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Research Article

Oncolytic Adenovirus: Viruses Engineered as Potent Vehicle in Anticancer Therapy and Vaccination

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Abbreviations Ad: Adenovirus; CRAd: Conditionally Replicative Adenoviruses: cFLIP: Cellular FLICE/caspase-8 Inhibitor Protein, HAdVs: Human Adenoviruses; OA: Oncolytic Adenovirus; TRAIL: Tumor necrosis factor Related Apoptosis Inducing Ligand; TSG: Tumor Suppressor Gene

Abstract

The prospect of harnessing the cytocidal properties of viruses for targeted attacks on cancer cells, has prompted an ongoing development in the design of manipulated adenoviral vectors. The adenovirus is efficient in gene transfer, and serves as vector in clinical gene therapy. But its genome contains genes that code for products that bind to and inactivate tumor suppressor gene products, and it has been demonstrated that the deletion of the early genes produces oncolytic adenoviruses that replicate in cancer cells and kill them. New forms of oncolytic viruses with deletions of some viral genes like E1B and being armed with anticancer cDNAs contributed to enhanced cytotoxicity of adenoviral vectors. The engineering of the adenoviral vector could be useful for the development of biotherapeutic agents, as it could expand the list of anti-oncogenes used in anticancer biotherapy.

Background

To date, the term "oncolytic virus" has been defined by a panel of experts as a non-pathogenic virus that specifically infects cancer cells and causes their destruction [1]. As oncolytic viruses are endowed with intrinsic anticancer activity, they are generally viewed as passive immunotherapeutics, which target the immune evasion of the malignancies and enhance antitumor immune surveillance.

The cytopathic effects exhibited by viruses after infecting cancer cells have led to their use in virotherapy. More than a century ago, it was observed that tumors spontaneously regressed in patients who had a viral infection, and this led to clinical trials that used bodily fluids containing human or animal viruses to treat cancer patients [2].

Since the discovery that some viruses are toxic and thus therapeutic to cancers, oncolytic viruses have evolved to recombinant viral strains with more specific killing potential to malignant cells, and they subsequently entered clinical trials. The recent developments have been highlighted on generation of conditional replication of the viruses in tumor, termed conditionally replicative adenoviruses (CRAd) [3,4], expression of transgenes with therapeutic effects, and targeting and delivery of oncolytic viruses.

As a human virus that exhibits tropism to virtually cells of all histologic origins, adenoviruses are used for gene transfer in the laboatories and disease therapy. The viruses have also been applied in anticancer biotherapy after being engineered to selectively replicate in the cancer cells after mutations and deletions of certain parts of the viral genome and/or by inserting target gene fragments into it to create armed adenoviruses. Oncolytic adenoviruses (OA) are genetically manipulated human adenoviruses (HAdVs) that have acquired a phenotype that enables them to infect and/or selectively replicate in tumor cells but are more restricted in normal cells [5].

Data show that manipulated adenoviruses kill cancer-derived cell lines but do not affect normal control cells, which are usually the immortalized cells of the same histologic origin [2]. To correct the genetic deficiency in malignancies, cDNA fragments that code for immunoregulators or pro-apoptotic cytokines, such as melanoma differentiation-associated gene-7(MDA-7)/IL-24 [3,4], tumor necrosis factor related apoptosis inducing ligand (TRAIL) [5], and tumor suppressor gene (TSG) cDNAs [6,7,8] or siRNA to silence anti-apoptotic genes, like survivin [9-11] have been incorporated into recombinant adenoviruses.

The adenoviral vectors expressing a tumor suppressor or cytotoxic/suicide proteins induce cell cycle arrest or a death cascade. We have constructed a recombinant adenovirus by incorpotation of a TSG frequently lost in human cancers to the viral genome by a homogenous recombination of the gragment on shuttle plasmid [12]. Expression of the transferred genes by intratumoral

injection remarkably reduced the tumor size of nude mice xenograted human nasopharyngeal carcinoma (NPC), and the in vitro study has shown that the transferred gene exertted tumor suppressive effects by enganging intracelllular signaling pathways to promote apoptosis and prevent cell cycle progression [13,14].

These strategies aim at introducing a new modality in anticancer biotherapy and have entered into preclinical studies and different phases of clinical trials. The present paper reviews the current advances in the field of oncolytic adenoviruses to assess their prospective utility as expression vectors for tumor suppressor genes (TSG) to be used in future anticancer biotherapy.

The adenoviral genomic composition and genetic basis of using adenovirus as anticancer therapy agent

Viruses encode for specific genes to kill the infected cells by multiple mechanisms including apoptosis triggering; it is induced directly by viral genomic products or through activation of death receptors and p53. HAdVs can infect a broad range of human cells with high efficiency and achieve high levels of transgene expression. Moreover, the viral genome adenovirus (Ad) is genetically stable and the inserted foreign genes are generally maintained without change through successive rounds of viral replication. These features make Ad vectors attractive in gene therapy.

The replication cycle of HAdVs is divided into two stages: the early stage and the late stage. During early stage, the viral proteins are expressed from six distinct early regions, to function as modifiers of the microenvironment so as to favor the replication of the adenoviruses. In the later stages of viral infection, the viral progeny is formed after assembly with structural proteins, viral load is increased to a threshold, and viruses then exploit apoptotic machinery to facilitate the efficient viral progeny release and spread.

The early regions of Ad genome include E1A, E1B, E2A, E2B, E3 and E4. In adenovirus, apoptosis inducing proteins include, E1A 12S and 13S proteins, E3, E4 of adenovirus [15]. The adenovirally encoded apoptotic regulators also function to escape inflammatory reactions and host's immune responses.

E1A, a pro-apoptotic adenoviral protein

E1A is a nuclear protein and a known regulator of gene expression. Although E1A does not bind to DNA directly, it interacts with a large number of cellular proteins functioning as transcription regulators [16]. Activation of c-Jun may be the result of transcriptional regulation, e.g. the coactivator p300 can participate in transcription of the jun gene [17,18].

CRAds based on the deletion of E1A has been generated, with introduction of a mutation in the pRb-binding domain of E1A. It has been shown to replicate in tumor cells with disrupted Rb signaling pathway [19,20]. These CRAds with manipulation in a single adenoviral antigen exhibited potential for cancer therapy, but they still replicate and cause some cytopathic effects in normal cells *in vitro* [21,22].

E1B, a viral genomic product that binds and interferes the activities of tumor suppressors

The 55K proteins encoded by early regions 1B (E1B-55K) from HAdV types 2, 5 and 12 contribute to complete cell transformation by antagonizing host apoptosis and growth arrest. In the case of Ad2/5 E1B-55K products, these growth-promoting activities correlate with their ability to direct transcriptional repression of p53-responsive promoters by binding to p53 [23]. Together with human papillomavirus (HPV) encoding p53 inhibitor protein E6, E1B also abrogates transcriptional induction of downstream pro-apoptotic factors by p53 through molecular interactions [24,25].

CRAd, which are capable of cancer-selective replication and oncolysis, have received widespread attention as potentially ideal tools for anticancer biotherapy. ONYX-015(dl1520), is a CRAd with E1B deletion based cancer selective replicative potential and a mutant adenovirus created by deletion in the gene coding for E1B that enables selective replication in malignant cells with dysfunctional p53 signaling pathway [26-29]. Clinical trails of ONYX-015 in combination with chemotherapy have yielded remarkably good efficacy and safety in patients with head and neck cancers [30]. As for hepatobiliary cancers, the clinical trial of intralesional ONYX- 015 showed sufficient safety but limited therapeutic effects [31]. These studies suggest that for biliary cancers, further efforts to develop CRAds are warranted, so as to exert more selective replication and effective oncolysis than ONYX-015.

Ad Max, the Ad5 based adenoviral vector we used as expressor of tumor suppressor BLU [12], is known to be disrupted in E1 and E3 [32,33]. E1B expression was still detected on immunoblotting. Several strains of siRNAs have been designed by searching for the AA dinucleotides in the full length sequence of the E1B 55K coding gene (nucleotides 2109→3509 in Human adenovirus C serotype 5, complete genome; AY339865.1, GenBank), Each AA and the 3' adjacent 19 nucleotides were chosen as potential siRNA target sites. They were listed in Table 1. The oligonucleotides were synthesized and tested for the potential of E1B silencing. Data showed that some of the fragments remarkably enhanced cytotoxicity when introduced to host cells (Figure 1). It has suggested that knockdown of E1B by RNA interference potentiates killing of tumor cells by adenovirally transferred BLU.

Figure 1 siRNAs against adenoviral E1B enhanced adenoviral cytotoxicity to NPC derived HNE1 cells. The cells previously infected with recombinant BLU Ad5 were transfected with anti E1B siRNAs designed as described in the text. Viability was assayed by co-incubating with CCK-8 kit and registered with an ELISA reader. The numbers below the x-axis depicted the species of oligonucleotides tested: 1. Scramble RNA; 2. AS22A5P; 3. AS22A5Q; 4. AS22A5R; 5. AS22A5S; 6. AS22A5T. Data was derived from at three independent tests.

The silencing of oncogene in cancer cells mediated by siRNA could be utilized as a therapeutical approach. When a fragment of

Table 1: Sequence of anti E1B siRNAs used in the present study.

AS 22A5P	Sense : GAUCAAGGAUAAUUGCGCUtt Antisense : AGCGCAAUUAUCCCUUGAUCtg
AS22A5Q	Sense : GCUUCUAUGGGUUUAACAAtt Antisense : UUGUUAAACCCAUAGAAGCtt
AS22A5R	Sense : GGCUCUAGCCGAUGAAGAUAtt Antisense : UAUCUUCAUCGCUAGAGCCaa
AS22A5S	Sense : AGAUGUAGCAUGAUAAAUAtt Antisense : UAUUUAUCAUGCUACAUCUaa
AS22A5T	Sense : AGACGAUUUUGACAAUUAtt Antisense : UUUUUGUCAAAUGCGUCUca

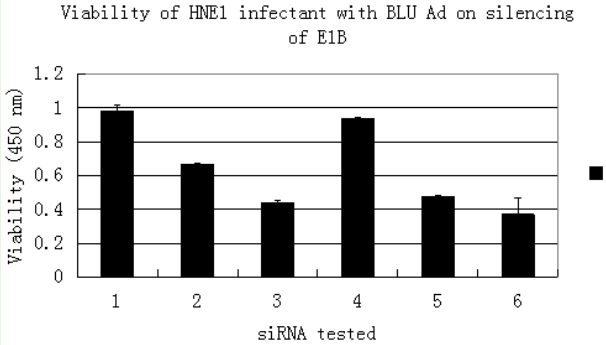

Figure 1: siRNAs against adenoviral E1B enhanced adenoviral cytotoxicity to NPC derived HNE1 cells. The cells previously infected with recombinant BLU Ad5 were transfected with anti E1B siRNAs designed as described in the text. Viability was assayed by co-incubating with CCK-8 kit and registered with an ELISA reader. The numbers below the x-axis depicted the species of oligonucleotides tested: 1. Scramble RNA; 2. AS22A5P; 3. AS22A5Q; 4. AS22A5R; 5. AS22A5S; 6. AS22A5T. Data was derived from at three independent tests.

short hairpin RNA (shRNA) against mutant K-ras was introduced to an oncolytic adenovirus ONYX-411, a 10-fold increase of the growth inhibition potency has been observed in cancer cells [34]. It has been noted that improvement of gene knockdown, curtail of emergence of viral escape mutants could be achieved by delivery of multiple shRNA delivery [35].

The E2 region of the viral genome encodes multiple proteins, including E2A functioning as a DNA binding protein (DBP). It possesses specific affinity for single stranded viral DNA, and the binding plays a role in the initiation and elongation of viral DNA synthesis during the early phase of Ad infection. During the late phase of infection, DBP plays a central role to activate the major late promoter (MLP) [36]. New generations of Ad5 based vectors have been introduced by additionally deleting viral genes, so as to attenuate their expression to avoid leakage problems. Vectors with E1, E2A/B, E3 and E4 deletions in different combinations showed low in vitro cytotoxicity and higher stability in vivo, but it remains to demonstrate whether the new second and third generation vectors significantly prolong transgene expression in vivo [37,38].

Immunoregulatory functions of E3 proteins

HAdVs are grouped into species A (HAdV-A) through species G (HAdV-G), and the level of sequence diversity is as high as 40% [39]. The size and composition of the E3 region differs considerably among Ad species, and the E3 transcription unit of HAdVs encodes proteins with immunoregulatory activities; Differential immunomodulatory functions encoded in early transcription unit 3 (E3) may play an important role in disease [40-42]. Common immune evasion functions in E3 proteins from species C have been described. Ads express species C E3 protein and enable the viruses to evade recognition and elimination by the host immune system by various mechanisms. An E3 protein, E3/19K retains MHC class I molecules and MHC class I-related chain A and B in the endoplasmic reticulum of infected cells, thereby suppressing recognition by cytotoxic T lymphocytes [43-46] and activation of natural killer (NK) cells [47,48]. It has been reported that an E3 protein E3/49K from species D targeted uninfected cells; it specifically binding lymphocytes and that cell surface protein tyrosine CD45 was identified as its receptor [49]. Such functions would be important for the utility of species D Ads as vectors for vaccination and gene therapy in humans, given that these have a number of favorable features [50]. Other common E3 proteins (E3/10.4K–14.5K) down-regulate various apoptosis receptors from the cell surface or affect TNF-α–induced signaling [45,51]. Disruption in this genomic portion may enhance host antiviral immunity against tumor cells harboring the mutant adenovirus during the therapy.

Adenovirus E4 open reading frame 4 (E4orf4)

The protein encoded by adenovirus E4 open reading frame 4 (E4orf4), protein (14-kDa) is a multifunctional viral regulator which functions to regulate gene expression at multiple steps, alternative splicing events, phosphorylation of viral and cellular proteins and protein translation [52-55]. Its expression of E4orf4 at high levels also induces caspase- and p53-independent, non-classical apoptosis in many human tumor cells [56,57]. Oncogenic transformation of primary cells sensitizes the host to cell death induced by E4orf4 induced.

Oncolytic adenoviruses: adenoviruses manipulated to be conditionally replicated in tumor cells

The replication of adenovirus depends on the entry of the host into the S phase of cell cycle. A tumor suppressor, retinoblastoma tumor suppressor (pRb) prevents the entry to S phase. Within the E1A molecule, there is a product of the retinoblastoma susceptibility gene binding site [58,59]. E1A gene is responsible for inactivation of several proteins, including retinoblastoma, allowing entry into S-phase. When E1A binds the protein pRb, transcription factor E2F1 dissociates and the host cells are prompted to enter the S phase. The adenovirus with deletion of the pRb-binding E1A has reduced replication potential in normal cells with intact pRb function, but the replicative ability in cancer cells is unaffected [60,61]. The Ad-delta-24 with the deletion of the binding motif with 24 amino acid residues shows an enhanced efficacy in treating glioma-carrying mutant pRb [62].

The oncolytic adenoviruses are converted from non-oncogenic adenoviruses, of serotypes 2 and 5 through certain manipulations. The mutant viruses have gone through three generations of development as defined by the mutations introduced [63]. And the modification of adenoviruses include:

Attenuation

In this category, adenoviral vectors of three generations have developed. During the process of viral replication, tumor cells are killed by ablation of the viral vectors. The first generation oncolytic Ads are administered in combination with chemotherapy and/or irradiation to achieve efficacy, wtih accetable level of safety. Oncolytic Ads of second generation are armed with therapeutic transgenes, to enhance the efficacy by increasing virion release, spread and effect of bystander. Oncolytic Ads of third generation are modified in viral capsid for transductional detrageting of normal cells but targeting cancer cells.

Targeting

A conditionally replicative adenovirus (CRAd) was created by deleting a 24 base pair deletion in the retinoblastoma

(Rb)-binding domain of the E1A protein (Ad5- Δ24E3). Whenever the pRb binding site on Adv early antigen interfring with cell cycle is manipulated, induction of S-phase in host cells is disabled [64], and retinoblastoma is silenced, this restricts Ad5-Δ24E3 to replication only in proliferating cells, such as tumour cells.

Manipulations introduced to Adv genome to produce oncolytic virions

Delta-24-RGD, a tumor-selective, replication-competent adenovirus with augmented cellular infectivity [65,66]. The deletion of RGD motif was introduced in a mutant viral E1A. The mutant AdV with Delta-24-RGD selectively replicates in tumor cells lyse the malignant cells in which pRb is inactivated. Delta-24-RGD's augmented infectivity is due to an insertion of an RGD-motif in the fiber knob, allowing for integrin-mediated infection, independent of cocksackie-adenovirus receptors (CAR) [67]. Intratumoral injection of Delta-24-RGD to xenografted human gliomas resulted in a remarkably longer survival than controls [68,69].

Onyx-015 as previously mentioned, was originally named Ad2/5 dl1520 [29,30,70]. It is an experimental oncolytic virus created by genetically engineering [29,30]. The E1B-55kDa gene has been deleted allowing the virus to selectively replicate in and lyse p53-deficient cancer cells [70]. ONYX-015 is an E1B-55kDa gene-deleted adenovirus which selectively replicates in and p53-deficient cancer cells and lyses them. Clinical trial suggested that tissue destruction was also highly selective, most in tumor lesions; significant tumor regression occurred in over 20% of evaluable patients; no toxicity to injected normal peritumoral tissues was demonstrated. That ONYX-015-induced necrosis in p53 mutant tumors more likely than were p53 wild-type tumors. The data implied a feasibility of modifying genome region coding for anti-apoptotic E1B as a promising approach to generate OA.

Adenovirus armed with anticancer cDNA: an ideal anticancer agent needs to be specific in action and should not harm the normal cells

To date, cancer is recognized as a disorder of multiple genetic defects, the development of which is aided by compromised immune surveillance and dysregulation in programmed cell death (PCD) [71,72]. Different abnormalities in PCD may promote carcinogenesis by hampering host immune surveillance, and also renders cancer cells resistant to antitumor therapies including chemo- and radiotherapy, as the cell killing in such context is mediated by activation of apoptosis. The inherited defects contributing to the immune evasion and resistance to therapy can be corrected by transfer of cDNAs with tumor suppressive activity. Cancer-targeting gene virotherapy (CTGVT) is an approach that uses an oncolytic adenoviral vector containing antitumor gene fragments as a combination of gene therapy and oncolytic adenovirus. When armed with some anticancer cDNA or siRNAs, coding for pro-drug converting enzymes, immunoregulatory cytokines, or pro-apoptotic proteins, an adenovirus could be adapted as a gene therapy vector.

Apoptosis is a precise cell death process inside the human body. Extrinsic or death receptor-induced apoptosis has been intensively studied. It is triggered by ligation of death receptors to death ligands [73]. One of the death ligands, TRAIL has gained much attention as a targeted therapeutic candidate and has therefore entered the Phase I clinical trial TRAIL has been known for its preferential killing of malignantly transformed cells, and low cytoxicity has been demonstrated [74-76]. The intratumor administration of an adenoviral recombinant of TRAIL has proved to possess therapeutic effect in the primary and metastatic models of xenografted human tumors in mice. The E1B deleted oncolytic adenovirus ZD55 armed with the death ligand TRAIL and a second mitochondrial activating component (Smac) contributed to the complete eradication of human hepatoma xenograft in nude mice [77].

The extrinsic apoptotic pathway is regulated by inhibitor proteins, including the cellular FLICE/caspase-8 inhibitor protein (cFLIP), which negatively regulates the activation of caspase-8 as a decoy binding partner [78]. The coding gene of cFLIP produces up to eleven isoforms of transcripts by alternative splicing, and three c-FLIP protein isoforms derived from distinct mRNA splice variants have been identified, namely c-FLIPL, c-FLIPS, and c-FLIPR [79]. The long c-FLIP isoform, c-FLIP$_L$, is structurally similar to procaspase-8, with two tandem DEDs at its N-terminus and a catalytically inactive caspase-like domain at its C-terminus [80]. It has been reported that silencing c-FLIP$_L$ in ovarian cancer cells with RNAi induced apoptosis leading to significantly decreased tumor development and reduced cellular proliferation in vivo [79,80]. This suggests that recombinant adenovirus incorporating anti FLIP RNAi may exert anticancer efficacy. One application of oncolytic adenovirus would be transfer of siRNA targeted against anti-apoptotic molecules to enhance host antitumor immunity.

We have shown that BLU/ZMYND10, encoded by a TSG mapped on a chromosomal region, 3p21, frequently lost in variety of human tumors inhibited NF-kB signaling, and hence the anti-apoptotic factor it transcriptionally induces, e.g. cIAP-2, cFLIP, to promote TRAIL trigering apoptosis when BLU is transferred by AdV 5 vector [13].

Thus tumor killing potential of the oncolytic adenoviruses could be enhanced by genetic modifications with insert of sequences coding for (1) enzymes converting an innocuous pro-drug into a cytotoxic agent [81,82]; (2) proteins that (at least theoretically) selectively induces a programmed death response in malignant cells, since tumor growth frequently depends on the defects in the apoptotic machinery [83]; or (3) short hairpin RNAs that interfere with the activities of factors required for the survival of transformed, but not normal cells [84].

The efficacy of adenoviral therapeutic recombinants

Several mutant genetic lesions within adenoviral genome leading to oncolytic activities have been described [85,86]. The production of oncolytic adenoviruses involves the deletion of the E1 through E4 genes, and disable the functions necessary for replication in normal cells but still permits replication of the mutant viruses with cytocidal effects in cancer cells [87].

ONYX-015 and ZD55 were both generated by deleting the E1B55 kd gene. In cancer patients, durable regressions are achieved in combination of recombinant adenovirus administration with chemotherapy (e.g., cisplatin) [88,89]. In line with this, the combined administration of ONYX-015 and cisplatin has provided potent antitumor activity, but further improvement of the oncolytic

adenovirus using a single virotherapeutic agent is expected. An armed therapeutic oncolytic adenovirus system, the ZD55 strain is not only devoid of the E1B 55-kd gene, similar to ONYX-015, but is also incorporated with foreign genes with antitumor activity in the viral genome, e.g. TRAIL. IL-24 or SOCS3 [6, 90, 91]. The ZD55 strain exhibits similar cytopathic effects and replication kinetics with ONYX-015 in vitro; however, the inserted gene is expressed and the expression level increases with replication of the virus. ZD55 not only replicates selectively in tumor cells and lyses them, but also releases the therapeutically active antitumor agents to the tumor microenvironment.

Manipulated oncolytic adenoviruses in which viral and/or cellular genes are placed under the control of artificial tumor-specific promoters have been developed and show promising results in anticancer therapy in animal models. Based on the fact that a fetal protein, alpha-fetoprotein (AFP) is switched on during the hepatocarcinogenesis, AFP promoter was used to control the expression of the E1A viral gene in hepatocellular carcinoma (HCC) cells. The manipulation achieved preferential replication of the pro-apoptotic E1A gene over-expressing Ad in AFP-producing HCC cells [92]. Midkine mRNA is over-expressed in osteosarcoma cell lines, and an oncolytic adenovirus has been constructed by inserting the midkine promoter upstream of the coding portion of the adenoviral genes, to drive the expression of the adenoviral antigens [83]. Infectivity and in vitro cytocidal effects of the engineered virus were significantly enhanced in target cells of osteosarcoma. These findings indicate that gene manipulation allows tailored virotherapy and facilitates more effective treatments for osteosarcoma.

NPC is a malignant tumor that is endemic to certain regions in the world, including Alaska, North Africa, southeast and southern China [93]. In its undifferentiated form NPC is tightly associated with latent infection of a lymphotropic human herpesvirus, the Epstein-Barr virus (EBV); therefore, EBV-dependent transcriptional targeting has been exploited in NPC biotherapy. The viral replication initiation site of EBV, OriP, was used to construct a conditionally replicating adenovirus, termed adv. OriP. E1A [17]. Extensive cell death was observed in EBV-positive NPC cells, but no cytoxicity was noted in a panel of EBV-negative cells, which were derived from NPC and fibroblasts from the nasopharynx. Adenoviral replication was demonstrated only in EBV-positive cells, and a combination of local irradiation and adenoviral administration caused the xenografted tumors to completely disappear within two weeks.

Similar effects were observed in metastatic gastrointestinal cancer treated with a virus in which the therapeutic genes were controlled by the carcinoembryonic antigen (CEA) promoter [94]. Recombinant adenoviruses combined with other tumor-specific promoters, like human TERT [95,96] or E2F [97] , showed effects in a broad range of cancer types.

Conclusion

1. As an efficient tool to transfer gene, adenovirus could be manipulated as a potent vector in anticancer therapy and vaccination by modifying its genomic components.
2. The successful construction of several strains of oncolytic adenoviruses has suggested that viral vectors specifically replicating in malignant cells, and armed with anticancer cDNA could achieve therapeutic effects. As a disease of genetic deficiency, cancer could be treated with a biotherapeutic approach by introducing different genes to correct a variety of functional defects.
3. Oncolytic adenoviruses with inserted anticancer cDNA would expand the list of therapeutic target genes and improve the efficacy of biotherapy.

Availability of data and material

The sequence of adenoviral E1B coding gene used in the present study is available in complete genome of Human adenovirus C serotype 5, AY339865.1 in Gen Bank, NCBI (Human adenovirus C serotype 5, complete genome - Nucleotide – NCBI.

http://www.ncbi.nlm.nih.gov/nuccore/33465830/).

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