Gene therapy and nasopharyngeal carcinoma*

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Summary

In 2003, a non-replicating adenoviral gene therapy product received the world's first government licence for the treatment of head and neck cancer. Two years later approval was granted to a replication-selective adenovirus for the treatment of nasopharyngeal carcinoma in combination with chemotherapy.

This review introduces the reader to gene therapy as an emerging treatment modality, and outlines its application to the management of nasopharyngeal carcinoma by examining recent pre-clinical and clinical research.

Key words: gene therapy, nasopharyngeal carcinoma, head and neck cancer

Introduction

Nasopharyngeal carcinoma (NPC) has a unique geographical and ethnic predisposition with high incidences in South East Asia (up to 30 per 100,000), [1] North Africa, and the Arctic [2]. Outside endemic areas the tumour is rare, with an incidence of less than 1 per 100,000 [3]. Interestingly emigrants from endemic areas remain at high risk of developing NPC, which diminishes with successive generations; [4] suggesting both environmental and genetic aetiological influences. Three well-defined factors for NPC have now been established: genetic susceptibility, early exposure to chemical carcinogens (especially Southern Chinese salted fish), and latent Epstein-Barr virus (EBV) infection [5]. NPC is traditionally divided into three histological subtypes: Type I Keratinising (squamous cell carcinomas), Type II Non-keratinising and Type III Undifferentiated [6]. However, this classification has subsequently been simplified into grade 1 squamous cell carcinomas and grade 2 undifferentiated carcinomas [7]. This latter classification correlates well with endemic area patient origin and EBV status - showing strong association with grade 2 cancers [8]. NPC is generally radiosensitive, and consequently the primary treatment modality is radiotherapy. Five year survival of patients with T1/T2 lesions (Ho classification [9]) range from 75% to 90%, falling to 50%-75% for T3/4 lesions [10]. Intensity modulated radiotherapy (IMRT) has improved local control rates to more than 90% [11]; as well as reducing toxicity to vital surrounding structures, with resultant improved side effects and quality of life outcomes [12]. Chemotherapy is a radiosensitiser, however its role, and in particular the timing of its delivery in relation to radiotherapy, has not been comprehensively established. The most convincing evidence is for concurrent chemoradiotherapy [13], although some promising results have been shown for neo-adjuvant chemotherapy [14,15]. The role of surgery, other than in diagnosis through biopsy, is in salvage for local and regional failure. Novel therapies, including gene therapy, have yet to be established in the management of patients with recurrent, residual or metastatic disease following treatment with conventional modalities.

Gene therapy

Gene therapy has been defined as 'the deliberate introduction of genetic material into patient's cells in order to treat or prevent a disease [16]'. Gene therapy was initially applied to inherited diseases with single gene mutations (monogenic); such as severe combined immunodeficiency disease (SCID), cystic fibrosis and haemophilia. However, permanent corrective gene

expression in such illnesses has been largely unsuccessful. This, together with the development of leukaemia in children with SCID and the death of a patient with ornithine transcarbamylase deficiency following viral gene therapy, has highlighted the potential risks of such treatments to researchers [17]. With this in mind, new applications for gene therapy, such as cancer and cardiovascular disease, were considered; where transient gene delivery would be more likely to be achieved, and provide a safe and clinically significant effect [18].

Cancer is a genetic disease, with normal cells undergoing multiple mutations, as part of an 'oncoevolutionary process,' to transform into malignant cells. These changes can be acquired during life, through exposure to carcinogens, or inherited. Mutations in the cell behaviour regulating genes (proto-oncogenes and tumour suppressor genes) lead to self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, ability to evade apoptosis, sustained angiogenesis, ability to invade tissues and metastasise - the hallmarks of cancer [19]. All of these changes are potential targets for gene therapy and has resulted in cancer gene therapy research dominating the gene therapy research field; with two-thirds of clinical trials for cancer applications [20]. However success at clinical trial has been disappointing mainly due to poor efficiency of gene transfer rather than a paucity of potential therapeutic genes [21]. Strategies to overcome this have focused on modulating the host immune response to the virus, optimising existing viruses and discovering/creating new viruses, and identifying tumour-associated genes that can improve viral potency.

Gene therapy strategies

there are three main strategies that characterise the type of gene expressed in gene therapy: corrective, cytoreductive and immunomodulatory. Cancer is caused by mutations in oncogenes and tumour suppressor genes (TSG) resulting in uncontrolled cell growth. Corrective gene therapy attempts to block oncogene or replace TSG function, thereby returning cells to normal cell growth. In over 50% of all cancers p53 TSG mutations are implicated, and is thus considered to be a potential magic bullet for corrective cancer gene therapy [22]. In 2003, the world's first government licensed gene therapy product featured a recombinant adenovirus expressing p53 (Gendicine; SiBiono Schenzhen, China) [23]. Oncogenes can be over-expressed or amplified in cancer; and their action can be blocked therapeutically at either the transcriptional or translational level, through RNA interference and catalytic ribozymes respectively.

Cytoreductive gene therapy aims to directly or indirectly kill cancer cells, rather than correct the underlying genetic defect. This can be achieved by introducing a transgene that can convert an inactive prodrug to a potent anti-cancer agent, for example the cytosine deaminase gene and 5-fluorocytosine (5-FC), which is converted to the highly toxic 5-fluorouracil (5-FU) [24]. Other strategies include inhibiting angiogenesis, through transgene expression of angiogenesis inhibitors such as endostatin (E10A) [25], or inducing apoptosis, by expressing exogenous p53 [23].

The host immune response detects tumour associated antigens (TAA), which allows CD8+ cytotoxic T cells to induce tumour lysis. Cancer cells subvert this immuno-surveillance function by reducing MHC class I expression and producing immune-suppressive cytokines [26]. Gene therapy can reverse this tumour-induced immuno-tolerance by increasing TAA presentation, upregulating MHC I expression or breaking down the immune suppressive environment through transduction of immune modulatory genes [21]. This has been achieved by using cytokines as transgenes, with resultant enhanced antitumour efficacy; for example adenovirus expressing granulocyte macrophage colony-stimulating factor (GM-CSF) in head and neck cancer animal models [27]. Furthermore clearance of the viral vector by the host immune response can also be reduced by this approach [28].

Vectors for gene therapy

The greatest limitation to cancer gene therapy becoming an effective everyday clinical treatment modality is the accuracy and efficiency of gene transfer to the target cells [21]. The ideal vector should be efficient, safe and tumour selective; preferentially affecting cancer cells and sparing normal cells. Tumour selectivity can be achieved by administering the vector directly into the tumour, or through engineering the vector physically or genetically. The latter strategy is more attractive as this would allow for systemic administration, which would be able to treat inaccessible or multiple lesions. However, to reach its target the vector would have to overcome clearance of circulating virus by complement and reticulo-endothelial cell-based mechanisms, which leads to phagocytosis of viral particles by macrophages or Kupffer cells [29].

The two main groups of vectors used in cancer gene therapy research are viral and non-viral vectors. Approximately 70% of clinical trials and the majority of research to date has used viral vectors [18]. Non-viral vectors use either physical transfection or particle-mediated systems to introduce foreign DNA into the target cells. They are generally less immunogenic than viral vectors which allows for repeated administration [30]. Additionally they are cheaper to produce and can carry more DNA. However their major disadvantage is low transfection rates.

Viruses are obligate intracellular pathogens, which require host cell machinery to complete their life cycle. They have evolved over millions of years to be highly effective at infecting, replicating within and generally lysing host cells. Viruses have also developed mechanisms that allow them to subvert the host immune system and thereby increase their infectivity, such as expressing interferon γ decoy receptors. Viral vectors commonly induce mild flu-like illnesses, but have the potential for causing acute life-threatening toxicity. Some 'oncolytic' viruses can also exert an anti-cancer effect directly themselves by selectively replicating and destroying cancer cells. Apart from different viral species, a common classification of viral vectors is whether they have the ability to replicate. Non-replicating viruses were the first vector systems for the delivery of foreign genes and most research to date has utilised such viruses [31]. However, the efficacy of gene transfer of these viruses is poor and for successful cancer treatment every tumour cell would have to be infected.

In contrast, replicating viruses allow more cancer cells to be infected as their progeny can go on to infect more cells and destroy the host cell as a direct consequence of their replication [31]. Replication-selective viruses have the ability to replicate in tumour cells, but not normal cells. Some viruses exhibit inherent tumour selectivity, such as Reovirus and Newcastle virus whereas other viruses can be genetically modified to improve tumour selectivity, such as Adenovirus and vaccinia virus. This can be achieved by inserting tumour-specific promoters, such as prostate-specific antigen (PSA) or deleting genes that are needed for viral replication in normal cells but not tumour cells, such as thymidine kinase.

ADENOVIRUS

The vast majority of NPC gene therapy research, indeed most cancer gene therapy research, has used adenovirus mediated strategies [32]. Adenoviruses are non-enveloped DNA viruses; first isolated in 1953 from human adenoids. The most significant advantage of adenovirus over other viral vectors is high *in vivo* transfection efficiency [33]. Furthermore, adenovirus is able to deliver therapeutic genes to dividing and non-dividing cells, and can be manufactured to high titres [34]. Also the virus remains episomal and does not integrate into the host cell genome, reducing the risk of insertional oncogenesis. However, adenoviruses are highly immunogenic and, hence, the risk of toxicity and even mortality with their clinical application is a concern [35]. Fortunately newer engineered viruses containing minimal viral genes ('gutless') exhibit far less toxicity. Adenoviruses have undergone extensive clinical trials in head and neck cancer, with Gendicine (p53 expressing replication deficient adenovirus) [36,37] and ONYX-015/H101 (E1B deleted replication selective adenovirus) [38-40] the most prominent constructs with established anti-tumour efficacy.

Therapeutic strategies – genetic targets

EBV

EBV is a ubiquitous herpes virus and consistently detected in NPC [41],with lower titres found in the differentiated compared to the undifferentiated forms. EBV is a latent infection in NPC, with the viral genome maintained as a circular episome that replicates once per cell cycle [42]. One viral protein, Epstein-Barr Nuclear Antigen 1 (EBNA1), and one region of the viral genome, origin of plasmid replication (oriP) have been identified as being necessary for viral replication [43]. EBNA1 activates viral DNA replication upon binding to specific sequences, termed the family of repeats (FR), within the oriP region of the EBV genome [44]. Additionally EBNA1 binding to the FR element causes downstream enhancement of gene expression, and this has been used as a gene therapy strategy, by restricting certain gene expression to EBV infected cells.

A plasmid has been constructed with the cytosine deaminase gene cloned downstream of the herpes simplex virus thymidine kinase promoter and FR sequence [45]. In EBV-positive cancer cells, transfection with the cytosine deaminase-containing plasmid in the presence of 5-FC abolished cell growth. This strategy has also been used to mediate expression of BRLF1 (RTA); which is an EBV protein able to regulate a switch in EBV replication from latent to lytic replication with resultant destruction of host cells [46]. Recombinant baculovirus constructed with a cytomegalovirus (CMV) promoter, OriP and EBNA1 gene was able to express RTA, induce EBV lytic replication and cell death; as well as inhibit growth in EBV-positive tumours in nude mice [43]. Further studies have examined Interferon-gamma (IFN-γ) delivery to NPC via a non-viral gene therapy vector under the control of an oriP-CMV promoter [47]. IFN-γ is a critical anti-viral and anti-tumour cytokine, and expression in this vector resulted in selective anti-proliferative effects on EBV-positive cancer cells *in vitro* and *in vivo*. An adenovirus in which p53 transgene expression was under the transcriptional regulation of oriP, resulted in selective gene expression in EBV-positive cells and apoptosis mediated cytotoxicity [48]. A further EBV positive tumour selectivity strategy employs a Virus-associated I (VAI) deleted adenovirus, which showed selective replication in EBV positive tumour cells and superior anti-tumour potency *in vivo* [49]. VAI RNAs are required for translation of adenoviral mRNA, and hence VAI deficient adenoviruses require a substitute source for viral replication. EBV-encoded small RNA1 is expressed in most EBV-associated human tumours and can act to replace the lost VAI RNAs of VAI deleted adenovirus. Another interesting transgene that has been investigated in an adenovirus backbone with oriP FR enhancer elements is Bim_S; which is a pro-apoptotic Bcl-2 family member [50]. The proportion of apoptotic cells following Bim_S expression was significantly increased and tumour regression was seen in mouse xenografts in combination with radiotherapy. The death receptor Fas and

its ligand FasL are extensively expressed in NPC [51], suggesting the Fas-mediated apoptotic pathway is intact. Adenovirus expressed FasL (oriP promoter) shows evidence of inducing apoptosis in EBV-positive cells, as well as tumour regression of NPC cell lines *in vivo*, in conjunction with radiotherapy. These results suggest that EBV-positive NPC can be effectively targeted by gene therapy strategies that exploit the oriP/EBNA1 relationship.

p53

p53 has been described as the 'guardian of the genome' and is the archetypal tumour suppressor gene; inducing cell cycle arrest and apoptosis in response to DNA damage. Low levels of p53 have been found in most head and neck cancers [52]; however, NPC has been demonstrated to have p53 over-expression in between 31 - 95% of primary tumours [53]. Mutations of p53 in NPC are rare, with the wild-type p53 induced apoptosis inactivated through loss of p14 and a mutated version of p63 (DN-p63) in NPC [54]. p14 is essential in maintaining p53 stability [55]. DN-p63 binds p53; thus preventing normal apoptosis activation [56]. In spite of this, there is evidence of therapeutic efficacy of p53 gene therapy for NPC; *in vitro*, in animal models and, more recently, in humans. *In vitro* studies have focused on adenoviral mediated p53 (adv.p53) gene therapy on NPC cells compared to control virus; [57, 58] in conjunction with radiotherapy [59,60] or chemotherapy [61]. Increased cytotoxicity and apoptosis was demonstrated in NPC cells treated with p53 expressing adenovirus, independent of EBV status [59]. In human NPC nude mice models, tumour regression was found in mouse treated with adv.p53 [58,62]. However, one study showed no improvement of adenovirus p53 treatment with radiotherapy compared to radiotherapy alone in nude mice bearing CNE-3 NPC xenografts [63]. This was felt to be due to poor transduction of NPC cells with adenovirus (< 15%). Therefore despite no clear rationale for p53 gene therapy in the treatment of NPC, convincing anti-tumour efficacy of this strategy has been demonstrated.

p16

p16 is a regulator of the G1 phase of the cell cycle and was one of the first members of INK4 (inhibitor of cyclin-dependent kinase 4 – CDK4) family to be identified [64]. Activation of various complexes of cyclins and cyclin-dependent kinases (CDK) permits the progression through cell cycle. p16, by competing for cyclin D binding with CDK4, results in G1 cell cycle arrest [65]. Inactivation of p16 is a common event in NPC (60-80% of primary tumours) and is associated with a worse prognosis, reduced radiosensitivity and higher rates of recurrence [66,67]. Inactivation is achieved through multiple mechanisms including homozygous deletion, promoter hypermethylation and point mutation [68]. Studies of other human p16 null cancers, including head and neck cancer, pancreas and lung [69-71], have shown that adenoviral mediated gene transfer has resulted in tumour growth inhibition and apoptosis [72]. It has been demonstrated *in vitro* that infection of a low p16 expressing NPC cell line (CNE-1) with a human p16 expressing replication-deficient adenovirus (adv.p16) resulted in significantly reduced cell survival; which was further reduced with the addition of radiotherapy. CNE-2Z NPC cell line has high endogenous p16 expression and showed significantly improved cell survival compared to CNE-1 with adv.p16 treatment [72]. NPC xenografts using the same cell lines *in vivo*, with and without treatment with p16 expressing adenovirus in SCID mice, showed no tumour growth in the low p16 expressing cell line (CNE-1) treated with adv.p16; compared to sustained tumour growth in the high p16 expressing cell line (CNE-2Z) treated with adv. p16. Cytotoxicity of adv.p16 appears to be multi-mechanistic with evidence of G1 arrest, senescence, apoptosis and necrosis. Furthermore, another group has demonstrated that p16 restoration of a different p16 null NPC cell line, NPC/HK-1, using a plasmid containing the p16 gene under control of a CMV promoter, was able to induce cell growth inhibition and G1 phase cell cycle arrest [73]. Therefore p16 corrective gene therapy in p16 deficient NPC would appear to be a promising therapeutic strategy.

Other strategies

Herpes simplex thymidine kinase cytoreductive gene therapy has been investigated in NPC under the control of human telomerase reverse transciptase (hTERT) promoter and CMV enhancer. hTERT expression is elevated in many nasopharyngeal tumours, and application of the non-toxic pro-drug ganciclovir converted to toxic ganciclovir triphosphate (catalysed by thymidine kinase) resulted in selective cytotoxicity to NPC cells *in vitro* and *in vivo* [74]. Further strategies using RNA interference of oncogenes and other pro-cancer genes, such as angiogenesis mediators, have been described with promising results. Adenovirus mediated transfer of multiple short hairpin RNA (shRNA) to silence vascular endothelial growth factor (VEGF), hTERT, and Bcl-xL oncogene has been demonstrated to induce growth suppression and apoptosis in human NPC cell lines i*n vitro* and *in vivo* [75].

Clinical trials of gene therapy for NPC

Recombinant replication-incompetent adenovirus with a human wild-type p53 (rAd-p53) replacing the E1 region has demonstrated safety and efficacy in clinical trials in patients with HNSCC [36,37]. rAd-p53 has also shown promising results alone or in conjunction with conventional treatments in other types of cancer, including lung, brain and bladder cancer [23]. In 2003, the Chinese Food and Drug Administration approved rAd-p53 (trademarked as Gendicine by Shenzhen SiBiono GeneTech [SiBiono; Shenzhen, China]) for the treatment of head and neck cancer. As such Gendicine became the world's first

gene therapy product approved by a governmental agency for the treatment of cancer.

Recently, a randomised controlled clinical trial has evaluated rAd-p53 intratumoural injections in conjunction with radiotherapy compared to radiotherapy alone in patients with NPC with spread to regional lymph nodes [76]. Tumours were biopsied before and after treatment; p53 protein, as well as downstream target proteins and vascular endothelial growth factor (VEGF) protein, were assessed using immunohistochemistry and reverse transcriptase polymerase chain reaction (RT-PCR). The tumour response to treatment was determined by computed tomography (CT). Complete response rates (disappearance of all target lesions) was 2.73 times higher in the rAd-p53 and radiotherapy group, compared to radiotherapy alone (66.7% compared to 24.4%). Five year locoregional failure rates were 2.7% for rAd-p53 and radiotherapy compared to 28% for radiotherapy alone. 7.5% and 11.7% higher five-year overall survival and disease-free survival rates were found with the combined treatment group respectively. Other than transient fever, no dose-limiting toxicity was encountered. Therefore rAd-p53 is a safe and efficacious treatment for NPC, as well as other head and neck cancers.

In 2005, H101 (Shanghai Sunway Biotech; Shanghai, China) a replication selective adenovirus became the world's first oncolytic virus product to be approved by a government agency for the treatment of NPC in combination with cisplatin-based chemotherapy. The virus features the same E1B-55kDa deletion to the ONYX-015 virus, which renders viral replication confined to cancer cells, which, unlike non-cancer cells, are able to efficiently export late viral RNA in the absence of E1A-55kDa [77]. A phase III randomised control trial compared intratumoural injection of H101 and cisplatin-based chemotherapy to chemotherapy alone. Overall response rates of 78.8% and 39.6% were recorded respectively, suggesting significant anti-tumour efficacy of the combined treatment [40].

Conclusion

Increased understanding of the molecular biology of cancer, as well as advances in techniques to engineer viruses with improved cancer selectivity and cancer killing properties, has established a government approved gene therapy product for the treatment of head and neck cancers, including NPC. Further research will develop treatments with improved anti-tumour potency, with clinical efficacy as monotherapies - without conjunctive contemporary modalities of radio- and chemo-therapy. Longer term goals of cancer treatment personalised to the individual patient's cancer genotype remains the ultimate aim of cancer gene therapy.

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