy in cancer have focused on five classes of tumor antigens: (1) tumor-specific antigens (MAGE-1, NY-ESO-1, TRAG-3, PSA); (2) mutated oncogene products (p53, K-ras, HER2, BCR/abl, WT-1); (3) reactivated embryonic gene products (CEA, AFP); (4) self-antigens overexpressed in tumors (MUC1, survivin); and (5) oncogenic virus antigens (EBV, HPV, HBV)[120,144]. Many antigen-specific vaccines that urge the host immune system to recognize the primary tumor have been developed, including recombinant viral and bacterial vaccines that encode tumor antigens, peptide-or protein-based vaccines that mixed with adjuvants, DNA-based vaccines expressing tumor antigens, and antigen-pulsed DC vaccines[120]. Up to now, at least 75 antigens were identified that had many of the study-defined characteristics of an ideal candidate antigen for cancer therapy. Many of these antigens are the focus of targeted therapy in clinical trials in cancer (Table 4)[144-149].

It is encouraging that many of the results of PC vaccine trials verified the safety and immunogenicity of these vaccines. In a mutated K-ras peptide vaccine clinical trial with 48 patients in PC, when combined with adjuvant GM-CSF, more than 50% of patients demonstrated a tumor-specific immune response and significantly improved median overall survival *vs* their non-responding counterparts[150]. A multi-institution double-blinded placebo-controlled trial of gastrin peptide vaccine in 154 patients with advanced-stage PC demonstrated a nearly 2-fold increase in the median overall survival in the treatment compared to the placebo group[151]. A phase Ⅰ / Ⅱ clinical trial of telomerase peptide vaccines also demonstrated prolonged survival in immune responders *vs* non-responders[152]. In a multi-institutional, open-label, dose-finding, phase Ⅱ trial of Algenpantucel-L, 70 patients with resected PC were administered by two different doses of the vaccine in combination with gemcitabine and 5-fluorouracil. Of the PC patients who received a higher dose of vaccine, 96% survived for at least one year in comparison to the historical control of 69%, showing a statistically significant difference[153].

Table 4 Antigenic sources used in pancreatic cancer vaccine trials

Peptide or protein vaccines	Mutant K-ras peptide
	WT1 peptide
	CAP1-6D
	G17DT
	HLA-A*0201 restricted VEGF receptor-1 and -2 peptides
	HLA-A24 restricted survivin-2B80-88 peptide (AYACNTSTL)
	Autologous heat shock protein HSPPC-96
	TELOVAC
Whole cell vaccines or dendritic cell vaccines	α-Gal transferase transfected allogeneic tumor cells (including Algenpantucel-L)
	GVAX
	MUC-1 pulsed autologous dendritic cells
Viral or bacterial vaccines	PANVAC-VF-MUC-1, CEA, and TRICOM transfected virus
	Mesothelin (CRS-207) transfected live-attenuated Listeria
DNA vaccines	VXMO1

VXMO1: VEGF receptor-2 DNA vaccine; GVAX: GM-CSF transfected allogeneic tumor cells; TELOVAC: Telomerase peptide GV1001; CAP1-6D: CEA peptide; WT1: Wilms tumor 1; G17DT: Gastrin peptide.

Although encouraging, results from single-agent immunotherapy clinical trials have been underwhelming. As more and more tumor antigens are identified, more specific and potent vaccines will be developed. The ideal vaccine will target multiple antigens that are crucial to the growth and progression of tumors. Perhaps combinatorial therapeutic approach, which includes chemotherapy, radiation, surgery, and immunotherapy, will result in even greater survival benefits for patients with PC[13].

APOPTOSIS RELATED GENES

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL or Apo-2 ligand (Apo-2L) is a type Ⅱ transmembrane protein belonging to the TNF superfamily and serves as an effective anticancer agent due to its cancer cell specificity and potent antitumor activity. TRAIL interacts with death receptor 4 (DR4) and DR5, which form the death-inducing signal complex (DISC) by binding to Fas associated death domain (FADD), thereby activates caspase-8 and results in activation of downstream caspases-3, -6, and -7, and apoptosis induction[154]. Recombinant human TRAIL has been developed as a novel anticancer agent and is being clinically evaluated for the treatment of both solid tumors and hematological malignancies, such as colorectal, melanoma, lung, ovarian cancers and non-Hodgkins lymphoma[155]. However, its use *in vivo* is limited by a short half-life in plasma due to a rapid clearance by the kidney.

Gene delivery of TRAIL into tumors may overcome this limitation. An adenoviral vector expressing TRAIL driven by a human telomerase reverse transcriptase (hTERT) promoter showed specific antitumor efficacy in PC cell lines *in vitro* and significantly suppressed tumor growth *in vivo*[156]. Systemic administration of this vector in combination with chemotherapy (gemcitabine) exhibited a synergistic effect in the induction of apoptosis[157]. TRAIL-engineered pancreas-derived mesenchymal stem cells (MSCs) were able to induce PC cell apoptosis *in vitro*[158]. Another study of adipose-derived MSCs that were transduced with the TRAIL gene showed that, when injected intravenously or subcutaneously into mice, these MSCs localized into tumors and mediated apoptosis without significant apparent toxicities to normal tissues[159]. These studies implied that MSCs may serve as a stable source of TRAIL delivery in PC therapy.

ONCOGENES

K-ras

Ras is the most common oncogene detected in human cancers. It comprises 3 families, H-ras, K-ras, and N-ras. Of these, the K-ras family is responsible for almost all of the PC mutations, with mutations in the other families occurring rarely. Studies suggested that K-ras, which is located on chromosome 12p13, is mutated in up to 95% of PC cases[16,160]. The *K-ras* encodes membrane-bound GTP-binding proteins, which can be activated by signaling partners to regulate many cellular functions, including cell growth, proliferation, and differentiation. Mutations of *K-ras* result in malfunction of GTPase and participate in the initiation or early phase of pancreatic tumorigenesis[71].

To blockade the Ras signaling pathway, cancer vaccines that stimulate immunity against mutant Ras proteins and antisense therapy that blocks the translation of mutant Ras gene are two common strategies. Antisense therapy involves the use of oligonucleotides, ribozymes and siRNAs. Retroviral delivery of K-ras siRNA to human tumor cells induced loss of expression of the K-ras gene, leading to loss of anchorage-independent growth and tumorigenicity *in vitro*[161]. Another study of K-ras siRNA delivered by electroporation demonstrated significant tumor growth inhibition both *in vitro* and *in vivo*. When K-ras siRNA is combined with gemcitabine, survival rate was significantly prolonged and the mean tumor volume was dramatically reduced when compared with single agents[162].

K-ras antisense oligodeoxynucleotide (K-ras-ASODN) was identified to successfully inhibit *K-ras* expression[163] and suppress the growth and invasiveness of PC cell lines *in vitro*[164]. In peritoneal dissemination models of PC, intraperitoneal injection of adenovirus expressing antisense K-ras RNA significantly suppressed the peritoneal growth with no significant systemic toxicity[165]. K-ras ASODN combined with type I insulin-like growth factor receptor (IGF-IR) antisense oligodeoxynucleotide (IGF-IR-ASODN) showed a significant inhibitory effect on tumor growth and induced apoptosis *in vitro* and *in vivo*, compared with each agent alone[166]. In a phase Ⅱ trial of pa-

tients with locally advanced and metastatic PC, ISIS 2503, a phosphorothioate oligonucleotide antisense inhibitor of human H-ras mRNA, showed a response rate of 10.4% and a median survival of 6.6 mo in combination with gemcitabine, which is promising but of unclear benefit[167]. Initial enthusiasm for this approach is currently diminishing following the failures of antisense inhibitors such as ISIS 3521 (a protein kinase C-alpha antisense oligonucleotide) and oblimersen (a bcl-2 antisense oligonucleotide) in lung cancer and melanoma, respectively.

LSM1

The cancer-associated Sm-like (CaSm) oncogene LSM1 has been reported to be overexpressed in 87% of PC cases[168]. An adenovirus expressing CaSm antisense RNA (Ad-alpha CaSm) reduced endogenous CaSm mRNA expression *in vitro*, and a single intratumoural dose of Ad-alpha CaSm inhibited tumor growth and extended survival time in an *in vivo* SCID mouse model of human PC. The antitumor effect was further enhanced by gemcitabine[168]. In a metastatic tumor model, systemic administration of Ad-alpha CaSm resulted in a significant decrease in the number of hepatic metastases and increased survival time through both direct and bystander effects[169].

HER-2/ErbB-2

Human epidermal growth factor receptor 2 (HER-2) is a transmembrane receptor tyrosine kinase of the ErbB family. It participates in the EGFR signaling pathway and has roles in cell proliferation, survival, motility, invasion and adhesion. Blocking of overexpressed HER-2 oncogene was able to improve survival in breast and gastroesophageal cancers. Some studies found that HER-2 amplification occurs in 2% of pancreatic ductal adenocarcinomas (PDACs) but has distinct features with implications for clinical practice, which represents an attractive target for anti-HER2 therapies[170]. However, clinical trials of anti-HER2 antibodies such as trastuzumab showed no benefits compared with standard chemotherapy and did not recommend further evaluation of anti-HER2 treatment in patients with metastatic PC[171].

MULTIDRUG RESISTANCE GENES

Drug resistance is a major cause of treatment failure in cancer chemotherapy. One of the important mechanisms of tumor multidrug resistance is increased drug efflux and decreased accumulation of drugs in the cell. Efflux transporters of the ATP-binding cassette (ABC) family such as ABCB1 (multidrug resistance 1, MDR1), the ABCC (multidrug resistance-associated protein, MRP) family, and ABCG2 (breast cancer resistance protein, BCRP) have been identified as major determinants of chemoresistance in tumor cells[172].

MDR1 is a classic paradigm in the ABC family and has been analyzed in detail in PC. Its product, P-glycoprotein, is a membrane protein that functions as an ATP-dependent exporter of drugs from cells. Some studies demonstrated a high rate (73.2%) of *MDR1* expression in PC[173,174], and other studies reported that MDR1 is associated with sensitivity to gemcitabine[175]. The MRP family consists of 9 members (MRP1-9) and is involved in exporting a variety of endogenous substrates as well as organic anions of xenobiotics, conferring cells resistance to cytotoxic and antiviral drugs[176]. It has been shown that the expression of MRP3 and MRP5 mRNAs was upregulated in PC and MRP3 was even correlated with tumor grade[176]. BCRP was first derived from a resistant breast cancer cell line, but is present in a wide range of human solid tumors, including PC[177]. One study proved that BCRP expression was frequent (73.1%) in PC, and high BCRP expression was a significant prognostic factor for early tumor recurrence and poor survival[178]. Conversely, a study demonstrated low BCRP mRNA levels in both normal pancreatic tissues and PC[176].

In fields of anti-*MDR* gene therapy for PC, some studies have showed that siRNAs against *MDR1* could specifically inhibit *MDR1* expression at the mRNA and protein levels and decreased resistance against daunorubicin in PC cell lines *in vitro*[179]. Similar results were found in another study of a hammerhead ribozyme against MDR1 mRNA[180]. However, *in vivo* studies and clinical trials are still scarce in this field and there is much room for advancement to validate their clinical applicability.

Another important approach of making use of the *MDR* gene is transducing the *MDR* gene into hematopoietic stem cells to strengthen the host's resistance to myelosuppression caused by chemotherapeutic drugs. An advantage of this approach is that it permits higher doses of chemotherapy without severe adverse effects, thus providing a better chance to achieve remission. *In vitro*, lentiviral or retroviral vectors encoding MGMT (P140K) and MDR1 or MRP1 resulted in significant survival advantage of human hematopoietic stem cells when undergoing intensification chemotherapy, compared with untransduced cells or either single vector alone[181-183]. In mouse models, *MDR1* gene modified hematopoietic cells also showed chemoprotective effect from various anticancer drugs[184,185]. So far, several clinical trials of MDR1 transfected autologous hematopoietic stem cell transplantation have been approved for the treatment of patients with breast cancer, ovarian cancer or leukemia. However, since the expression rate of the *MDR1* gene in PC is very high, this system may not be useful for treating this cancer.

PROLIFERATION RELATED GENES

VEGF

As mentioned above, the VEGF signal pathway plays a leading role in tumor angiogenesis. Therefore, knockdown of VEGF gene expression is a promising way in anti-angiogenesis therapy in PC. Materials in this approach include antisense oligonucleotides, ribozymes and siRNAs.

One study of antisense oligodeoxynucleotide of

VEGF-C showed that it decreased the expression levels of VEGF-C and inhibited lymphangiogenesis in nude mice with orthotopically xenografted human PC, but had no significant effect on angiogenesis[186]. This result was verified in another study with short hairpin RNA (shRNA) targeting *VEGF-C*, and the VEGF-C shRNA significantly inhibited cell proliferation and tumor growth *in vivo*[187]. On the other hand, antisense oligonucleotide of VEGF was proved to significantly decrease neoangiogenesis and vascular permeability in orthotopic xenograft models; furthermore, it reduced tumor growth and metastasis and improved survival[188]. Systemic or intratumoral injection of VEGF specific siRNAs also led to the significant reduction in the subcutaneous tumor growth through down-regulating VEGF expression and decreasing microvascular density[189,190]. A study with hammerhead ribozymes against VEGF gene transcripts showed inhibition of tumor growth and liver metastasis of a PC cell line *in vivo*[191]. However, no clinical trial in this approach has been reported in PC.

Human telomerase reverse transcriptase

Telomere is a region of repetitive nucleotide sequences at each end of a chromatid, which plays a critical role in maintaining chromosome stability. Telomere is shortened progressively during normal cell division. When its length becomes critically short, it triggers replicative senescence or apoptosis. Telomerase is a ribonucleoprotein polymerase that maintains the length of telomere. Human telomerase complex consists of telomerase reverse transcriptase (hTERT), telomerase RNA (hTR or TERC), telomerase associated protein-1 (TEP-1), hsp90 and p23, of which the RNA subunit and hTERT constitute the core of telomerase. It has been tested that hTERT is the limiting component of telomerase and its expression levels parallel to those of telomerase activity[192,193]. Generally, telomerase activity is detectable only in germ line cells and certain stem cells but is repressed in somatic cells. Upregulated telomerase activity is associated with promotion of tumorigenesis, neoplastic growth and metastasis of human cancer[194,195]. In fact, approximately 85% of human cancers exhibit reactivation of telomerase activity, which is even as high as 92%-95% in PC[196,197]. Recently, one new viewpoint declares that hTERT is also implicated in DNA repair and regulation of the expression of genes that control cell proliferation, which promotes tumorigenesis independent on the stability of telomere[198]. In a nutshell, hTERT is an important proliferation-related factor and can serve as a tumor marker and a prognostic indicator.

In vitro, hTERT antisense oligonucleotide (hTERT-ASODN) could down-regulate expression of hTERT mRNA and increase cell apoptosis rate in a concentration- and time-dependent manner in PC cell lines[199,200]. Therefore, consecutive transfections were performed in order to inhibit telomerase activity and result in a continuous reduction in cell viability[201]. Cell cycle analysis indicated that the cells were mainly arrested at the G0/G1 phase with the treatment of hTERT-ASODN. In addition, hTERT ASODN synergized with gemcitabine to exert an anti-proliferation effect[200]. Similar results were demonstrated in another study of hTERT-siRNA transfection in pancreatic cancer cell line Capan-2, and the inhibitory effect was associated with the downregulation of *Bcl-2* and cyclooxygenase-2 (*COX-2*)[202]. Hammerhead ribozyme targeting hTR was also found to depress telomerase activity, and ribozyme targeting hTERT mRNA showed stronger inhibition. Since the level of hTERT mRNA expression is less than that of hTR expression in cancer cells, hTERT might be a more useful therapeutic target[203]. An hTERT peptide vaccine GV1001 was tested in a phase Ⅰ/Ⅱ study of 48 patients with unresectable PC. The patients received intradermal injection in combination with GM-CSF. In the end, 24 of 38 evaluable patients demonstrated immune responses with the highest percentage (75%) in the intermediate dose group. Approximately 8.6 mo of mean survival for this group was significantly longer and one-year survival rate was 25%[152]. These promising results have led to commencement of another phase Ⅲ trial of GV1001.

COX-2

COX-2 is a key enzyme of the metabolic process of arachidonic acid and an early response protein that is induced rapidly by growth factors, tumor promoters, oncogenes, and carcinogens. It plays an important role in tumorigenesis and angiogenesis. It has been shown that COX-2 mRNA and protein expression was highly upregulated in up to 90% of PC cases but was undetectable in nontumorous pancreatic tissue[204,205], and this indicated that COX-2 may be a potential target for treatment of PC. One study showed that COX-2 siRNA transfection could inhibit cell proliferation, induce cell apoptosis and regulate cell cycle of a PC cell line *in vitro* and decrease its tumorigenicity when inoculated subcutaneously into nude mice[206]. Nevertheless, inhibition of COX-2 is more applied in anti-inflammation treatment as nonsteroidal anti-inflammatory drugs (selective inhibitors of COX-2) than gene therapy.

Clinical research of gene therapy

Up to July 2013, 1970 gene therapy clinical trials have been completed, are ongoing or have been approved worldwide. Of these, 1264 (64.2%) have been made for treatment of cancers, including lung, gynecological, skin, urological, neurological and gastrointestinal tumors, as well as hematological malignancies and pediatric tumors. In this part, we summarized the finished and ongoing clinical trials of PC gene therapy worldwide (Tables 5 and 6) and the administration routes they employed (Figure 3). Meanwhile, since a variety of cancers have common characteristics, such as mutation of tumor suppressor genes or oncogenes and overexpression of tumor antigens, one therapeutic transgene can be effective for different tumors. In fact, many clinical trials are indicated for several cancers simultaneously, like all solid tumors

Table 5 Closed clinical trials of gene therapy in pancreatic cancer

Trial ID and investigator	Phase	Clinical indication	Patients (*n*)	Transgenes	Vectors and target cells	Administration route	Combined therapy	Results
DE-0009 Löhr *et al*[67]	Ⅰ / Ⅱ	Inoperable PC	14	Cytochrome p450	Naked/plasmid DNA transfected allogeneic human 293 embryonic kidney cells	Inject into the tumor vasculature *via* supraselective angiography	In combination with low-dose ifosfamide	4 patients showed tumor regression, the other ten individuals remained stable. Median survival was doubled compared with historic controls. 1-yr survival rate was three times better
DE-0024 Pecher *et al*[207]	Ⅰ / Ⅱ	Advanced breast cancer, PC and gallbladder carcinoma	10 (2 PC)	MUC-1	Naked/plasmid DNA transfected autologus dendritic cells	Subcutaneous injection	None	9 patients showed signs of progression. Only one remained stable for 3 mo until she was transferred to another therapy. 3 of 10 patients developed vaccine-specific delayed-type hypersensitivity reaction (DTH). 4 of 10 patients showed increased mucinspecific INF-gamma-secreting CD8+ T cells
DE-0063 Kubuschok *et al*[208]	Ⅰ	PC	3 healthy donors and 1 PC patient	Mutated ras oncoprotein	EB virus transformed autologous lymphoblastoid cells	Subcutaneous injection	None	All the subjects showed strong vaccine-induced muRas-specific cytotoxic T lymphocytes
DE-0083 Niethammer *et al*[209]	Ⅰ	Advanced PC	45	VEGFR-2	Naked/plasmid DNA (oral DNA vaccine)	Oral administration	In combination with gemcitabine	Not reported
ES-0004 Mazzolini *et al*[210]	Ⅰ	Liver cancer, PC, colorectal cancer	17 (3 PC)	Interleukin-12 (IL-12)	Adenovirus transfected autologous dendritic cells	Intratumoral injection	None	Treatment was well tolerated. 11 of 17 were assessable for response. A partial response was observed in 1 case with PC. Stable disease was observed in 2 patients and progression in 8 patients
FR-0018 Gilly *et al*[211]	Ⅰ / Ⅱ	Unresectable digestive cancer	6 (3 PC)	Interleukin-2 (IL-2)	Adenovirus	Intratumoral injection	None	Good safety. But final results of tumor responses were not reported
US-0853 Le *et al*[212]	Ⅰ	Ovarian cancer, PC, lung cancer, mesothelioma	28	Mesothelin	Listeria monocytogenes	Intravenous injection	1: Live attenuated Listeria vaccine (n = 9); 2: live attenuated mesothein expressing Listeria vaccine (n = 17)	In arm 2, Listeriolysin O and mesothelin-specific T-cell responses were seen and 37% of subjects lived ⩾ 15 mo
US-0700 Galanis *et al*[213]	Ⅰ	Gemcitabine-refractory, metastatic PC	12	Cyclin G1	Retrovirus	Intravenous injection	None	Good safety. But there was no evidence of anti-tumor activity
Kaufman *et al*[214]	Ⅰ	Advanced PC	10	CEA, MUC-1, and TRICOM (including B7.1, ICAM-1, LFA-3)	Poxvirus	Subcutaneous injection	In combination with GM-CSF	Antigen-specific T-cell responses in 5 of 8 evaluated patients. 15.1 mo of median survival in responders *vs* 3.9 mo in non-responders. Overall median survival is 6.3 mo
Jaffee *et al*[141]	Ⅰ	Resected PC	14	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	In combination with standard chemoradiation	3 of 14 patients developed DTH, and 3 patients had increased disease-free survival time (at least 25 mo after diagnosis)
US-0475 Lutz *et al*[215]	Ⅱ	Resected PC	60	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	In combination with standard chemoradiation	The median disease-free survival is 17.3 mo with median survival of 24.8 mo. Induction of CD8+ mesothelin-specific T cells correlated with disease-free survival
Laheru *et al*[142]	A pilot study	Advanced PC	50	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	A: vaccine alone (n = 20) B: vaccine plus cyclophosphamide (n = 30)	Cohort B showed enhanced mesothelin-specific T-cell responses. Median survival values in cohort A and cohort B were 2.3 and 4.3 mo
Le *et al*[143]	Ⅰ b	Advanced PC	30	CM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	1: Ipilimumab alone (n = 15); 2: Ipilimumab plus vaccine (n = 15)	Median overall survival was 3.6 mo for arm 1 and 5.7 mo for arm 2. Mesothelin-specific T cells responses were associated with increased disease-free survival in arm 2

Table 6 Ongoing clinical trials of gene therapy in pancreatic cancer

Phase	Transgenes
I	Oncolytic adenovirus and oncolytic herpesvirus[1]
	Somatostatin receptor 2 (sst2), Deoxycitidine kinase :: uridylmonophosphate kinase (dck::umk)
	Somatostatin receptor 2 (sst2)
	Mesothelin-scFv with signaling domains comprised of TCR , CD28, and 4-1BB (CD137) cDNA
	GM-CSF
	PANVAC (CEA, MUC-1, and TRICOM)
	Cytosine deaminase, Herpes simplex virus thymidine kinase (HSV-TK)
	Cyclin G1
	p53
	BikDD (DOTAP-cholesterol mediated gene transfection)
	Mutated Ras
I / II	Cytochrome p450
	AEG 35156: antisense oligonucleotide to X-linked inhibitor of apoptosis protein (XIAP)
	Herpes simplex virus thymidine kinase (HSV-TK)
	Diphtheria Toxin A Chain (DTA) gene with H19 promoter
	Alpha-(1,3) galactosyltransferase
II	CEA, MUC-1, and PANVAC
	Mutated Ras
	Alpha-(1,3) galactosyltransferase
III	Alpha-(1,3) galactosyltransferase
	PANVAC (CEA, MUC-1, and TRICOM)[2]

[1]Clinical trials of oncolytic viruses were completed or are ongoing in Spain and Japan, and their details were not clear; [2]This clinical trial has closed, but details and results have not been published yet.

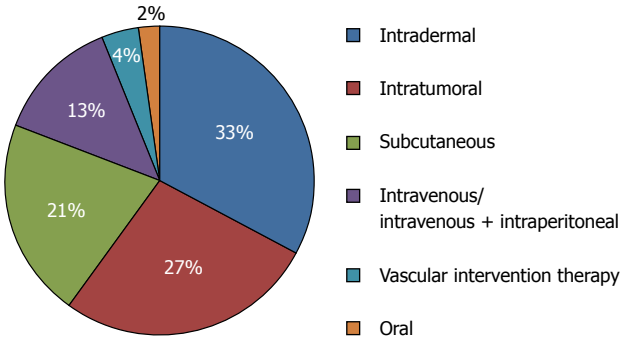

Figure 3 Clinical trials regarding administration routes of gene therapy in pancreatic cancer.

or advanced tumors. Therefore, here we also collected such kind of trials and obtained a list of them (Table 7). It is worth noting that our analysis is mainly based on the records in The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide website (http://www.wiley.co.uk/genmed/clinical), so trials in some countries (*e.g.*, Japan) are not available.

Table 7 Clinical trials of unspecific tumor gene therapy that may involve pancreatic cancer

Phase	Clinical indication	Transgenes
II	Adenocarcinoma	CEA, TCRzeta and CD28
I	Cachexia	GHRH
I	CEA- or HER-2-expressing malignancies	HER-2, CEA
I	CEA-expressing malignancies	CEA
I / II	CEA-expressing malignancies	CAP-1 peptide from CEA
I / II	CEA-expressing malignancies	T cell receptor alpha and beta chains cDNA
I	CEA-expressing malignancies	CEA, B7.1, ICAM-1, LFA-3, GM-CSF
I	CEA-expressing malignancies	Anti-CEA-SFv-Zeta T cell receptor
I	CEA-expressing malignancies	B7.1 (CD80)
II	Advanced cancer with overexpression of p53	Anti-p53 T cell receptor
I	MUC-1- expressing tumors	MUC-1, IL-2
I	Advanced cancer	Cytochrome P450
I	Advanced cancer	Endostatin
I	Advanced cancer	Heat shock protein 70
I	Advanced cancer	T cell receptor alpha and beta chain
I	Advanced cancer	Bifunctional shRNA specific for stathmin 1 oncoprotein
I / II	Advanced cancer	GM-CSF
I	Advanced cancer	IL-12
I / II	Advanced cancer	CYP1B1
I	Advanced cancer	GM-CSF, CD154 (CD40-ligand)
I / II	Advanced cancer	IL-2
I	Advanced cancer	p53
I	Advanced cancer	AMEP
I	Advanced cancer	B7.1 (CD80) ,ICAM-1, LFA-3
I / II	Advanced cancer	Cytosine deaminase
I / II	Advanced cancer	CEA
I	Solid tumors	Tumor antigen
I	Solid tumors	Interferon-gamma
I	Solid tumors	TNF
I	Solid tumors	Brachyury oncoprotein
I	Solid tumors	Human telomerase reverse transcriptase
I / II	Solid tumors	Oncolytic virus (no transgene)
I	Solid tumors	Retinoblastoma 94
I	Solid tumors	p53
I	Solid tumors	O6-methylguanine DNA methyltransferase (MGMT)
I	Solid tumors	GM-CSF
I	Solid tumors	GM-CSF, bi-shRNA-furin
I	Solid tumors	GM-CSF, TGF-beta 2 antisense
I	Solid tumors	GM-CSF, humanized *Escherichia coli* beta-galactosidase

CEA: Carcinoembryonic antigen; IL-2: Interleukin-2; AMEP: Antiangiogenic metargidin peptide; CYP1B1: Cytochrome P450 isoenzyme 1B1; TNF: Tumor necrosis factor; hTERT: Human telomerase reverse transcriptase; GHRH: Human growth hormone releasing hormone.

Conclusions and prospects

Over the past decades, plenty of experimental works and clinical trials have demonstrated the safety and efficacy of cancer gene therapy. Strategies encompass tumor suppressor, suicide, anti-angiogenesis and immune related genes, as well as activated oncogenes and multidrug resistance genes. Proliferation and apoptosis related signal pathways are also possible targets. However, among these therapeutic targets, only a small part were tested in clinical settings and proved to be effective, and even for them, outcomes were far from curative and treatments have to be combined with standard chemo- or radio-therapy for maximum benefits.

In this field, tumor suppressor gene *p53* and mutant

oncogene *K-ras* are two members that have been most deeply studied in all kinds of cancers and have yielded encouraging results *in vitro* and in animal models. Unfortunately, these findings have not been translated to improve outcomes in clinical trials. In suicide strategy, HSV-TK, CD and cytochrome p450 have been proved to be efficacious. A closed clinical trial of cytochrome p450 showed exciting results when combined with ifosfamide, and more trials are ongoing. As to anti-angiogenesis approach, VEGFR is of course the best choice, clinical trials of peptide vaccine derived from VEGFR-2 and DNA vaccine targeting VEGFR-2 did show benefits, but as a monotherapy it is not a "magic bullet" for all tumor patients since anti-angiogenesis is far more effective in preventing tumor growth than causing regression of established tumors, thus this method may be more suitable for patients with minimal residual disease. Nowadays immunotherapy has become the mainstream direction of studies in searching new cancer treatment regimens, and the cooperation of gene therapy and immunotherapy has created even more inspiring achievements, such as adoptive transfer of genetically modified T-cells or DCs, DNA vaccines (CEA, MUC-1, B7.1, ICAM-1, LFA-3), and direct transfer of cytokine genes (IL-2, IL-12, GM-CSF, INF-gamma). Of course, combining different targets or combination of gene therapy and traditional chemoradiotherapy may lead to improved efficacy of gene therapy in PC.

Considering that low efficiency of gene transfer is the main obstacle in application and popularization of gene therapy, the selection of high-efficient and tumor-targeted vectors should be emphasized. In general, viral vectors offer higher transduction efficiency and long-term gene expression and this is why more than two-thirds of clinical trials reported employ viral vectors. Recently, oncolytic viruses that infect and replicate selectively in cancer cells have become the hotspot in this field and show a bright future. Oncolytic viruses produced from initially infected cancer cells can spread to surrounding cancer tissue and distant tissue, thus intratumoural injections could be efficient, even for the treatment of disseminated tumors. Systemic administration of oncolytic viruses has also shown good safety and this is of great interest particularly in PC, since it is difficult to inject viruses into primary lesions directly in PC patients.

Gene therapy of cancer is not yet applied in routine clinical practice, but good safety records and validated clinical benefits make it a good candidate for all clinical settings including neo-adjuvant, adjuvant and palliative treatment. Despite these promising therapeutic strategies, greatest advance in the treatment of PC may still come from improved early detection and diagnosis.

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