1 Gene Therapy for Pancreatic Cancer

Han Hsi Wong · Nicholas R. Lemoine

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Abstract Pancreatic cancer has high morbidity and mortality and remains one of the most difficult cancers to treat. Clearly there is a strong need for the development of novel therapeutic approaches. Since the first clinical trial in 1990, gene therapy has improved and expanded tremendously, largely due to the advancement in molecular technology. It involves the introduction of exogenous nucleic acids to express, restore, or inhibit a gene of interest to reverse or destroy the malignant phenotype of cancer cells. Recent understanding of the genetics and pathogenesis of pancreatic cancer has generated a large number of studies targeting these abnormalities, albeit with variable levels of success.

In this chapter, gene delivery systems (viral and nonviral), molecular targets, and gene therapy approaches in the context of pancreatic cancer treatment are discussed. These include the RNA-directed strategies, dominant-negative mutants, gene restoration, gene-directed prodrug activation therapy, oncolytic viruses, and immunotherapy. In each section the genetic and molecular aberrations of pancreatic cancer are introduced, followed by results from laboratory studies and subsequent clinical trials, where available.

1. Introduction

Pancreatic cancer is a devastating disease and extremely difficult to treat with conventional therapies. Outcome has not improved substantially over the past 25 years, with overall 5-year survival remaining dismally poor at 5%. Its pathogenesis is one of the best characterized of the common malignancies. Multiple genetic mutations have been identified as the precursor to the development of pancreatic cancer and these can be targeted for therapeutic interventions [1] (*Table 1*). These mutations result in some of the hallmark features of malignancy, such as disinhibited growth, avoidance of apoptosis, evasion of host immune response, sustained angiogenesis, tissue invasion, and metastasis. Due to the genetic basis of the disease and recent advancement in molecular techniques, targeted gene therapy has become an area of intense research, used either alone or in combination with standard therapies. The latter has the advantages of improving treatment outcomes, reducing toxicity caused by standard radiotherapy and chemotherapy, and overcoming the problem of cross-resistance.

The term gene therapy encompasses a wide range of treatment approaches that involve the introduction of exogenous nucleic acids into cells to treat the causes of a particular disease (*Table 2*). The first gene therapy trial was conducted in 1990 on a 4-year-old girl with adenosine deaminase deficiency (ADA), where a normal copy of the ADA gene was transferred into her peripheral lymphocytes using a retroviral vector. Since then gene therapy has expanded towards treating other inherited genetic disorders such as severe combined immunodeficiency (SCID), hemophilia and cystic fibrosis. Improvement in molecular technology has made it possible to include more complex diseases such as cancer. By expressing, restoring or inhibiting a particular gene of interest, the intention is to prevent or reverse the growth of cancer cells.

2. Gene Delivery System

These include viral and nonviral methods (*Table 3*). The most efficient delivery system is that of viral vectors and hence it is most commonly used in cancer gene therapy protocols. In addition to exploiting the efficiency with which viruses carry genes into the cells, some viruses

Table 1

Genetic and molecular targets for pancreatic cancer gene therapy

	Frequency of mutation/expression (%)
Akt2	20
Bcl-2	23
Bcl-xL	90
BIRC5 (survivin)	77–94
c-Met-encoded HGF receptor	61–87
CaSm (LSM1)	87
CCK-B receptor	95
CEA	85–90
EGFR	69
FAK	48–75
Gastrin precursors & gastrin	23–91
IGF-1R	64
K-ras	75–90
Mesothelin	90–100
MUC1	90
Notch3	69–74
$p16^{INK4A}$ (MTS1)	85
p53	50–75
pRb	6
Shh	70
Smad4	55
Telomerase	92–95
TP53INP1	86
TβRI	1
TβRII	4
VEGF	93

Table 2

Conditions required for effective gene therapy

• The genetic defect is known and specific to the disease
• The use of an efficient gene delivery system
• Targeted delivery with sparing of surrounding normal cells
• The new genetic material can be sustained in or expressed by the cell
• Treatment can be given in vivo
• Effective alone or in combination with other treatments
• Safe and has minimal toxicity

Table 3

Advantages and disadvantages of different gene delivery systems

Vector	Advantages	Disadvantages
Viral	Tumor selectivity possible	Packaging cell line needed for production, safety concerns
Adenovirus	High gene transfer efficiency in replicating and nonreplicating cells, nonintegrating to host genome, high-titer production, good cloning capacity with "gutless" vector, oncolytic	Transient gene expression, low cloning capacity, immunogenic, down-regulation of attachment receptors in many cancer cell types
Retrovirus	Moderate gene transfer efficiency, long-term expression, low immunogenicity	Inability to transduce nonreplicating cells, low cloning capacity, risk of insertional mutagenesis, low-titer production
Adeno-associated virus	High gene transfer efficiency in replicating and nonreplicating cells, long-term expression, nonimmunogenic	Low cloning capacity, risk of insertional mutagenesis, low-titer production, contamination with helper virus
Lentivirus	High gene transfer efficiency in replicating and nonreplicating cells, long-term expression, low immunogenicity, high-titer production	Low cloning capacity, risk of insertional mutagenesis
Nonviral Naked DNA, liposome, polymer and dendrimer, hybrid)	Large transgene, ease of manufacturing and administration, safe and noninfectious, no risk of recombination, limited immunogenicity	Low efficiency, transient gene expression, no tumor-specificity (although tumor-targeting possible e.g. with modified polymers or hybrid vectors)
Mesenchymal stem cell	High efficiency of gene expression, nonimmunogenic, multipotentiality, targeted delivery to tumor site	Limited experience, complex process, need prior ex vivo transduction with viral vector (and hence associated problems), high cost

have the benefit of being able to replicate in and destroy cancer cells (oncolytic viruses). Tumor selectivity is made possible by modification of the viral coat (transductional targeting) or by exploiting tumor- or tissue-selective gene promoters to drive expression within the viral genome (transcriptional targeting). Its disadvantages include the limited size of foreign DNA that can be incorporated, the need for packaging cell lines for its production, its potential toxicity and immunogenicity. Nonviral or physical methods on the other hand have advantages in terms of manufacturing, ease of handling, no risk of recombination, low immunogenicity and large DNA insert, but are far less effective and generally lack targeting specificity. Mesenchymal stem cells have recently been studied as a novel gene delivery system.

2.1 Adenovirus

Human adenoviruses belong to the family Adenoviridae and were first isolated from human adenoid tissue, from which the name was derived. Fifty-one serotypes of adenoviruses have been identified so far, and divided into species A to F, based on DNA homology and agglutination properties. Adenoviruses type 2 (Ad2) and 5 (Ad5) belong to subgroup C and are the most studied and characterized viruses used both as a gene transfer vector and oncolytic agent. They are nonenveloped, icosahedral, double-stranded DNA viruses, about 70–90 nm in diameter, with a linear DNA of approximately 34–48 kb in size. They are genetically stable, amenable to high-titer production and purification (up to 10^{13} particles/ml), and highly efficient at entering the cell (both quiescent and replicating) and nucleus with the resulting expression of the gene of interest. Its DNA is transcribed episomally, without integrating into the host cell's genome. Upto 7.5 kb of foreign DNA can be inserted with deletions of its E1 and E3 genes. The E1A gene product forces quiescent cells into S phase so that viral DNA can be replicated, and induces the expression of other viral genes. Deletion of E1A renders the virus replication incompetent, but is still able to transfer the foreign gene effectively. The E3 gene is involved in immune-response evasion and virus release from cells and is therefore dispensable (although some of its properties make it attractive for retention in oncolytic applications). An extension of this is the "gutless" (or helper-dependent) adenovirus in which most of the viral genome is deleted, leaving only essential genes for vector propagation and packaging, thus allowing for up to 36 kb of cloning capacities.

The disadvantages of adenoviral vectors include the induction of (dose-dependent) host immune responses. In 1999, an 18-year-old patient with partial ornithine transcarbamylase deficiency died after receiving an adenovirus-based gene therapy, although in this exceptional case, a very high dose was delivered directly into the liver in a patient with evident liver dysfunction. While this might be perceived as a disadvantage, it could be beneficial in cancer treatment as it might stimulate anti-tumor immunity. Except for members of subgroup B, adenoviruses enter cells by their attachment to the Coxsackie and Adenovirus Receptor (CAR) on the cell surface. However, CAR is often downregulated in many tumor types, rendering them resistant to adenoviral infection. Furthermore, CAR is a transmembrane component of tight junctions and might limit viral infection across epithelial surfaces. Ad5, the prototype adenoviral vector, also has a high prevalence of neutralizing antibodies in the normal population (45–90%). Several approaches have been explored to overcome these problems, including the use of bispecific molecules or antibodies that bind to the fiber or capsid of the adenovirus and redirect it to a different receptor, Ad5 bearing the fibers of other adenoviral subgroups, and genetic modification of the viral particle. More recently, the use of adenoviruses of subgroup B, such as Ad11 or Ad35 that attach to CD46 instead of CAR, has generated a lot of interest in cancer gene therapy.

Because the expression of therapeutic genes using adenoviral vector is transient, the aim of treatment protocols has largely been directed toward lysing the cancer cells.

2.2 Retrovirus

Retroviruses are enveloped viruses that contain a single-stranded RNA genome. Selected gene deletions make the virus nonreplicative, create a cloning capacity of 8 kb and also lower its immunogenicity. The genes include *env* (envelope – encodes surface protein), *gag* (group-

specific antigen – encodes core and structural proteins) and *pol* (polymerase – encodes integrase, protease and reverse transcriptase). Modification of the *env* gene can also increase its specificity to target cells. After infection, its RNA is transcribed by reverse transcriptase into DNA, which becomes integrated randomly into the host cell's genome, allowing for long-term expression of the transgene of interest. Gene delivery to pancreatic cancer cells has been achieved with oncoretroviral vectors such as the Moloney murine leukemia virus.

The limitations of retroviruses include their inability to transduce nonreplicating cells. As only a fraction of the cells are actively dividing at any given time, the gene transfer efficiency is much reduced. Furthermore, production in high titers has been a major issue, with the highest achievable at only 10^8 particles/ml. The integration of viral DNA into the host cell's chromosomes can result in insertional mutagenesis, predisposing to the development of malignancy. In fact there have been reports that patients with X-linked SCID treated with retrovirus develop leukemia at a significant frequency.

2.3 Adeno-Associated Virus

Adeno-associated viruses (AAVs) are small, nonenveloped, single-stranded DNA parvoviruses. In order to replicate efficiently, they require co-infection with a helper virus, normally adenoviruses or herpesviruses. The AAV genome contains the cap and rep genes that encode structural and viral replication proteins respectively. Deletions of these genes render the virus nonreplicative and also create a space of 4.5 kb for foreign DNA insertion. The wild-type virus integrates into the host genome at a specific site on chromosome 19, a property determined by its rep gene. Engineered viruses thus have abolished genome-integrating ability.

AAVs are nonpathogenic and nonimmunogenic, have broad tropism for many cell types, and are efficient in transducing both replicating and quiescent cells. In some cells long-term expression of transgenes has been achieved. Recombinant AAVs are produced with a helper virus and a packaging cell line capable of supplying the cap and rep gene products. However, high-titer production is limited due to the toxic effect of rep proteins to both the packaging cell line and helper virus. Its low cloning capacity can be overcome by co-infection with two AAVs, each carrying half the desired gene, with the larger transgene eventually formed in the host cell by homologous recombination or splicing.

Herpes simplex virus ICP6-deleted mutant has been shown to enhance AAV-vector expression in vitro and in vivo for pancreatic cancer cells [2].

2.4 Lentivirus

Lentiviruses are a group of retroviruses to which the causative agents of acquired immunodeficiency syndrome (AIDS), the human immunodeficiency viruses (HIV-1 and -2) belong. It is a promising class of vector because unlike retroviruses, it can integrate its complementary DNA in both replicating and nondividing cells, although this occurs at random chromosomal sites. Lentiviruses are able to achieve this due to the presence of accessory gene products, which form a pre-integration complex with the DNA allowing it to enter the nucleus (other vectors must wait for the nuclear membrane to break down during cell division). Other advantages include its low immunogenicity, ease of high-titer production and the stable expression of transgenes (up to 6 months in pancreatic cancer cells using the equine infectious

anemia virus [3]). Safety concerns have led to the development of self-inactivating HIV-1 in which the proviral integrants lack enhancer-promoter activity within their long terminal repeats, thereby reducing the risk of clonal dominance and insertional mutagenesis [4]. In this case, the gene of interest is expressed from an exogenous viral or cellular promoter that is inserted into the lentiviral vector.

Lentiviral-mediated RNA interference targeting the high mobility group A1 (HMGA1, architectural proteins that are overexpressed in pancreatic cancer) has been studied in pancreatic cancer cell lines, where it increased the cellular sensitivity to gemcitabine [5].

2.5 Naked DNA

Naked DNA in the form of plasmid can be produced easily from bacteria. Although the insert size is potentially limitless, the delivery efficiency is very low, there is no tumor-specificity and the transgene expression is brief. The CpG motifs in the plasmid DNA can induce a limited host immune response.

The simplest method of delivery is by direct injection using a needle and syringe. Various techniques have been invented to improve uptake by means of increasing the permeability of the cell membrane. These include electroporation (using low-strength electromagnetic fields) and ultrasound. Hydrodynamic injection involves high-pressure, rapid injection of large volumes of genetic material. It improves uptake possibly by pressure on the endothelium, or by transient localized occlusion of blood vessels such as the hepatic portal vein. The gene gun approach makes use of DNA coated with heavy metals (e.g. gold) delivered at high speed using a vacuum pump or helium propellant, allowing direct penetration through cell membranes.

2.6 Liposome

Liposomes are spherical lipid bilayers that mimic biological membranes. Cationic liposome is positively charged and the negatively charged DNA binds to it by electrostatic interaction, forming the so-called lipoplex. The excess positive charge on these liposomes allows efficient interaction with the cell membrane, enabling endocytosis with subsequent delivery of DNA to the nucleus. Dissociation of DNA from liposomes occurs probably by the effect of anionic lipids within the cell. Although safe and easy to make, this system results in only transient transgene expression, lacks targeting specificity and may suffer from formulation instability. Transfer of a reporter gene using liposomes into the pancreas has been successful in a rat model via local artery and pancreatic duct infusion [6]. Transgene expression was limited to up to 28 days. Intraperitoneal injection of lipoplexes was also effective in treating peritoneal dissemination of pancreatic cancer in mice [7,8].

2.7 Polymer and Dendrimer

Cationic polymers such as poly-L-lysine (PLL), polyethyleneimine (PEI), and oligopeptides can form polyplexes with DNA. They can exist as linear or branched polymers of varying lengths. Dendrimers are a type of highly branched synthetic polymers with a spherical shape. Longer polymers are more efficient at condensing DNA and resistant to degradation. However

over-condensed DNA may impede its release and become trapped in endosomes, resulting in lysosomal degradation. The most commonly used PEI has a high density of amine groups. These attract protons and consequently water, causing rupture of the endosome and facilitating the release of DNA. Improvements to the specificity of polyplexes have been undertaken, for example by the incorporation of a receptor-specific antibody or ligand protein.

2.8 Hybrid

These have been developed to overcome the individual shortcomings of both viral and nonviral vectors. One example is the virosomes – liposomes that contain viral antigens (e.g. influenza virus) embedded in their membranes. These have the advantage of improving cellular binding of particles, as well as stimulating the host cell anti-tumor immune response.

2.9 Mesenchymal Stem Cell

Mesenchymal stem cells (MSCs) are bone marrow-derived nonhematopoietic precursor cells that normally contribute to the maintenance and regeneration of connective tissues through engraftment. They were first suggested by the German pathologist Cohnheim in 1867; he studied wound healing and found that many cells in the wound came from the bloodstream, and therefore must have originated from the bone marrow. They can be aspirated relatively easily from the bone marrow, expanded in vitro, and transfected with exogenous genes. The multipotentiality of MSCs has made them an attractive vector for gene therapy for diseases of various origins.

Besides malignant cells, the tumor environment also contains blood vessels, immune cells and stromal fibroblasts. This closely resembles wound healing and scar formation as observed by Cohnheim, whereby signals (such as growth factors and chemokines) could attract and mediate the engraftment and proliferation of MSCs. Indeed MSCs have already been used for targeted delivery of therapeutic genes to the tumor environment in animal models, including for glioma, melanoma and breast cancer [9]. Recently, lentivirus-transduced MSCs have shown promising results in targeting human orthotopic pancreatic tumor xenografts in nude mice [10].

3. Molecular Targets for Gene Therapy in Pancreatic Cancer

3.1 Ras

The epidermal growth factor receptor (EGFR, also known as human EGF receptor 1 – HER1 or ErbB1) is a transmembrane glycoprotein with an intracellular tyrosine kinase domain. Binding of its ligands (EGF and transforming growth factor-α (TGF-α)) leads to phosphorylation of tyrosine residues on the intracellular domain, activating downstream signaling cascades. An orally active EGFR tyrosine kinase inhibitor, erlotinib (Tarceva or OSI-774), has already shown survival benefit in combination with gemcitabine for patients with advanced pancreatic cancer (median survival (MS) of 6.24 compared to 5.91 months in controls, with 1-year survivals of 23 and 17% respectively) [11]. The United States Food and Drug

Administration had already approved its use in 2005, but European registration is restricted to those with metastatic disease.

The K-ras oncogene (homologous to the ras gene of Kirsten murine sarcoma virus) mutation is found in 75–90% of pancreatic cancers, mostly at codon 12 but also at codons 13 and 61. The gene encodes a 21-kDa membrane-bound guanosine triphosphate (GTP)-binding protein involved in growth factor-mediated signal transduction pathways. It can be activated through the overexpression or activation of ras-activating signaling partners such as EGFR. The best characterized effector pathway in ras function is the Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK; MEK) cascade. Mutations result in impaired GTPhosphatase (GTPase) activity, causing it to be locked in the GTP-bound state and thus activating downstream signaling cascades. Activation of this oncogene is involved in the initiation or early phase of carcinogenesis. Another less characterized member of the ras family, H-ras (homologous to the ras gene of Harvey murine sarcoma virus), also plays a role in promoting tumor growth. The H-ras-ERK cascade is activated by TGF-α in pancreatic cancer cells with K-ras mutations.

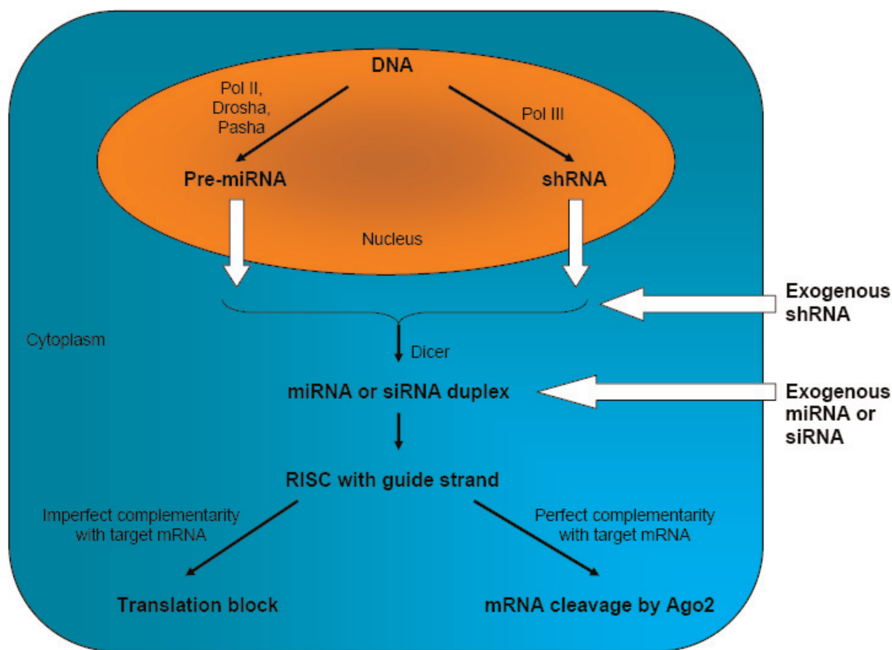
3.1.1 RNA-Directed Strategies

RNA-directed strategies are promising approaches in pancreatic cancer gene therapy. These include the ribozymes, antisense therapy and RNA interference (RNAi). They are also called "knock-down" strategies because selected gene expressions are specifically inhibited. Ribozymes (from ribonucleic acid enzyme) were first discovered in the 1980s in the ciliated protozoan *Tetrahymena thermophila*. They are catalytic RNA molecules that bind to sequence-specific mRNA and cleave its phosphodiester backbone, most commonly at the GUC triplet sequence. Adenovirus-mediated transfer of anti-K-ras ribozyme can induce growth suppression and apoptosis of human pancreatic cancer cells in mice [12,13].

Antisense therapy involves the administration of oligonucleotides (short sequence of nucleic acids) with sequences complementary to regions of specific mRNA strands, thereby blocking their translation. Transfection with a plasmid encoding antisense K-ras RNA has been shown to inhibit the growth of pancreatic cancer cell lines both in vitro and in vivo [14]. Liposome-mediated transfer was also effective in a mouse model [8]. In a phase II trial of the antisense inhibitor of H-ras, ISIS-2503 in combination with gemcitabine, patients with locally advanced or metastatic pancreatic adenocarcinoma showed a response rate of 10.4% and an MS of 6.6 months (5% and 5.7 months respectively for gemcitabine alone) [15].

The gene silencing process of RNAi involves the manufacture of short, double-stranded RNAs (<30 base pair), termed microRNAs (miRNAs) or small interfering RNAs (siRNAs), by the cytoplasmic RNase enzyme Dicer (*Fig. 1*). These RNA duplexes subsequently bind to a multi-protein nuclease complex to form the RNA-induced silencing complex (RISC). ATP-dependent unwinding of the siRNA duplex activates the RISC, which identifies complementary mRNA. This results in translation block or cleavage of the mRNA by the RNA endonuclease Ago2. Synthetic siRNAs or miRNAs can be introduced directly or by transcription of small hairpin RNAs (shRNAs) by RNA polymerase III. RNAi is a relatively new technology compared to the antisense system, demonstrating better potency, specificity and efficiency. Unfortunately, it has not yet reached the stage of entering clinical trials. Retroviral delivery of siRNAs targeting K-ras inhibited the growth of pancreatic cancer cells both in vitro and in vivo [16]. Inhibition of the mutant K-ras^{v12} was shown while other ras isoforms were unaffected,

Fig. 1
Mechanisms of RNA-mediated gene silencing. MicroRNAs (miRNAs) are transcribed from host DNA by polymerase II (Pol II), then processed by the RNAse III enzyme Drosha and its cofactor Pasha into 60–110 nucleotide pre-miRNA hairpins in the nucleus. Small hairpin RNAs (shRNAs) are transcribed by polymerase III (Pol III). These RNA intermediates are exported to the cytoplasm by Exportin 5 and its GTP-binding cofactor Ran. These are processed by the RNAse III Dicer into transient miRNA or small interfering RNA (siRNA) duplexes (~22 nucleotides) which subsequently bind to the double-stranded RNA binding protein R2D2 forming the RISC (RNA-induced silencing complex). Unwinding of the duplex is likely to involve an ATP-dependent RNA helicase. Only a single strand of RNA, called the guide strain, remains incorporated in the RISC, selected by the Argonaute protein Ago2, an endonucleolytic component of the RISC. The fate of the target mRNA depends on the degree of complementarity with the guide strand. miRNAs, shRNAs and siRNAs can be introduced exogenously in gene therapy.



demonstrating the extraordinary specificity of siRNA as the wild-type and mutant ras differs only in a single codon. By combining the oncolytic and gene-carrying capabilities of adenovirus, a replication-selective oncolytic virus (ONYX-411 or dl922-947 – see below) carrying K-ras^{v12} siRNA was almost twice as effective in causing pancreatic tumor growth reduction in vivo than using ONYX-411 alone [17].

3.1.2 Dominant-Negative Mutant

Dominant-negative mutants are gene products that affect the function of the “normal” products by blocking the elements by which they interact. One example is the N116Y, derived

from the v-H-ras oncogene by substituting asparagine with tyrosine at codon 116, the GTP-binding domain. N116Y prevents the activation of oncogenic ras protein with which it competes for a guanine nucleotide exchange factor. Delivery by an adenoviral vector effectively reduced the number of hepatic metastases following intrasplenic injection of the PCI-43 pancreatic cancer cells in mice [18].

3.2 Phosphatidylinositol-3-Kinase/Akt Pathway

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway plays a role in cell proliferation, survival and resistance to apoptosis. Akt2 (also known as protein kinase B-β) is amplified in 20% of pancreatic cancers and its suppression by antisense RNA reduced growth and tumorigenicity [19]. Upon activation by EGFR or ras, PI3K activates Akt which in turn has multiple downstream targets. Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) dephosphorylates the 3'OH group phosphorylated by PI3K, acting as a tumor suppressor.

RASN17 is a dominant-negative mutant of ras that inhibits PI3K/Akt pathway upstream of PI3K, whilst AAA-AKT is a dominant-negative mutant of Akt. Overexpression of these inhibitors induced apoptosis and abolished anchorage-independent growth of pancreatic cancer cells. Adenoviral vector carrying RASN17 also produced significant anti-tumor effect in vivo [20].

3.3 Gastrin-Cholecystokinin Receptor Pathway

Gastrin is a peptide hormone secreted by G cells of the gastric antrum and duodenum, and it can act as a growth factor for gastric, colonic and pancreatic cancers. Cholecystokinin-B (CCK-B) receptor, gastrin precursors, and the fully-processed amidated gastrin are expressed in 95, 55–91 and 23% of pancreatic cancers respectively, but not in the normal pancreas. Both CCK-B and its splice variant CCK-C receptors bind to CCK and gastrin. BxPC-3 pancreatic cancer cells transfected with the antisense DNA for CCK-C receptor showed a 65% decrease in cell numbers compared to control. When performed in vivo, tumors of treated nude mice were 75% smaller in volume and 83% reduced in weight [21].

3.4 Insulin-Like Growth Factor and Focal Adhesion Kinase

Insulin-like growth factor (IGF) and its receptors have been extensively studied in cancers of the colon, breast and prostate. In particular IGF-1 receptor (IGF-1R), a transmembrane receptor tyrosine kinase, has anti-apoptotic and growth promoting effects via multiple signaling pathways. IGF-1R is overexpressed in 64% of pancreatic cancer cells. Adenoviral vectors carrying either the IGF-1R dominant negative inhibitor or shRNA for IGF-1R can inhibit pancreatic cancer growth both in vitro and in vivo, together with increased sensitivity to chemotherapy and radiation-induced apoptosis [22].

Focal adhesion kinase (FAK), expressed in 48–75% of pancreatic cancers, is a nonreceptor cytoplasmic tyrosine kinase involved in the regulation of cellular signaling, migration, apoptosis and cell cycle progression. It is also associated with invasive potential. Gene silencing

with RNAi promoted anoikis and inhibited metastasis of human pancreatic cancer in an animal model, as well as enhancing gemcitabine chemosensitivity [23].

3.5 Smad4 and TGF-β

Mutation or deletion of the Smad4 (or MADH4 – mothers against decapentaplegic homologue 4) gene, originally designated as the tumor suppressor gene DPC4 (deleted in pancreatic carcinoma, locus 4) on chromosome 18q, occurs in 55% of pancreatic cancers. It is a member of the Smad family of transcription factors, and Smad4 inactivation potentiates tumor growth, angiogenesis and invasion and is associated with poor prognosis. The formation of a heteromeric complex between TGF-β ligand and TGF-β type I and type II receptors (TβRI and TβRII) leads to phosphorylation of cytoplasmic Smad2 and Smad3, which in turn form heteromeric complexes with Smad4. Restoration of the Smad4 gene using an adenoviral vector showed inhibition of pancreatic tumor growth in mice [24]. Suppression of tumor growth was mediated in part by the down-regulation of vascular endothelial growth factor (VEGF) and expression of gelatinases (involved in tumor growth, invasion, angiogenesis and promotion).

TGF-β plays a complex role as it is tumor suppressive in epithelial cells, but can also promote invasion and metastasis during the later stages of carcinoma progression. Mutations of TβRI and TβRII are found in 1% and 4% of pancreatic cancers respectively. Knockdown of Smad4 resulted in TGF-β-induced cell cycle arrest and migration but not in TGF-β-induced epithelial-mesenchymal transition, which makes cells more migratory and invasive. The antisense oligonucleotide specific for human TGF-β2 mRNA, AP 12009, was initially tested for use in high-grade glioma. It significantly reduced TGF-β2 secretion in human pancreatic cancer cell lines, decreased proliferation, blocked tumor migration, and reversed TGF-β2-mediated immune suppression [25]. Ongoing phase I/II studies for the treatment of pancreatic carcinoma, malignant melanoma and colorectal carcinoma have been reported to show promising results, with one advanced pancreatic cancer patient still alive 72 weeks after having experienced a complete response [26].

3.6 Hedgehog Signaling

Hedgehog (Hh) signaling specifies the pattern and structure of many tissues during embryonic development. There are three mammalian Hh proteins, namely Sonic (Shh), Indian (Ihh) and Desert Hh (Dhh) respectively. Activation of the Hh pathway is controlled by two transmembrane proteins, the tumor suppressor Patched (Ptc) or the oncogenic Smoothened (Smo). Shh is expressed in 70% of human pancreatic adenocarcinomas. Ihh expression is increased 35-fold in pancreatic cancer compared to normal tissues. Mechanisms of tumorigenesis include its effects on the cell cycle regulators cyclin D1 and p21, protection from apoptosis via the PI3K/Akt signaling and stabilization of Bcl-2 and Bcl-xL (antiapoptotic proteins found in 23 and 90% of invasive ductal adenocarcinomas of the pancreas, respectively), as well as its collaboration with activated K-ras.

GLI-1 is a transcription factor that mediates the Shh pathway. Its inhibition by a synthetic miRNA was reported to suppress proliferation and induce apoptosis in MIAPaCa-2 cells [27].

3.7 Notch Signaling

Notch signaling is important in the development of organs, affecting tissue proliferation, differentiation and apoptosis. There are four known Notch genes in mammals that encode for heterodimeric transmembrane receptors. Its ligands are from two families of proteins known as "Delta" and "Jagged" respectively. Activation leads to proteolytic cleavage of the Notch receptors by γ-secretase, releasing the cytoplasmic domain which migrates to the nucleus and binds to transcription factors such as CSL (CBF-1 in mammals, suppressor of hairless in Drosophila and LAG-1 in Caenorhabditis elegans).

Notch signaling is a downstream event of ras, EGFR and TGF-α in pancreatic tumorigenesis. It also promotes tumor neovascularization. Down-regulation of Notch1 with siRNA resulted in inhibition of cell growth and induction of apoptosis in pancreatic cancer cells, with subsequent reduction in cell invasion in vitro by the inactivation of nuclear factor κB, VEGF and matrix metalloproteinase-9 [28,29]. Notch3 is found in around 70% of pancreatic cancers and is associated with a more aggressive tumor phenotype. Inhibition by siRNA downregulated Bcl-xL, while γ-secretase inhibitors (GSI and L-685,458) resulted in decreased proliferation of pancreatic cancer cells in vitro [30].

3.8 Muc1

MUC1 (mucin-1, CD227) is a polymorphic, glycosylated type I transmembrane protein expressed on glandular epithelium including the pancreas, breast, lung and gastrointestinal tract. It is overexpressed in 90% of pancreatic cancers and aberrantly glycosylated. It inhibits cell-cell and cell-stroma interactions and functions as a signal transducer in the progression of cancer, including tumor invasion and metastasis. Evidence suggests that circulating anti-MUC1-IgG antibodies is a favorable prognostic factor for pancreatic cancer. Downregulation of MUC1 expression in S2–013 human pancreatic cancer cell line by RNAi significantly decreased proliferation in vitro and in nude mice [31]. Implantation of these cells into the caecum or pancreas showed significant reduction of lymph node, pulmonary and peritoneal metastases. Similarly, siRNA towards MUC1 reduced cell proliferation and enhanced sensitivity to genotoxic drugs in MIAPaCa-2 and Capan-1 cells [32].

3.9 Telomerase

Telomerase is a reverse transcriptase that contains an RNA template used to synthesize telomeric repeats onto chromosomal ends. Activation of telomerase and its maintenance of telomeres play a role in immortalization of human cancer cells, as telomeres shrink after each cell division. Telomerase activity is found in 92–95% of pancreatic cancers, and is associated with increased potential of invasion and metastasis and poor prognosis. Upregulation of telomerase may also be responsible for the development of chemotherapy resistance. The large phase III TeloVac trial using the telomerase peptide vaccine GV1001 in patients with advanced pancreatic cancer is currently ongoing.

Adenovirus-mediated transduction of p53 gene has been shown to inhibit telomerase activity in human pancreatic cancer cell lines, independent of its effect on apoptosis, cell growth and cycle arrest [33]. Antisense to the RNA component of telomerase seemed to

increase susceptibility of PANC-1 cells to cisplatin [34]. Telomerase reverse transcriptase antisense oligonucleotide was found to inhibit the proliferation of BxPC-3 cells in vitro by decreasing telomerase activity and increasing apoptosis [35].

3.10 Oncogenes and tumor Suppressor Genes

3.10.1 P53

The tumor suppressor gene p53 on chromosome 17p is inactivated by mutation in 50–75% of pancreatic adenocarcinomas. It encodes a 53-kDa transcription factor that is upregulated and activated upon stress, such as viral infection and DNA damage (*Fig. 2*). It induces the expression of proteins responsible for DNA repair, apoptosis and G1 cell cycle arrest. It is normally maintained at a very low level by mdm2 (murine double minute; also called hdm2 in humans) which targets p53 for ubiquitin-mediated degradation. Stress or mitogenic signals increase the level of $p14^{ARF}$ (an alternate reading frame product of $p16^{INK4A}$) which in turn inhibits mdm2, leading to the stabilization and activation of p53. Recently it was discovered that the DNA damage-induced p53 response is dispensable for tumor suppression, but instead $p19^{ARF}$ (murine equivalent of human $p14^{ARF}$), induced by oncogenic disruption of the cell cycle, plays a crucial role.

The first gene therapy for the treatment of cancer was approved in China in 2004, where Gendicine, a replication-defective Ad5 expressing p53 is used for squamous cell carcinoma of the head and neck. In pancreatic cancer, transfer of p53 using a similar vector inhibited the growth of cancer cell lines in vitro and in a mouse xenograft model [36]. A retroviral p53-expressing vector inhibited the growth of primary as well as peritoneal deposits of BxPC-3 in mice [37]. Reintroduction of p53 using an adenoviral vector to cells previously treated with gemcitabine increased cytotoxicity both in vitro and in vivo, although this effect was not seen with cisplatin [38].

Thoc1/p84 (also known as hHpr1 or p84N5) is a protein that localizes in the subnuclear regions associated with RNA processing and binds to the retinoblastoma protein. A study reported that infection of pancreatic cancer with adenovirus encoding p53 and Thoc1/p84 inhibited growth in vitro and in vivo to a greater extent than treatment with either one alone [39].

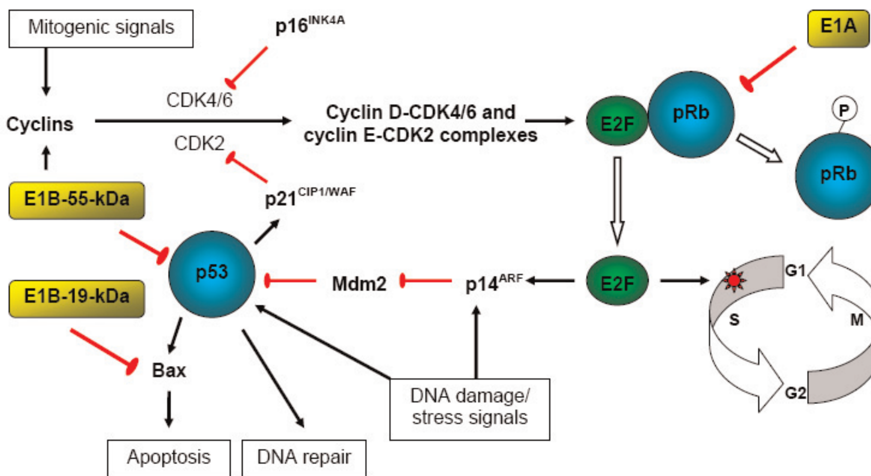
3.10.2 Retinoblastoma Protein, $P16^{ink4a}$ And $P21^{cip1/Waf1}$

The retinoblastoma protein (pRb) is a tumor suppressor that regulates the G1 to S checkpoint of the cell cycle (*Fig. 2*). In response to mitogenic signals, cyclin D is upregulated, which in turn activates cyclin D-dependent kinases 4 and 6 (CDK4/6). This leads to the phosphorylation of pRb, resulting in the release of transcription factors of the E2F family, to induce the expression of genes needed for DNA synthesis. pRb can also be phosphorylated by cyclin E-dependent kinase 2 (CDK2) in late G1 phase. $p16^{INK4A}$ (inhibitor of CDK4A) binds and inactivates CDK4/6, while $p21^{CIP1/WAF1}$ (CDK interacting protein 1/wild-type p53-associated fragment 1), induced by p53, inhibits CDK2.

The $p16^{INK4A}$ (multiple tumor suppressor 1 – MTS1) gene on chromosome 9 is deleted in 85% of pancreatic adenocarcinomas. p16-mediated cytotoxicity is tightly associated with the

Fig. 2

Mechanisms of cell cycle control. Retinoblastoma protein (pRb) is normally hypophosphorylated and binds to transcription factors of the E2F family, regulating the G1 to S checkpoint of the cell cycle. On encountering mitogenic signals, up-regulation of cyclins enables cyclin-dependent kinases (CDKs) to phosphorylate pRb, releasing E2F that subsequently induces the expression of genes needed for DNA synthesis and thus progression of cell cycle. E2F also leads to the transcription of $p14^{ARF}$ that inhibits mdm2 (murine double minute). Mdm2 normally exhibits ubiquitin ligase activity directed towards p53, resulting in its degradation. p53 is a transcription factor that is upregulated and activated by stress signals such as viral infection and DNA damage. It serves to activate the transcription of genes coding for proteins that induce apoptosis (Bax), cell cycle arrest ($p21^{CIP1/WAF}$ via its inhibition of CDK2) or DNA repair. Inactivation of pRb will thus lead to the activation of p53 to act as a safety mechanism. $p16^{INK4A}$ is a tumor suppressor that inactivates CDK4/6. The adenoviral early gene products serve to induce cell cycle progression and prevent apoptosis. E1B-19-kDa and E1B-55-kDa bind to and inhibit Bax and p53 respectively. E1B-55-kDa could also induce the expression of cyclin E. E1A binds to pRb to release E2F. E1A also promotes the acetylation of pRb by p300/CBP (cyclic adenosine monophosphate response element-binding binding protein), causing pRb to associate with mdm2 to inhibit p53.



presence of functional pRb. This is advantageous for gene therapy in pancreatic cancer as only 6% showed mutant pRb. Replication-defective adenoviruses containing p16 or p21 can significantly suppress the growth of pancreatic cancer cells in vitro [40,41].

3.10.3 P73

p73 on chromosome 1p is a proapoptotic gene of the p53-gene family observed in 45.6% of pancreatic adenocarcinomas. Overexpression of p73 is inversely linked to lymph node metastasis and tumor size. It can induce cell cycle arrest and apoptosis in a p53-analogous manner. An adenoviral vector carrying p73 was capable of killing several pancreatic cancer cell lines, including those that were completely resistant to p53-mediated apoptosis [42].

3.10.4 Cancer-Associated Sm-Like Protein

CaSm, also known as human Sm-like protein (LSM1), is overexpressed in the majority of pancreatic cancers and encodes a 133-amino-acid protein that contains two Sm motifs found in the common small nuclear RNA proteins and the LSm family of proteins. The LSm family of proteins are involved in mRNA decapping and degradation. Antisense CaSm RNA can alter the transformed phenotype of pancreatic cancer cells in vitro by reducing their ability to form large colonies in soft agar [43]. Using an adenoviral vector this antisense RNA inhibited in vivo tumor growth primarily by disrupting cell cycle progression, and the anti-tumor effect was further enhanced by gemcitabine [44].

3.10.5 Birc5 (Survivin)

Survivin is a 16.5-kDa protein encoded by a gene known as BIRC5 (baculoviral inhibitor of apoptosis repeat-containing 5) on chromosome 17. It functions as an antiapoptotic and cell cycle regulatory protein by blocking the common downstream effectors of both the intrinsic mitochondrial and the extrinsic membrane death receptor pathways. Survivin is expressed in more than 80% of pancreatic cancers and some premalignant lesions, but not in nonneoplastic pancreatic tissues, and is associated with poor clinical outcome.

When pancreatic cancer cell lines were irradiated, survivin mRNA expression was upregulated to induce increased radioresistance. siRNA treatment was found to improve radiosensitivity and induce apoptosis of pancreatic cancer cells [45,46]. Survivin antisense oligonucleotide was able to produce significant anti-tumor activity in a human xenograft tumor model in mice [47].

3.11 Angiogenesis

Solid tumor growth is dependent on angiogenesis, a process involving the vascular endothelial growth factor (VEGF) family of proteins and receptors. VEGF is a glycoprotein that promotes endothelial cell survival, mitogenesis, migration, differentiation, and vascular permeability. It is overexpressed in over 90% of pancreatic cancers and is associated with increased microvessel density, tumor progression and poor prognosis. The VEGF receptors, VEGFR-1 (fibromyalgia syndrome-like tyrosine kinase-1) and VEGFR-2 (fetal liver kinase-1 or kinase insert domain receptor) are also overexpressed in the vasculature of tumors that express VEGF. MIAPaCa-2 human pancreatic cancer cells transfected with anti-VEGF ribozyme demonstrated suppression of growth and metastatic potential [48].

3.11.1 Soluble Vegfr-2

Soluble forms of VEGFR-1 and VEGFR-2 could inhibit VEGF-dependent tumor angiogenesis, first by sequestering VEGF, and secondly by forming a heteromeric complex with their wild-type receptor, thus acting as a dominant negative. A recombinant adenovirus encoding a soluble form of VEGFR-2 showed a significant anti-tumor effect when injected intravenously into mice bearing pancreatic cancer cells [49]. A truncated dominant negative mutant of

VEGFR-2, when delivered via a replication-defective retrovirus, also resulted in tumor growth inhibition in vivo [50].

3.11.2 As-3

AS-3 is a VEGF antisense oligonucleotide that has been tested in mice with human pancreatic cancer cells implanted into the pancreas [51]. AS-3 normalized plasma VEGF level, decreased angiogenesis, reduced tumor growth and metastasis with subsequent improved survival. None of the treated animals developed ascites, suggesting a reduction in vascular permeability caused by decreased VEGF.

3.11.3 Natural Killer Transcript 4

Natural killer transcript 4 (NK4) is a synthetic competitive antagonist of hepatocyte growth factor (HGF) and an angiogenesis inhibitor. HGF binds to the c-Met-encoded receptor, which is overexpressed in 61–87% of pancreatic cancers. HGF is infrequently expressed by pancreatic cancer cells, but tumor-associated fibroblasts do produce HGF. HGF promotes growth and enhances cell motility and extracellular matrix breakdown, leading to invasion and metastasis of cancer cells. In pancreatic cancer mouse tumor model, NK4 suppressed tumor progression by inhibiting both angiogenesis and HGF-mediated invasion/metastasis [52].

An NK4-expressing adenoviral vector potently inhibited invasion of cancer cells in response to HGF [53]. Intrasplenic injection of this virus suppressed the number and growth of hepatic metastases, while intraperitoneal injection inhibited the development of AsPC-1 tumor in a mouse peritoneal dissemination model [54,55]. In mice with orthotopically implanted SUIT-2 tumors, peritumoral injection in combination with gemcitabine significantly reduced tumor volume, compared to either one alone [56]. Complete suppression of peritoneal dissemination and liver metastases was also noted, leading to improved survival of the animals.

3.12 Microrna

MicroRNAs (miRNAs) are small (~22 nucleotides), endogenous, noncoding RNA molecules that regulate gene expression important for developmental and physiologic processes, e.g. miR-375 in pancreatic islet cell development and the regulation of insulin secretion. The first miRNA, line-4, was described by Victor Ambros and colleagues in 1993 in the nematode *Caenorhabditis elegans*. Since then, approximately 450 miRNAs have been identified in mammalian cells but this could be up to 1000, although only a limited number has been defined functionally. They all have the common function of negatively regulating gene expression posttranscriptionally.

The first hint that miRNAs are involved in human cancer came from the pathogenesis of B-cell chronic lymphocytic lymphoma, where deletion at the 13q14 locus results in the loss of miR-15a and miR-16. It is now known that miRNAs can be both oncogenic and tumor suppressive, depending on their target mRNAs, and are therefore known as “oncomirs.” For example in C. elegans, the miRNA let7 suppresses the expression of let-60, the ras equivalent in humans. Expression profiling showed that 100 miRNA precursors are aberrantly expressed in

pancreatic cancer or desmoplasia [57]. Examples include the upregulation of the oncogenic miR-21, miR-155 and miR-106a, as well as the down-regulation of the tumor suppressive miR-34a and miR-127. miR-21 has been shown to target the tumor suppressor PTEN, tropomyosin 1 and programmed cell death 4 in various cancers. miR-155 represses the expression of tumor protein p53-induced nuclear protein 1 (TP53INP1, a proapoptotic stress-induced p53 gene target that is reduced in 86% of pancreatic cancers) [58]. Restoration of TP53INP1 can inhibit tumor growth in vivo. miR-106a negatively regulates pRb. miR-34a is encoded by a p53-responsive gene. miR-127 targets Bcl-6, an oncogenic protein that suppresses p53 expression and modulates DNA damage-induced apoptosis. Its level is reduced in the pancreatic cancer cell line CFPAC-1 [59].

miRNA-based cancer gene therapy has the theoretical advantage of targeting multiple targets that are controlled by an individual miRNA by virtue of its post-transcriptional modulation. Therapeutic strategies include the reconstitution of tumor-suppressive miRNAs and the knockdown of oncogenic miRNAs by anti-miRNA oligonucleotides. The latter can be given in its 2'-O-methyl-modified (or "antagomirs" when delivered in the form of liposomal complex) or locked-nucleic-acid form that increases stability, efficacy and affinity to target RNAs. Alternatively one could use coding vectors instead of oligonucleotides. Studies of these treatment approaches have been limited in pancreatic cancer but promising in others such as breast cancer and glioma.

4. Gene-Directed Prodrug Activation Therapy

Also known as suicide gene therapy, it involves the delivery of a gene to the tumor that will lead to the expression of an enzyme. A prodrug is subsequently administered that is activated selectively by this enzyme.

4.1 Herpes Simplex Virus Thymidine Kinase/Ganciclovir

The herpes simplex virus thymidine kinase (HSV-tk)/ganciclovir approach is the most well-known example. HSV-tk is able to monophosphorylate the guanosine analogue ganciclovir, which is subsequently converted by cellular guanylate kinases to the triphosphorylated forms, blocking DNA synthesis and inducing cell death. It also produces the so-called "bystander effect", whereby HSV-tk-transduced tumor cells are toxic to nearby neighboring unmodified tumor cells by the diffusion of the cytotoxin. Adenoviral delivery of this gene together with ganciclovir has been shown to be effective in nude mice bearing human pancreatic cancer cells [60]. Retroviral transduction of HSV-tk has limited efficacy [61]. A combination of these two appeared to be more effective in tumor reduction compared to either one alone [62]. Liposome-mediated transfer of HSV-tk caused tumor regression in nude mice with peritoneal dissemination of the human pancreatic cancer cells PSN-1 [7].

4.2 Cytosine Deaminase

Cytosine deaminase (CD) is a bacterial enzyme that converts the prodrug 5-fluorocytosine (5-FC) into the cytotoxic and radiosensitising agent 5-fluorouracil (5-FU). Retrovirally delivered

CD gene (linked to the oncogene ErbB2 promoter) with 5-FC administration, enhanced cell killing of ErbB2-positive pancreatic cancer cells [63]. Both in vitro and in vivo growth inhibition have been demonstrated using adenoviral vector [64]. A phase I trial of this virus, injected intratumorally under ultrasound guidance, is being tested with chemoradiotherapy in patients with nonmetastatic pancreatic adenocarcinoma.

FCY1 and FUR1 are genes that encode CD and uracil phosphoribosyltransferase (UPRT) respectively. They are derived from the yeast *Saccharomyces cerevisiae*. As some cells are relatively resistant to 5-FU, UPRT has an additional advantage because it catalyses the conversion of 5-FU into the toxic metabolite 5-fluorouridine-5'-monophosphate. However, an in vitro study of these genes transfected using plasmid vectors into human pancreatic cancer cell lines was disappointing [65]. FCY1 alone was ineffective, and in combination with FUR1 only some showed increased sensitivity to 5-FC. In another study of an E1B-55-kDa-deleted adenovirus carrying the UPRT gene together with 5-FU, mice with peritoneal dissemination of AsPC-1 showed dramatic improvement without toxicity to normal tissues [66].

4.3 Nitroreductase

The Escherichia coli enzyme nitroreductase (NTR) is able to reduce the prodrug CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide) to 2- and 4-hydroxylamino derivatives; the latter then reacts with cellular thioesters to generate a potent alkylating agent capable of cross-linking DNA. In contrast to HSV-tk/ganciclovir and CD that work by inhibiting DNA synthesis, NTR/CB1954 is also toxic to nonreplicating tumor cells. Introduction of this gene using a retroviral vector resulted in increased sensitivity to CB1954 (up to 500-fold) in pancreatic cancer cell lines in vitro, with the associated "bystander effect" [67]. In vivo studies of human pancreatic cancer xenografts in nude mice with either retroviral or adenoviral vector have shown promising results, demonstrating significant improvements in MS [68,69]. No clinical trial has yet been done in patients with pancreatic cancer.

5. Oncolytic Virus

In 1912 de Pace discovered that a patient with uterine cervical carcinoma experienced tumor regression after an attenuated rabies vaccination, suggesting a role for virus in cancer therapy. Viruses have the intrinsic ability to replicate in and lyse cells. In addition to direct lysis, viruses can kill cells by their heavy biochemical demand, the expression of toxic proteins, and the induction of both inflammatory cytokines and T cell-mediated immunity. The term "replication-selective oncolytic viruses" applies to viruses that are able to replicate specifically in and destroy tumor cells, and this property is either genetically engineered (e.g. adenovirus, HSV, vaccinia virus and polio virus) or inherent (e.g. reovirus, Newcastle disease virus, vesicular stomatitis virus and autonomous parvovirus). The former can be achieved either by deletion of viral genes that are critical for viral replication in normal cells but are dispensable in neoplastic cells, or by placement of tumor-specific promoters upstream of these genes.

Clinical trials of several naturally-occurring oncolytic viruses were started back in the 1950s. Using viruses in a monotherapy setting, however, is not enough to cause complete tumor regression. Treatment efficacy could be improved by combining with conventional therapies, inserting therapeutic genes, and improving the viral oncolytic potency and specificity.

5.1 Adenovirus

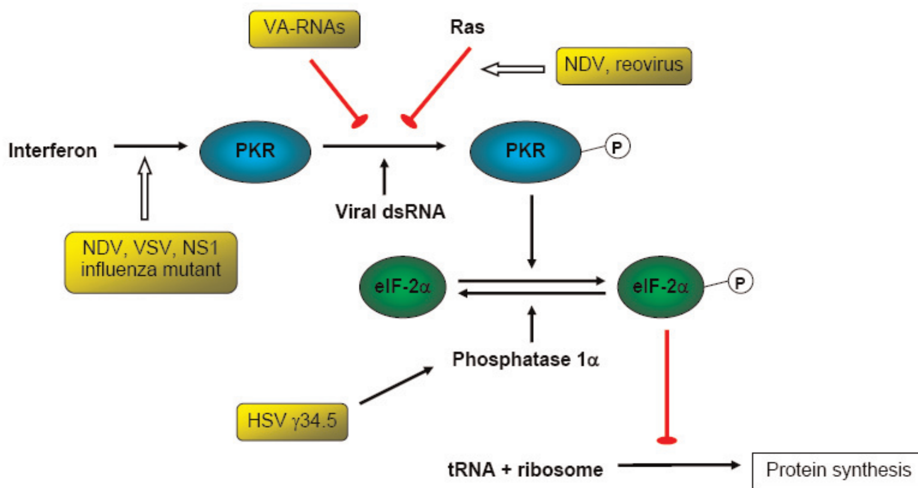
The adenoviral genome is of particular interest in cancer gene therapy because genetic modifications could significantly enhance its oncolytic potency and tumor selectivity. When adenovirus infects a cell, its E1A gene product binds to pRb, enabling the cell cycle to proceed so that viral DNA replication could occur (*Fig. 2*). Because replicating cancer cells are often found in the S phase of the cell cycle, E1A-CR2-deleted Ad5 mutant (ONYX-411 or dl922–947) can selectively replicate in and destroy replicating cancer cells, but not normal resting cells. The E1B-19-kDa protein is a homologue of the cellular antiapoptotic protein Bcl-2. It prolongs cell survival by inhibition of the extrinsic death receptor and the intrinsic mitochondrial apoptotic signaling pathways. E1B-19-kDa interacts with and inhibits the p53-inducible and death-promoting Bax protein (*Fig. 2*). It can also prevent Fas-mediated apoptosis. E1B-19-kDa-deleted adenovirus has demonstrated tumor necrosis factor (TNF)-enhanced cancer selectivity, due to genetic blocks in apoptotic pathways in cancer cells, in addition to enhanced viral spread and anti-tumor potency. The adenoviral VA-RNAs are RNA polymerase III transcripts that are obligatory for efficient translation of viral and cellular mRNAs by blocking the double-stranded RNA-activated protein kinase (PKR) (*Fig. 3*). Because Epstein-Barr virus (EBV) also expresses similar RNAs (EBV-encoded RNAs 1 and 2), they can complement VA-RNA-1-deleted adenovirus (dl331) enabling it to selectively replicate in EBV-associated tumors such as Burkitt's lymphoma and nasopharyngeal carcinoma. Finally the adenovirus death protein (ADP), responsible for efficient lysis and release of viral progenies from infected cells, can be overexpressed in mutant virus leading to increased cell lysis and viral spread.

ONYX-015 (dl1520) is a replication-selective Ad5 with E1B-55-kDa gene deletion. The E1B-55-kDa protein is able to bind and inactivate p53, an essential step for effective viral replication (*Fig. 2*). It was thought that as most tumors have lost the function of the p53 pathway, deletion of E1B-55-kDa gene would enable the virus to selectively replicate in cancer cells but not in normal cells. Promising laboratory results have led ONYX-015 to be the first replication-selective oncolytic virus to enter clinical trials and a similar virus, H101 was approved in China in 2006 as the world's first oncolytic virus for head and neck cancer therapy. For pancreatic cancer, two clinical trials of ONYX-015 failed to show any objective response. In a phase I trial, ONYX-015 was administered via CT-guided (22 patients) or intraoperative injection (1 patient) into pancreatic primary tumors every 4 weeks until tumor progression [70]. Six patients showed 25–49% tumor regression, 11 were stable, and 5 showed tumor progression. A phase I/II study of 21 patients was done to evaluate the use of endoscopic ultrasound-guided intratumoral injection of advanced pancreatic carcinomas with ONYX-015 and then in combination with systemic gemcitabine [71]. Two had partial progression, 2 had minor response, 6 had stable disease, and 11 progressed or had to go off the study because of treatment toxicity. Viral replication was not detectable on fine needle biopsy of the tumors, unlike other trials for head and neck cancers, liver metastases of colorectal carcinoma, and ovarian cancer.

It is important to note that the interaction between E1B-55-kDa and p53 is more complex than originally thought, because ONYX-015 could replicate in some tumor cells that retain the wild-type p53. Tumor selectivity of ONYX-015 has been shown to be determined not by p53, but by the export of late viral RNA, a function requiring E1B-55-kDa in normal but not in tumor cells [72]. Recent evidence also suggests that E1B-55-kDa could regulate the cell cycle by

Fig. 3

Role of the double-stranded RNA-activated protein kinase (PKR) pathway. Interferons (IFNs) are produced by infected cells and result in an intracellular cascade leading to the up-regulation of PKR. The eukaryotic initiation factor-2 (eIF-2) is a GTP-binding protein that has three subunits, namely α, β and γ. On binding to viral double-stranded RNA (dsRNA), PKR autophosphorylates thus activating its kinase activity, which in turn phosphorylates eIF-2α. Phosphorylated eIF-2α sequesters eIF-2B, a guanine nucleotide exchange factor. Without eIF-2B, the guanosine diphosphate (GDP) bound to eIF-2 cannot be exchanged for GTP. As a result eIF-2 is unable to bring the initiator transfer RNA (tRNA) to the 40S ribosomal subunits, thereby shutting off protein synthesis. This IFN/PKR pathway therefore serves as a natural host defense system against viral infection. To overcome this, herpes simplex virus (HSV) expresses γ34.5 which interacts with cellular phosphatase 1α to dephosphorylate eIF-2α, leading to synthesis of protein needed for viral replication. Inactivated-IFN and activated-ras pathways are frequently found in tumor cells (the latter could inhibit PKR). A few naturally found viruses can replicate selectively in these cancer cells, including the Newcastle disease virus (NDV), reovirus and vesicular stomatitis virus (VSV). Influenza virus deleted for gene encoding for NS1 can selectively replicate in tumors with a defective IFN pathway. Adenovirus can produce virus-associated (VA)-RNAs that inhibit PKR.



inducing cyclin E, whereby cyclin E overexpression in cancer cells would allow for the efficient replication of ONYX-015 [73].

5.2 Herpes Simplex Virus

Herpes simplex virus (HSV) is a large, enveloped, double-stranded DNA virus with a genome size of approximately 152kb. There are two serotypes of HSV, namely HSV-1 and HSV-2. HSV-1 with deletion of its thymidine kinase gene (HSV-tk) became the first genetically engineered replication-selective oncolytic virus to be tested in the laboratory in 1991. HSV-tk is normally

needed for nucleic acid metabolism; therefore mutant virus is dependent on endogenous levels of this enzyme, which is found in high levels in replicating cells.

A number of HSV mutants have been tested on pancreatic cancer cells. G207 is a replication-selective mutant of HSV-1 with deletions at both γ34.5 locus and disruption of its unique long (UL) 39 gene. γ34.5 prevents the shut-off of host protein synthesis in infected cells by interacting with cellular phosphatase 1α to dephosphorylate eIF-2α, leading to the production of more progeny viruses from infected cells (➲ *Fig. 3*). UL39 normally encodes for the infected cell protein (ICP) 6, the large subunit of ribonucleotide reductase required in the biosynthesis of DNA. Given their functions, γ34.5- and UL39-deleted mutants are unable to replicate in normal cells but they can do so in actively dividing tumor cells. NV1020 is derived from HSV-1 and contains deletions in the endogenous HSV-tk and in one of the two γ34.5 genes. NV1020, however, contains an exogenous copy of the HSV-tk gene derived from HSV-2, thus maintaining its sensitivity to aciclovir and ganciclovir, which would otherwise be lost with the disrupted HSV-tk gene. Both G207 and NV1020 were equally effective in lysing human pancreatic cancer cell lines in vitro [74]. In Hs766T flank tumors in athymic mice, tumor eradication was achieved in 25% of the animals with G207 and 40% with NV1020 respectively.

OncoVEX$^{GM\text{-}CSF}$ is a recombinant HSV-1 with γ34.5 and ICP47 gene deletions, together with the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene inserted. ICP47 normally binds to TAP (transporter associated with antigen processing) to prevent the delivery of peptides into the endoplasmic reticulum, where they bind to major histocompatability complex (MHC) class I molecules. The genetic alterations of OncoVEX$^{GM\text{-}CSF}$ mean that it could replicate selectively in tumor cells while boosting the anti-tumor immune response. It will be tested in a phase I trial in patients with unresectable pancreatic cancer.

The hrR3 (UL39 deleted, HSV-tk conserved) virus combined both oncolytic viral and suicide gene therapies. When tested on nude mice bearing peritoneal dissemination of the human pancreatic cancer cells SW1990, long term survival was seen in 70% of mice receiving hrR3 and ganciclovir, 40% receiving hrR3 alone, and none of the untreated animals [75]. Interestingly in a study of hrR3 and R3616 (γ34.5 deleted), there was more cytotoxicity to pancreatic cancer cells in vitro when each of the virus was given in combination with gemcitabine, although viral replication was inhibited [76]. In a murine model with peritoneal dissemination, R3616 with gemcitabine had a greater effect than R3616 alone, while hrR3 with gemcitabine had a weaker effect than hrR3 alone. This demonstrates the complex interactions between viruses, cells and cytotoxic drugs.

The unique short (US) 3 gene encodes a serine/threonine protein kinase that protects cells from apoptosis. US3 locus-deficient HSV-2 mutant (L1BR1) showed significantly better anti-tumor effect in vivo compared to hrR3 and R3616 [77]. It also showed the lowest replication capacity in normal human hepatocytes, and enhanced tumor apoptosis in vitro in combination with cisplatin and 5-FU.

FusOn-H2 is a mutant HSV-2 with deletion of the protein kinase domain of the viral ICP10 gene. This domain is normally required for HSV-2 replication, where it binds and phosphorylates ras, leading to activation of the ras/MAPK/ERK pathway, as well as the expression and stabilization of the transcription factor c-Fos. FusOn-H2 can therefore replicate selectively in pancreatic cancer cells in which the majority have an activated ras signaling pathway. It has demonstrated impressive in vivo results both at tumor eradication and prevention of local metastasis in mice [78].

The agent d120.surv is a recombinant HSV-1 that contains a survivin promoter driving the expression of ICP4, a major transactivating factor for viral genes. This makes replication of the virus restricted to survivin-expressing cancer cells. In vitro cytotoxicity was significantly higher in AsPC-1 (high survivin expression) compared to PANC-1 (low survivin expression) [79].

5.3 Reovirus

Reovirus is a nonenveloped, double-stranded RNA virus that normally causes subclinical infection in humans. Replication of reovirus requires an activated ras signaling pathway, as the phosphorylation of PKR is blocked in these cells (*Fig. 3*). In a human pancreatic cancer murine xenograft model, local intratumoral injection inhibited tumor growth, while a systemic anti-tumor effect was also observed in a bilateral model [80]. Viral replication was detected in the tumor but not in the surrounding normal tissue.

6. Immunotherapy

In the first attempt at cancer immunotherapy, William Coley in the 1890s injected bacterial components into the tumors of his patients, activating the immune system which contributed to tumor cell rejection. In 1967, Lindenmann and Klein discovered that vaccination of mice with influenza virus-infected tumor cells showed anti-tumor response to noninfected parental cells, suggesting that the immunogenicity of host cell components was greatly increased by incorporation into the makeup of the virus. Compelling evidence now suggests that the immune system plays an important role in the control of malignancy. Immunotherapy can be divided into passive or active. Passive immunotherapy includes the use of anti-tumor agents that have been generated in vitro, such as the use of antibodies or effector cells, whereas active immunotherapy aims to stimulate an anti-tumor response in vivo by means of vaccination. The failure of cetuximab (an anti-EGFR antibody) and bevacizumab (an anti-VEGF antibody) in recent phase III trials of pancreatic cancer patients has been discouraging. Nonetheless antibodies (either inhibitory, immunotoxin or radioconjugate) against other targets, as well as active immunotherapy, continue to hold promise in pancreatic cancer treatment (see chapter on "Vaccine Therapy and Immunotherapy"). This section will focus on the genetic approaches in pancreatic cancer immunotherapy.

6.1 TNF-α

TNF-α is a multifunctional cytokine that has anti-tumor ability. TNFerade Biologic (TNFerade) is a replication-deficient adenovirus carrying the gene for human TNF-α, regulated by a radiation-inducible promoter Early Growth Response (Egr-1). The latter would ensure maximal gene expression when infected tissue is irradiated. TNFerade was effective in combination with radiation in a number of human xenograft models, including glioma, prostate, esophageal and radiation-resistant laryngeal cancers. The multicenter phase II/III Pancreatic Cancer Clinical Trial with TNFerade is currently ongoing and involves patients with locally advanced pancreatic cancer. Patients were given radiotherapy and 5-FU with or without CT-guided

transabdominal injection of TNFerade. Preliminary data of 51 patients revealed that the 1-year survival increased from 28 to 70.5% with the addition of TNFerade, with MS of 335 and 515 days respectively [81].

6.2 Gm-csf

GM-CSF is one of a few cytokines that have shown significant anti-tumor effect in vivo. It is an important growth factor for granulocytes and monocytes, and has a crucial role in the growth and differentiation of dendritic cells (DCs). Retroviral transduction of the GM-CSF gene inhibited in vivo growth of human pancreatic cancer cells, and is associated with increased survival of nude mice even in a mature T cell-deficient condition [82].

Jaffee et al. conducted a phase I study using allogeneic GM-CSF-secreting whole-cell tumor vaccine (transfected using a plasmid vector) for pancreatic cancer [83]. This is based on the concept that the localization of GM-CSF in the implanted tumor environment, together with the shared tumor antigen expressed by the primary cancer, would effectively induce an anti-tumor immune response. Three of the 8 patients who received the higher vaccine dose developed post-vaccination delayed-type hypersensitivity responses associated with increased disease-free survival time. In a recently completed phase II study of 60 patients with resected pancreatic adenocarcinoma, patients who received vaccine cells together with 5-FU and radiotherapy have a reported MS of 26 months, with a 1- and 2-year survival of 88 and 76% respectively [84].

6.3 Interleukins

Murine pancreatic cancer cells genetically altered to express interleukin (IL)-2 could induce an anti-tumor immune response against established parental tumors, resulting in tumor regression and long-lasting immunity [85]. Human pancreatic tumors transduced retrovirally with IL-2 or IL-4 demonstrated inhibited growth in vivo [86]. In mice bearing the PANC-1 human pancreatic cells, the combination of an E1B-55-kDa-deleted adenovirus (AxE1AdB) and an E1-deleted adenoviral vector carrying the IL-2 gene (AxCAhIL2) resulted in 110 times more IL-2 production than with AxCAhIL2 alone [87]. Complete regression of established tumors was observed. In this case AxE1AdB acted as a helper virus, augmenting the effect of the replication-deficient AxCAhIL2. In a phase I trial using a nonreplicative adenovirus encoding IL-12 for patients with advanced digestive tumors, seven had pancreatic cancer. Overall the treatment was well-tolerated but had only mild anti-tumor effects [88].

6.4 Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA) glycoprotein is expressed at a low level in normal colonic epithelium but is overexpressed in many malignant diseases, including those of the colon, rectum, stomach and pancreas (85–90%). Its serum level is sometimes used as a marker for the diagnosis of pancreatic cancer, with a sensitivity of 25–40% and a specificity of 70–90%.

TRICOM is a poxvirus-based vaccine containing tumor-associated antigens in combination with a TRIaid of COstimulatory Molecules (B7–1, Intercellular Adhesion Molecule-1,

Leucocyte Function-Associated Antigen-3). The aim is to enhance tumor-specific T cell response. Marshall et al. conducted a phase I study of 58 patients using the replication-defective fowlpox recombinant (rF)-CEA(6D)-TRICOM and recombinant vaccinia (rV)-CEA(6D)-TRICOM vaccines, with or without GM-CSF [89]. CEA(6D) contains a modification of the HLA-A2 CEA CAP-1 epitope to enhance its immunogenicity. Only one patient had pancreatic cancer – she was previously diagnosed, had had radiotherapy with chemosensitization, followed by ALVAC-CEA (replication-defective avipox containing the CEA gene) vaccine because of disease progression [90]. She remained stable for 6 months after ALVAC-CEA but progressed with rising CA 19-9 and pain, unresponsive to chemotherapy. After two vaccinations with (rF)-CEA(6D)-TRICOM both CA 19-9, pain decreased for over a year. Enhanced CEA-specific T cell responses were noted in the majority of patients. In spite of this a phase III trial of 255 patients using PANVAC-VF (vaccine consisted of recombinant vaccinia and fowlpox viruses co-expressing CEA, MUC-1 and TRICOM) failed to improve overall survival compared to palliative chemotherapy or best supportive care [91].

DCs present tumor antigens in association with the MHC class I and II molecules, resulting in the expression and upregulation of cytokines and co-stimulatory molecules, which in turn initiate antigen-specific T cell responses. In one study, autologous, monocyte-derived DCs loaded with mRNA of CEA were given to 3 patients with resected pancreatic cancer for 6 months, following neoadjuvant chemoradiotherapy [92]. All patients remained disease-free for more than 30 months from diagnosis.

6.5 Mesothelin

Mesothelin is a 40-kDa protein present on normal mesothelial cells of the pericardium, pleura and peritoneum, but is overexpressed in mesotheliomas, and pancreatic and ovarian cancers. It is detected in 90–100% of pancreatic adenocarcinomas. Mesothelin DNA vaccine in combination with the anti-glucocorticoid-induced TNF receptor antibody (anti-GITR) has been tested in mice with syngeneic mesothelin-expressing pancreatic cancer, with impressive results [93]. Half of the animals treated with mesothelin were tumor-free 25 days after tumor injection compared to 0% of nontreated mice. This increased to 94% with the addition of anti-GITR. The agonist anti-GITR served to enhance the T cell-mediated response of the vaccine.

6.6 Survivin

Vaccination with survivin DNA prolonged survival in murine pancreatic and lymphoma tumor models, associated with slower tumor growth and increased lymphocyte infiltration [94].

6.7 Muc1

In a phase I/II trial, 10 patients with advanced breast, pancreatic or papillary cancer were vaccinated with autologous DCs transfected liposomally with MUC1 cDNA [95]. Four patients showed a 2- to 10- fold increase in the frequency of mucin-specific IFN-γ-secreting CD8 + T cells, suggesting an immune response.

Key Research Points

- Gene therapy aims to express, restore or inhibit a particular gene of interest.
- Each gene delivery system (viral or nonviral) has its individual benefits and shortcomings.
- Viral vectors have high transduction efficiencies and are the most commonly used.
- Mesenchymal stem cells are promising delivery vectors but need further study.
- Multiple genetic and molecular aberrations have been targeted, such as signaling pathways and oncogenes.
- RNA-directed strategies, particularly RNA interference, have demonstrated impressive targeting specificity.
- Approaches such as dominant-negative mutants, gene restoration, gene-directed prodrug activation therapy, oncolytic viruses, inhibition of angiogenesis and immunotherapy have demonstrated variable laboratory and clinical outcomes.
- Successes in animal models are not always reflected in humans.

Future Scientific Directions

- Continuing research on the molecular and genetic changes in pancreatic cancer as targets for gene therapy, e.g. miRNAs
- Refinement of gene delivery systems, especially viral vectors and mesenchymal stem cells
- Harnessing the powerful anti-tumor capacity of the immune system
- Development of multi-targeted and systemic therapeutic strategies for better treatment efficacy
- Better diagnostic techniques – a role for "preventative" gene therapy?

Clinical Implications

- Gene therapy is a promising therapeutic approach for pancreatic cancer although still in its early stage.
- Good results have been demonstrated in animal models, but results from a limited number of clinical trials were less encouraging.
- Multimodality treatments, particularly with conventional therapies, are still the way forward, resulting in better efficacy, reduced toxicity and cross-resistance.
- Current gene therapeutic protocols are mainly delivered intratumorally, limiting their use in many pancreatic cancer patients.
- Systemic delivery to treat metastatic or occult disease is feasible, but remains to be fully explored.

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