A fiber modified adenovirus vector that targets to the EphrinA2 receptor reveals enhanced gene transfer to *ex vivo* pancreatic cancer

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Abstract. Pancreatic cancer is an aggressive malignancy with a dismal prognosis. To improve treatment options new treatments, such as adenoviral (Ad) gene therapy are necessary. However, low expression of the coxsackie and adenovirus receptor (CAR) in pancreatic cancer cells (PC) limits the therapeutic efficacy of these vectors. The aim of this study was to improve transduction of PC by recombinant adenoviruses by inserting peptides into the HI loop that binds to receptors highly expressed on pancreatic cancer and were shown to target these carcinomas *in vivo*. We report the successful incorporation into the HI loop of peptide Tyr-Ser-Ala (YSA), a peptide ligand targeting the EphrinA2 (EphA2) receptor, and K237, a peptide targeting to the vascular endothelial growth factor receptor-II (VEGFRII). Subsequently, we showed that both peptides enhanced the transduction of a number of human PC lines that abundantly express the targeted receptor. Additional competition studies confirmed that the YSA peptide redirects Ad-YSA from CAR and specifically targets the EphA2 receptor. Due to this transduction efficiency of Ad-YSA is increased not only in human pancreatic cancer cell lines but more importantly also in pancreatic cancer resection specimens. Since the YSA peptide has been shown to specifically target pancreatic cancer in patients, it may be expected that Ad-YSA will also display increased tropism for this tumour.

Introduction

Adenocarcinoma of the pancreas is a devastating disease. Due to late diagnosis tumour resection is available for only 20% of the patients (1). Furthermore, even after resection, the 5-year survival rate is less than 20% (2). As a consequence the annual death rate almost equals the annual incidence (3). The majority of patients with non-resectable disease receive palliative treatment. Gemcitabine-based chemotherapy regimens are the treatment of choice and offer relief of complaints, but responses are limited (4). Other recently developed drugs that target matrix metalloproteinase activity (5) or inhibit the K-*ras* pathway (6) failed to show long-term efficacy. Therefore, there is a great need for the development of novel approaches including gene therapy.

Initial anti-cancer gene therapy strategies, aimed at restoring tumour-suppressor gene function, down regulation of activated oncogenes or introduction of suicide genes, were safe but failed to show efficacy. Therefore, at present gene therapy is aimed at enhancing immune responses against the tumour or to induce cancer cell lysis using an oncolytic-virus (7,8). For the latter approach, adenoviruses that selectively replicate in tumour cells seem promising (9). These Conditionally Replicative Adenovirus (CRAd) vectors use differences in cell cycle control between tumour cells and normal cells to replicate selectively in tumour tissue (10,11). ONYX-015 (dl1520) was the first CRAd to enter clinical trials of anti-tumour gene therapy and proved to be well tolerated with acceptable toxicity upon various administration routes (12,13). Combined with chemotherapy, considerable anti-tumour efficacy was observed in the clinical setting (12,14). Two clinical trials addressed the safety and efficacy of ONYX-015 in PC patients, specifically. CT-guided injection of doses up to $2x10^{12}$ viral particles (VP) into the primary tumour was well tolerated. However, no anti-tumour response was demonstrated in patients (15). Endoscopic intra-tumoural injection of ONYX-015, with some patients receiving up to 80 injections, also failed to induce an anti-tumour response (16). In both studies virus replication was not detectable in contrast to earlier experiments *in vitro* and in animal studies. Subsequent studies including intra-tumoural injections showed that tumour cell transduction hampers effective initiation of the Ad replication cycle. Low expression of the coxsackie and adenovirus

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receptor (CAR) on tumour cells *in vivo* appeared a major determinant of poor susceptibility to Ad infection *in vivo* (17-19). Since PC cells are quite refractory to wild-type Ad infection (20), limited viral entry will severely impair the therapeutic efficacy of these vectors. Redirecting Ad from CAR to PC specific antigens can overcome this problem.

A well established method to redirect adenoviral vectors is insertion of a specific peptide motif in the HI-loop of the fiber knob (21). For PC, insertion of an Arg-Gly-Asp (RGD) peptide motif that efficiently binds to av-integrins has been used. Av-integrins are strongly up regulated in PC and RGD retargeting provided increased adenoviral gene transfer to these cancers *in vivo* (22). The widespread expression of integrins, however, also enhances the transduction of non-tumour cells by Ad-RGD (23). This will decrease the specificity of adenovirus cancer gene therapy and may enhance toxicity. We therefore aimed to redirect adenoviral vectors to proteins specifically exposed on PC to enhance adenovirus entry into these cancer cells *in vivo*. Receptor-specific peptide ligands that can home to PC tumour *in vivo* seem the most suitable targets for this approach. We have selected four promising targets for PC: a) the neurotensin receptor (NTR) (24-26), for which neurotensin can be used as a targeting peptide, b) the vascular endothelial growth factor receptor-II (VEGFR-II/KDR), (27-29) that is targeted by the K237 peptide (30), c) Thomsen-Friedenreich antigen (TF) (31-33), for which p30 and p30/1 have been identified as targeting moieties (34,35), d) the EphrinA2 receptor (EphA2) (36) for which YSA (Tyr-Ser-Ala) and SWL (Ser-Trp-Leu) peptides have been identified as ligands (37).

To generate vectors that specifically home to PC we inserted these targeting moieties into the HI-loop of the adenovirus fiber knob. The effect of this retargeting on adenovirus transduction efficacy was studied in several human PC cell lines *in vitro*. To assess the efficiency of gene delivery in a system more comparable to the PC *in vivo*, we subsequently transduced the most promising vectors to pancreatic cancer slices obtained from resection material. We reveal that Ad-YSA reveals an increased transduction efficiency not only to in vitro growing PC cells and cell lines but also to *ex vivo* tissue slices of freshly obtained pancreatic cancer from patients.

Materials and methods

Cells. HEK293 cells and the established PC cell lines (Capan-1 and Hs766-T) were obtained from the American Type Culture Collection, Rockville, MD; BxPC-3 and MIAPaCa-2 were obtained from Boehringer Ingelheim, (Belgium). The near primary pancreatic carcinoma cell lines (p6.3 and p10.5, <10 passages) were obtained from Dr E. Jaffee, Johns Hopkins University School of Medicine, Baltimore, MD. The cells were cultured in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (heat inactivated) and L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) all from Cambrex Bio Science (Walkersville). Primary human hepatocytes were isolated as described before (38). Hepatocytes were cultured in Williams' E medium supplemented with 4% (v/v) heat inactivated fetal bovine serum, glutamine (2 mM), 0.1 μM dexamethasone, 20 mU/ml insulin (20 mU/ml), ornithine (2 mM), and penicillin (100 U/ml), streptomycin (100 μg/ml) and ampotericin (Fungizone 2.5 μg/ml). Human fibroblasts (passage <10) were a gift from the department of Genetic Metabolic diseases AMC, Amsterdam. Human umbilical vein endothelial cells (HUVECs, passage 1-3) were isolated as described (39) and cultured in Medium-199 (Gibco-BRL, Paisley, Scotland), supplemented with 20% (v/v) fetal bovine serum, 50 μg/ml heparin (Sigma, St. Louis, MO), 6-25 μg/ml endothelial cell growth supplement (ECGS; Sigma), and 100 U/ml penicillin/streptomycin (Gibco-BRL). All cell lines were cultured at 37˚C in 10% CO_2 atmosphere.

Receptor expression. Expression of the Thomsen-Friedenreich antigen and neurotensin receptor was determined by indirect immunofluorescence. Cells ($2.0x10^5$) cells were plated in 12-well plates and allowed to adhere overnight. Cells were released with enzyme free cell dissociation buffer (Invitrogen), washed with ice cold PBS, followed by incubation for 1 h on ice with 1 μg/ml Thomsen-Friedenreich (clone A78-G/A7, Neomarkers, Fremont) or NTR antibody (clone B-N6, kindly provided by Dr J.M. Ovigne, Diaclone, Besancon, France) in PBS. Cells were washed with PBS thrice and incubated for 1 h on ice with 1:1000 goat anti mouse Alexa Fluor 488 (Molecular Probes, Eugene). The isotype negative control antibody IgM (Neomarkers, Fremont) was used as negative control. The ratio between the mean fluorescence intensity of the cell population of interest to that of the negative control was determined. Flow cytometry was carried out by using a FACScan system (Becton-Dickinson, Franklin Lakes, NJ, USA). Cell lysates were prepared in RIPA lysis buffer including a cocktail of protease inhibitors (1:250, Roche). The protein concentration was determined with a BCA assay kit (Sigma). Cell lysates (25 μg) were separated with 8% (EphA2) or 6% (VEGFR-II) SDS/PAGE and transferred to nitrocellulose membranes. Aspecific binding to membranes was blocked by pre-incubation with 4% milkpowder in PBS for 1 h at R.T. Incubation with antibodies was performed o/n in PBS with 0.05% Tween. For EphA2 a 1:400 dilution of the anti-EphA2 clone D7 was used (Sigma). For VEGFR-II a 1:500 dilution of the anti-KDR (Sigma) was used and a 1:100 dilution of the anti VEGFR-II (Santa Cruz). Goat anti mouse IgG HRP (ITK) diluted 1:1000 was used as secondary antibody. Membranes were developed using the Lumi-lightPLUS Western blotting Substrate, Roche, Mannheim, Germany, kit according to the manufacturer's instructions.

Plasmids. The HI-loop region of E1-, E3-deleted adenovirus vectors were modified using pAdHM15 (40). pAdHM15 was digested with PI-SceI and Ceu-I and ligated with the cytomegalovirus (CMV) immediate-early promoter enhanced green fluorescent protein (GFP) cassette (20). pAdHM15-CMV-eGFP was digested with BstB1 and ligated with the following sets of annealed primers: RGD for: CGAAGTGTG ACTGCCGCGGTGACTGTTTCTG; RGD rev: TTCACACT GACGGCGCCTCTGACAAAGACGC; inserted RGD peptide: CCRGDCC. NT for: CGAAGGAGCTGTACGAGA ACAAGCCCCGCCGCCCCTACATCCTG; NT rev: CGAA GGATGTAGGGGCGGCGGGGCTTGTTCTCGTACAGCT CCTT; inserted NT peptide: ELYENKPRRPYIL; P30 for: CGAAGCACGGCCGCTTCATCCTGCCCTGGTGGTACG

CCTTCAGCCCCTC; P30rev: CGGAGGGGCTGAAGG CGTACCACCAGGGCAGGATGAAGCGGCCGTGCTT. Inserted p30 peptide: HGRFILPWWYAFSPS; P30-1 for: CGAAGATCGTGTGGCACCGCTGGTACGCCTGGAGC CCCGCCAGCCGCATCT. P30-1 rev: CGAGATGCGGC TGGCGGGGCTCCAGGAGTACCGGCGGTGCCGCACG ATCTT. Inserted p30-1 peptide: IVWHRWYAWSPASRI; K237 for: CGAAGCACACCATGTACTACCACCACT ACCAGCACCACCT. K237 rev: CGAGGTGGTGCTGGTA GTGGTGGTAGTAGCGGGTGTGCTT. Inserted k237 peptide: HTMYYHHYQHHL; YSA for: CGAAGTACAG CGCCTACCCCGACGGCGTGCCCATGATGT. YSA rev: CGACATCATGGGCACGCTGTCGGGGTAGGCGCTGT ACTT. Inserted YSA peptide: YSAYPDSVPMMS; SWL for: CGAAGAGCTGGCTGGCCTACCCCGGCGCCGTGA GCTACCGCT. SWL rev: CGAGCGGTAGCTCACGGC GCCGGGGTAGGCCAGCCAGCTCTT; Inserted SWL peptide: SWLAYPGAVSYR. Upon ligation and transformation we checked if the recombinant constructs were correct by restriction enzyme analysis and PCR. Constructs depicting the correct bands were subsequently sequenced to exclude mutations.

Virus generation, propagation and analysis. Recombinant adenoviral vectors were generated by transfection of HEK 293 cells with *Pac*I-linearized Ad-CMV-eGFP-RGD, Ad-CMV-eGFP-p30, Ad-CMV-eGFP-p30-1, Ad-CMV-eGFP-NT, Ad-CMV-eGFP-K237, Ad-CMV-eGFP-YSA and Ad-CMV-eGFP-SWL. Ad-Wt, Ad-RGD and Ad-SWL were rescued efficiently. Ad-K237 and Ad-YSA were rescued with reduced efficiency as indicated by a longer period between transfection and the occurrence of plaques. Ad-p30 and Ad-p30-1 could not be rescued despite several attempts and using 911 cells in addition to 293 cells.

All viruses isolated upon transfection were propagated on HEK293 cells, purified and concentrated by performing two cesium chloride gradients. Virus preparations were dialyzed against 1 lt of PBS two times. After the last dialysis glycerol was added to a final concentration of 10% (v/v) and virus preps were aliquoted, and stored at -80˚C. Virus DNA was verified for proper incorporation of peptide coding sequences using the primers fiber-forward: CAAACGCTGTTGGATTTATG and fiber-reverse: GTGTAAGAGGATGTGGCAAAT for sequencing. The amplified fiber region of wild-type and recombinant virus corresponded to approximately 190 and 210 bp, respectively, indicating proper insertion of peptide sequences. Adenovirus protein was separated by SDS-page under boiling and non-boiling conditions. The anti-fiber monoclonal 4D2 antibody (NeoMarkers, Fremont, CA) was used to test for fiber trimerization efficiency of all retargeted vectors as described before (41). All retargeted vectors showed proper trimerization (not shown).

Infectious titers were determined using the end-point dilution assay. The number of genomic copies was determined by quantitative real-time polymerase chain reaction by using the primers against hexon DNA [forward: gacatgactttcgagct cgatcccatgga, reverse: ccggccgagaagggtgtgcgcaggta (42)].

Transduction experiments. Recombinant fiber knob 5 was expressed in *E.coli* for blocking experiments, using the pQE30.KNOB5 expression vector (kindly provided by Dr J.T. Douglas, Birmingham, AL) (43). To assess adenovirus infection efficacy, $5.0x10^4$ cells were plated in 48-well plates and allowed to adhere overnight at 37˚C. Blocking experiments were performed by incubation of cells with blocking agents in PBS for 20 min at room temperature. CAR, VEGFR-II and EphA2 were blocked by 5 μg recombinant fiber knob, and the synthetic peptides K237 and YSA (Eurogentec, Seraing, Belgium), respectively. Transduction was performed at R.T. in DMEM with 2% FBS with 500 and 1000 genomic copies per cell. After 30 min medium was removed and cells were washed with DMEM/2% FBS and cells were incubated for 24 h in complete media. The percentage of GFP positive cells was determined by flow cytometry (Becton-Dickinson FACS reader).

Primary tissue slices. Fresh human pancreatic specimens were obtained from patients undergoing a pancreaticoduodenectomy (Whipple's procedure) for pancreatic head tumours (pancreatic cancer slices) and carcinomas of the bile duct or ampulla of Vater (normal pancreas slices). Tumour pieces were embedded in 3% low melting point agarose/PBS and loaded into a MD4000 Krumdieck tissue slicer (Alabama Research & Development, Munford, AL). Samples were sliced at a thickness of approximately 250 μm (estimated $2.5x10^5$ cells) while submerged in oxygenated ice-cold Krebs. Slices were incubated in 12-well tissue culture plates (1 slice per well) in 1 ml complete culture medium supplemented with insulin, transferrin and selenium (ITS mix, Invitrogen), MEM vitamins and MEM amino acids (Invitrogen), 20 mM Hepes (pH 7.4). After 1 h, medium was replaced and slices were infected with $1.0x10^8$ or $5.0x10^8$ genomic copies of each Adenoviral construct. To correct for slice viability, size and differences in CAR expression, co infection with equivalent amounts of Ad-CMV-dsRED was performed. Upon addition of the virus, tissue culture plates were placed in an Innova4300 incubator (New Brunswick Scientific Co., Edison, NJ) that was humidified and gassed with 95% O_2 and continuously shaken back and forth (90 times/min) at 37˚C. Virus was removed and fresh medium was added after 36 h. Slices were removed from culture after 72 h and lysed overnight at 4˚C in 60 μl RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for reporter gene quantification or fixated in 4% formaldehyde for histochemistry. For reporter gene expression measurements, virus-infected slices were freeze-thawed once and total fluorescence of GFP and dsRED was measured with a NOVOstar fluorometer (BMG Labtechnologies, Offenburg, Germany). For immunohistochemistry slices fixed in 4% formaldehyde were embedded in paraplast and sections of 7 μm were made.

Immunohistochemistry. H&E stained sections were assessed by an experienced pathologist for viability and the presence of tumour cells. Tissue expression of CAR and EphA2 was studied using material obtained from the department of Pathology.

For immunostaining of CAR, cryo-sections were fixated in 80% acetone and treated with 3% sodium azide to block endogenous peroxidase activity. Then the slides were blocked with 2.5% normal goat serum and 2.5% fetal calf serum in

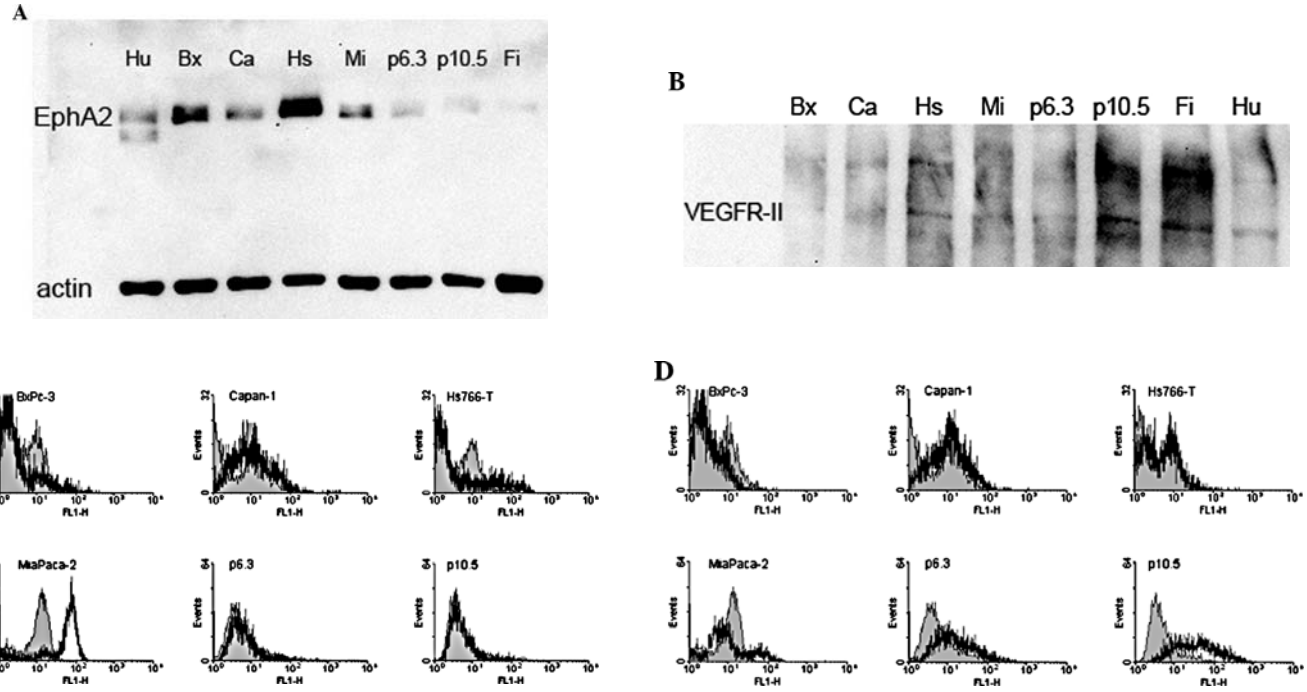

Figure 1. Expression of the EphrineA2 receptor, vascular endothelial growth factor receptor-II, neurotensin receptor and Thomsen-Friedenreich antigen on human pancreatic cancer and normal cells. Cell lysate (25 μg) of the human pancreatic cancer cell lines BxPc-3 (Bx), Capan-1 (Ca), Hs766-T (Hs), MIAPaCa-2 (Mi), p6.3, p10.5, human breast fibroblasts (Fi) and human umbilical vein endothelial cells (Hu) were separated by SDS-page gel electrophoresis and blotted. (A) The EphA2 receptor was detected using the monoclonal clone D7. (B) The VEGFR-II was detected using the anti-KDR mono-clonal. Detection of the human actin protein was used to show equal loading on the Western blotting. (C) The neurotensin receptor was detected with the NTR monoclonal antibody using FACS analysis. (D) The Thomsen-Friedenreich receptor was detected using the monoclonal antibody A78-G/A7 for FACS analysis. For both antibodies an isotype control antibody served as negative control (shaded peaks).

PBS/Triton 0.1% for 90 min at R.T. CAR detection was subsequently performed using a 1:500 dilution of monoclonal antibody to CAR (Upstate, clone RmcB).

Immunohistochemistry for all other antigens was performed on 4% PFA fixed slices embedded in paraplast. Endogenous peroxidase was blocked with 3% peroxide. EphA2 antigen retrieval was performed by steaming in 10 mM Tris (pH 9.4)/1 mM EDTA for 10 min at 120˚C. The slides were cooled in running tap water and blocked in 2.5% normal goat serum and 2.5% normal human serum in PBS/Triton 0.1% prior to incubation overnight at 4˚C with EphA2 antibody (1:400 dilution, Santa Cruz, clone sc-924), or with anti-GFP (1:1000, clone JL-8, Clontech, Palo Alto, CA). Presence of primary antibody was detected using the Powervision system (ImmunoLogic, Duiven, The Netherlands). Tissues were washed and incubated with streptavidin-horseradish peroxidase conjugate (Dako). Tissues were stained with 3.3'-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. All tissue slice sections and immunolabeling staining were assessed by an experienced gastroenterological pathologist for tumour differentiation grade, localization and frequency of antigen expression, viability of slices, and the presence of tumour cells.

Results

Receptor expression on primary and established human cells. To achieve retargeting of adenovirus we focused on alternative receptors that have been detected on pancreatic cancer cells. Based on literature data we selected four potential targets. To confirm presence of these receptors in PC we studied their presence in a panel of human pancreatic cancer cells, normal human primary fibroblasts and endothelial cells (HUVECs) using Western blotting for EphA2 and VEGFR-II (Fig. 1A and B) or FACS analysis [neurotensin receptor-1 (NTR-1)] and Thomsen-Friedenreich antigen (TF) (Fig. 1C and D).

The EphA2 (MW 140 kDa) was differentially expressed on all pancreatic cancer cells with lowest expression on PC tumour cells p6.3 and p10.5. Human fibroblasts lacked EphA2 expression but human endothelial cells did express EphA2 (Fig. 1A). VEGFR-II (MW 200-230 kDa) was expressed on all pancreatic cancer cells as well as normal human fibroblasts and HUVECs (Fig. 1B). The neurotensin receptor was only expressed on MIAPaCa-2 cells (Fig. 1C). The Thomsen-Friedenreich antigen was detected only on the MIAPaCa-2 cell lines and pancreatic cancer cell lines p6.3 and p10.5. From these results we conclude that based on their expression patterns all four receptors are suitable for redirecting Ad to human PC.

Transduction of human pancreatic cancer cell lines in vitro. Insertion of targeting sequences for the VEGFR-II, EphA2 and NT into the HI loop resulted in viable viral vectors that could be propagated on 293 cells. Constructs with an insertion of the p30 sequence that binds to the TF antigen did not result in detectable adenovirus replication. Since repeated attempts failed we assume that the HI-loop can not harbour this peptide without loss of proper secondary and/or tertiary structure of the fiber protein.

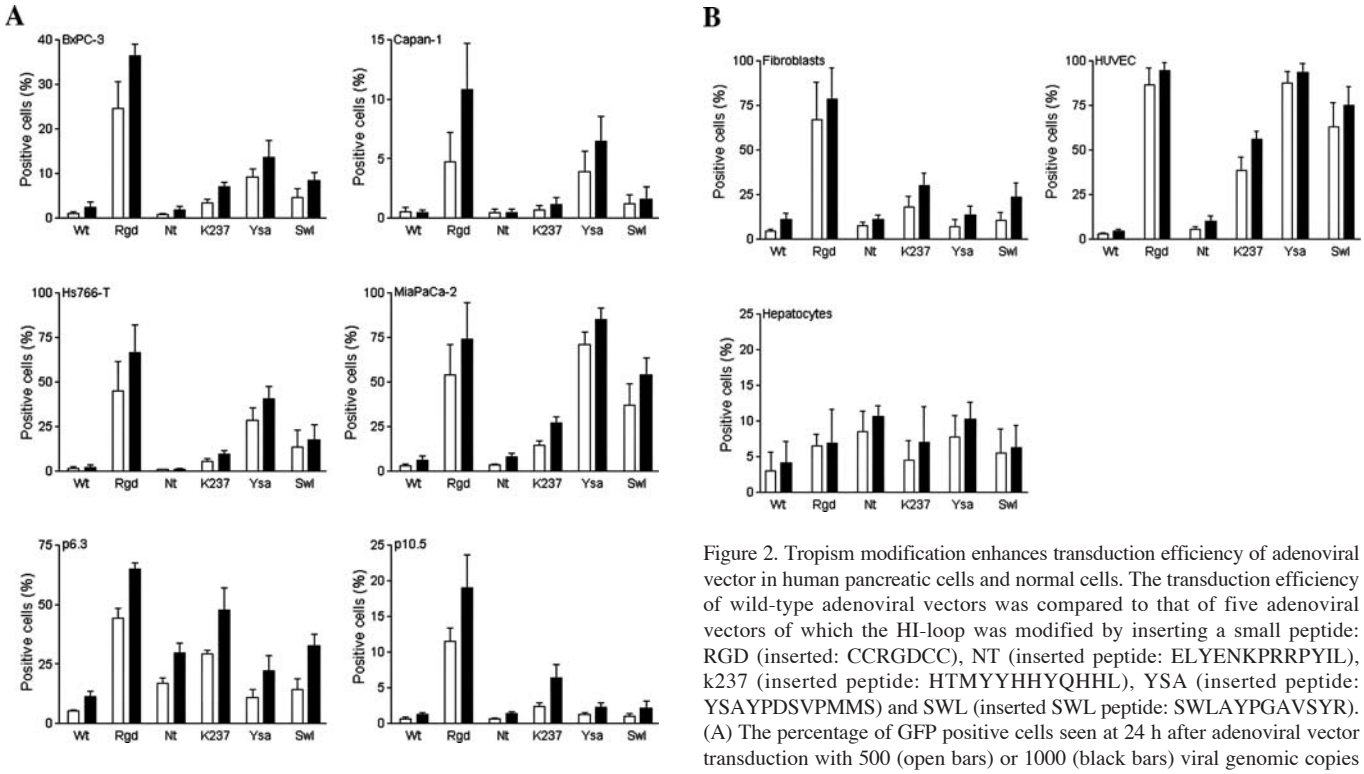

Figure 2. Tropism modification enhances transduction efficiency of adenoviral vector in human pancreatic cells and normal cells. The transduction efficiency of wild-type adenoviral vectors was compared to that of five adenoviral vectors of which the HI-loop was modified by inserting a small peptide: RGD (inserted: CCRGDCC), NT (inserted peptide: ELYENKPRRPYIL), k237 (inserted peptide: HTMYYHHYQHHL), YSA (inserted peptide: YSAYPDSVPMMS) and SWL (inserted SWL peptide: SWLAYPGAVSYR). (A) The percentage of GFP positive cells seen at 24 h after adenoviral vector transduction with 500 (open bars) or 1000 (black bars) viral genomic copies per cell is given for human pancreatic cancer cells MIAPaCa-2, BxPC-3, Hs766-T, Capan-1, p6.3 and p10.5. (B) The percentage of positive cells seen at 24 h in primary human umbilical vein endothelial cells, human primary fibroblast and human primary hepatocytes. In all experiments infections were performed in the presence of 5 μg recombinant fiber knob to block CAR mediated entry. Values represent the mean ± standard deviation of 2 or 3 independent experiments.

Table I. Vector preparation for studies in cells.

Vector	Genomic copies/ml	Infectious particles/ml	GC/pfu ratio
Ad-wt	$4.6x10^{10}$	$2.0x10^{8}$	230
Ad-RGD	$8.8x10^{10}$	$3.2x10^{8}$	275
Ad-NT	$1.0x10^{11}$	$2.5x10^{8}$	400
Ad-K237	$6.4x10^{11}$	$5.0x10^{9}$	128
Ad-YSA	$3.9x10^{11}$	$1.6x10^{9}$	244
Ad-SWL	$5.1x10^{10}$	$5.0x10^{8}$	102

Vector preparation used for slice experiments.

Vector	Genomic copies/ml	Infectious particles/ml	GC/pfu ratio
Ad-wt	$3.2x10^{11}$	$1.5x10^{9}$	203
Ad-RGD	$1.4x10^{11}$	$1.0x10^{9}$	139
Ad-NT	$1.0x10^{11}$	$2.5x10^{8}$	400
Ad-K237	$6.4x10^{11}$	$5.0x10^{9}$	128
Ad-dsRED	$2.4x10^{12}$	$2.4x10^{10}$	100
Ad-YSA	$3.9x10^{11}$	$1.6x10^{9}$	244
Ad-SWL	$5.1x10^{10}$	$5.0x10^{8}$	102

Physical properties of adenovirus stocks for *in vitro* and *ex vivo* transduction experiments. Ad-CMV-eGFP vectors were propagated with no peptide (wt) or the following peptide in the HI-loop of fiber knob: CCRGDCC (Ad-RGD), neurotensin (Ad-NT), K237 (Ad-K237), YSA (Ad-YSA), SWL (Ad-SWL). Ad-CMV-dsRED served as a control vector for tissue slice experiments. The amount of genomic copies and infectious particles (gc/pfu) was determined as described in Materials and methods.

To ensure that all redirected viral batches were of comparable quality we determined their infectivity on 293 cells and we assessed the number of genome copies using a qPCR method (Table I). For all subsequent experiments only vector preparations with a genomic copies to plaque forming unit ratio (gc/pfu) below 400 were used.

Redirecting recombinant adenoviruses with peptides inserted into the HI loop targets the receptors present on PC. To determine the effect of redirecting adenovirus from CAR to specific receptors on pancreatic tumour cells we transduced several human PC cell lines and non-malignant human cells with our modified adenoviral constructs (Fig. 2). Wild-type virus Ad-wt (wt-Ad-CMV-eGFP) transduced cells entirely via CAR, as demonstrated by a complete block of transduction upon addition of recombinant fiber knob to the medium (not shown). As a positive control for modified virus, we used a construct with the RGD motif in the HI loop. In all cancer cell lines tested as well as in normal human fibroblasts and HUVECs, the transduction efficiency of the RGD vector was the highest, which indicates that the RGD motif does not provide specific transduction of PC cells.

The high expression of the neurotensin receptor suggested that it could be a specific target for PC transduction. Insertion of the high affinity natural ligand for this receptor, (ELYENKPRRPYIL), did not improve transduction of MIAPaCa-2 cells although these cells abundantly express NTR-1 (Fig. 1C) In contrast, the transduction efficiency of

the pancreatic cancer cell line p6.3 was enhanced. It seems likely that this is due to non-specific binding since this PC cell line does not express the NTR (Fig. 1C).

The peptide K237 binds VEGFR-II *in vitro* with low affinity and inhibits tumour growth and metastasis *in vivo*. We reasoned that expression into the HI-loop of fiber knob targets adenovirus to VEGFR-II. Incubation of Ad-K237 with pancreatic cancer cells showed indeed an increase in transduction efficiency in all PC cell lines and fibroblasts. The targeting indexes are moderate (4 to 5-fold). Only in the PC cell line p6.3 and in endothelial cells targeting indexes of 10 were obtained. The increased transduction efficiency suggests that insertion of the K237 peptide in the HI loop indeed provides uptake of adenovirus via VEGFR-II.

Since the EphA2 receptor is expressed in several tumours, including pancreatic cancer, it is an attractive target. The EphA2 mimetic YSA peptide selectively targets the EphA2 receptor with high affinity (Kd=186 nM). This high affinity allows specific delivery of cytotoxic agents to blood vessels of diseased tissues by this peptide. The SWL peptide also binds to the EphA2 receptor albeit with a lower affinity (Kd=678 nM). Because of their specificity both peptides seemed good candidates to redirect adenovirus to the EphA2 receptor that has an enhanced expression on pancreatic cancer cells. All established pancreatic cancer cell lines could indeed be targeted by Ad-YSA. The targeting index was 7- to 20-fold, which is almost comparable to that obtained by Ad-RGD. The transduction efficiencies were lower in the PC cell lines p6.3 and p10.5 that also have the lowest expression of the Eph2 receptor (Fig. 1A). Insertion of the SWL peptide was found to be less effective which may be due to the lower affinity of this peptide for the EphA2 receptor (37). Human primary fibroblasts do not express EphA2 and indeed were found to be refractory to Ad-YSA transduction, while transduction of human primary hepatocytes was only 2-fold higher compared to Ad-wt. Human HUVEC cells that do express this receptor (37) are efficiently transduced by YSA and SWL redirected vectors. The close correlation seen between transduction efficiency and Eph2 expression suggests that insertion of the YSA peptide in the HI-loop redirects adenovirus to this receptor. These results reveal that the Ad-YSA and the Ad-K237 display a specific increase in transduction efficiency of human PC cell lines and therefore seem promising for targeting pancreatic cancer.

Synthetic targeting peptides inhibit transduction of human PC cell lines by Ad-YSA and Ad-K237. To confirm specific uptake of redirected adenovirus we performed competition experiments with YSA and K237 peptides (Fig. 3). Pancreatic cancer cell lines MIAPaCa-2 and Hs766-T expressing the EphA2 and VEGR-II receptor were pre-incubated with each of the synthetic peptides and fiber knob, to block CAR-dependent uptake. Transduction of Ad-RGD was unaffected by the addition of either peptide, indicating that the peptides do not interfere with integrin mediated uptake.

In both PC cell lines, transduction by Ad-YSA was strongly inhibited by addition of the YSA peptide. Pre-incubation with 500 μM YSA, impaired Ad-YSA transduction of MIAPaCa-2 by 60% and of Hs766-T by 93%. This strong competition indicates that infection of these cells is indeed mediated by

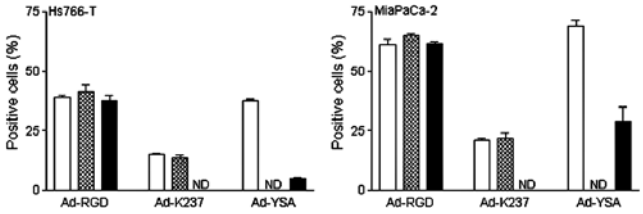

Figure 3. Insertion of YSA peptide in the HI loop mediates specific adenoviral transduction of human pancreatic cancer cells. The human pancreatic cancer cell lines MIAPaCa-2 and Hs766-T were incubated with Ad-RGD, Ad-K237 and Ad-YSA (MOI=500 viral genome copies per cell) after pre incubation at room temperature with 5 μg fiber knob (all instances) and 750 μM K237 peptide (dashed bars), 500 μM YSA peptide (solid bars), or no peptide (open bars). Subsequently, cells were washed and virus was allowed to adhere to the cells at 37˚C for 60 min. Cells were washed again and the percentage of GFP expressing cells was determined by FACS 24 h later. Values represent the mean ± standard deviation of triplicate experiments. ND; not determined.

the YSA peptide inserted into the HI loop. Since others have shown that specific binding of the YSA peptide to the EphA2 receptor results in efficient homing to pancreatic cancers *in vivo*, Ad-YSA seems a promising candidate for specific targeting to this tumour type.

In contrast, transduction of both cancer cell lines by Ad-K237 is not inhibited by addition of the K237 peptide. Even the presence of 500 μM K237 peptide did not lower transduction levels. Also the transduction of human endothelial cells was not inhibited by this peptide. The lack of competition inhibition by this targeting peptide suggests that entry of the Ad-K237 takes place via a non-specific mechanism and thus not by the VEGFR-II. Apparently the K237 peptide behaves differently from the synthetic K237 peptide most likely due to structural constraints within the HI loop.

Transduction of normal pancreas and pancreatic cancer tissue slices (ex vivo) by recombinant adenoviruses. Several studies have shown that transduction efficiency of adenovirus in cell culture experiments does not predict its efficacy *in vivo*. We developed a system for transduction of pancreatic explants where both normal tissue and cancer tissue can be cultured for up to 72 h without major loss of viability (44). We compared the transduction efficiency of adenoviral vectors redirected by RGD, K237 and YSA peptides in tissue slices of normal pancreas and pancreatic cancer. All slices were co-infected with an equal amount of Ad-CMV-dsRED (dsRED derived from normal vector) as an internal control to correct for differences in viability, size and composition.

The ratio of GFP to dsRED fluorescence provides a measure of transduction efficiency of the modified vector normalized for the efficiency of the wild-type vector. Co-infection of a non-modified GFP expression vector and the dsRED control vector should yield a ratio of 1. With the viral batches used in this study, this ratio was not always 1 due to the lower gc/pfu ratio of the dsRED vector (Table I). Slices that displayed no detectable dsRED and GFP expression were considered non-viable and were excluded (10-15% of all slices). The transduction efficiency of pancreas cancer by our tropism modified adenovirus expressing GFP was compared to that of unmodified wt-adenovirus expressing dsRED.

Table II. Characteristics of patient specimens and outcome of adenovirus targeting.

				Wt-GFP/ dsRED		RGD-GFP/ dsRED		K237-GFP/ dsRED		YSA-GFP/ dsRED	
ID	Differentiation	CAR	EphA2	Mean	SD	Mean	SD	Mean	SD	Mean	SD
T.1	Poor	<10% ++	>50% ++	1.00	0.08	5.25*	1.42	1.92	0.58	3.00*	0.30
T.2	Poor	>50% +	nd	1.00	0.27	3.11	1.69	1.48	1.08	2.64	0.94
T.3	Moderate	>50% +++	>50% ++	1.00	0.31	7.41*	7.01	2.19	0.47	2.61	1.19
T.4	Poor	<50% ++	>50% +	1.00	0.31	3.04*	0.60	3.39*	0.68	5.35*	2.42
T.5	Well	<10%	>50% +++	1.00	0.31	4.04*	1.69	2.82*	1.95	7.24*	1.13
T.6	Moderate	<50% ++	nd	1.00	0.31	1.90*	0.82	1.25	0.03	2.51*	0.24
T.7	Moderate	<10%	>50% ++	1.00	0.31	2.33*	0.89	2.04*	0.28	3.60*	0.40
T.8	Poor to moderate	nd	>50% +	1.00	0.31	nd	nd	nd	nd	3.56*	0.22
T.9	Poor to Moderate	<10%	>50% +	1.00	0.31	nd	nd	nd	nd	2.50*	0.14
T.10	Moderate	nd	>50% ++	1.00	0.31	nd	nd	nd	nd	1.21	0.53
T.11	Moderate	nd	>50% +	1.00	0.31	nd	nd	nd	nd	4.47*	0.62
T.12	Liver metastases	nd	nd	1.00	0.31	nd	nd	nd	nd	4.00*	0.49
N.1	Pancreatitis	nd	nd	1.00	0.22	nd	nd	nd	nd	1.72	0.39
N.2	Normal pancreas	nd	nd	1.00	0.02	nd	nd	0.42*	0.05	1.28*	0.06
N.3	Fibrosing pancreatitis	nd	nd	1.00	0.18	1.24	0.09	1.14	0.29	2.44*	0.58
N.4	Normal pancreas	>50% ++	>50% +++	1.00	0.27	2.45*	1.10	1.15	0.51	1.84	0.20
N.5	Normal pancreas	nd	nd	1.00	0.05	nd	nd	nd	nd	1.54*	0.11

Human pancreatic cancer (T.1-T.11) or pancreatitis (N.1 and N.3) was resected according to Whipple's procedure or during bypass surgery for palliation (T.12). Normal pancreas was obtained from Whipple specimens from patients with cholangiocarcinoma or papillary cancer (N.2, N.4 and N.5). The tissue was sliced typically yielding 15-50 oval slices that were infected with the following adenoviruses: Wt-GFP, RGD-GFP, K237-GFP and YSA-GFP. An equal amount of Wt-dsRED was included to serve as an internal control. Typically, 4-6 slices were infected per virus. All studies were performed with $1.0x10^8$ (normal pancreas) to $5.0x10^8$ (tumour) gc per slice (~10 pfu/cell) for sufficient reporter protein expression to allow detection in the fluorimeter. Three days after initiation of the experiment the slices were lysed and total fluorescence was determined fluorometrically. Non-infected slices served as negative control. The fluorescence values per slice were used to calculate the GFP/dsRED ratio per slice and the mean values ± standard deviation (SD) of these ratios were calculated. Wt-GFP/dsRED was normalized to 1 in order to serve as a reference value for calculation of the targeting index of fiber modified vectors compared to Wt-GFP. A two-way ANOVA, with patient and adenovirus type as factors, showed that both in the tumor and in the normal pancreas, the targeting per virus was patient dependent. Therefore, one-way ANOVA analysis per patient was used to determine whether the targeting index was significantly enhanced ($^*p<0.05$) compared to Wt-GFP. Finally, the mean targeting indexes of YSA-GFP between tumour (n=12) and normal pancreas (n=5) were compared to each other using the independent t-test (see text). CAR and EphA2 were immunolabeled immunohistochemically by using the original surgical specimens. We scored receptor expression according to the following system: <10%, <50% or >50% positive cells; weak (+), moderate (++) or strong (+++); nd, not determined. Two-way ANOVA analysis showed no interaction between EphA2 receptor expression in tumour explants and targeting efficacy of Ad-YSA. All studies were performed with the same batches of virus to exclude differences in targeting index due to variations in batch quality.

The difference in expression of these two reporter genes is presented as the targeting index (Table II). The targeting index of Ad-RGD, Ad-K237 and Ad-YSA ranged from 1.9 to 7.4; 1.2 to 3.3; and 1.2 to 7.2, respectively. Our data show that the variation in transduction efficiency between slices was considerable. Some cancers were more susceptible to Ad-RGD relative to Ad-YSA (T.3) and vice versa (T.5). This is not unexpected and reflects the physical heterogeneity of PC. The maximal targeting indexes of Ad-RGD and Ad-YSA were lower in the *ex vivo* setup (7.4 and 7.2-fold, respectively) compared to the *in vitro* experiments. Herein, maximal infectivity enhancement was 30- and 20-fold, respectively (Fig. 2A).

In line with the previous experiments on PC cell lines, insertion of K237 resulted in minimal targeting towards PC (average targeting index =2), indicating low affinity or aspecific binding to pancreatic cancer cells. Pooled analysis of all infected explants showed that the targeting index of Ad-YSA was 3.55±1.58.

To investigate if the modified vectors efficiently transduce normal pancreas cells we determined the transduction efficiency of the redirected vectors in normal pancreas tissue slices (Table II). Normal pancreatic slices were viable for at least three days without major morphological deteriorations (44). Ad-wt easily transduced normal pancreas. The targeting indexes of normal pancreas and of pancreas with benign disease such as fibrosis and pancreatitis were <2.5, <1.2, <2.5 for Ad-RGD, Ad-K237 and Ad-YSA, respectively. For Ad-YSA, the enhancement in transduction was significant in 3 out of 5 patients with the highest targeting index in fibrosing pancreatitis.

Hence, Ad-YSA seems to enhance infectivity of normal pancreas. However taken together the targeting indices by Ad-YSA in normal pancreas (1.76±0.43) was significantly lower than in pancreatic cancer (3.55±1.58; p=0.027), which suggest that insertion of the YSA peptide does provide specific targeting to pancreatic cancers.

Pre-clinical and clinical studies suggest that pancreatic cancer is refractory to adenoviral infection, indicating the requirement of tumour redirected vectors. In these studies we found consistent transduction of *ex vivo* pancreatic cancer slices by Ad-wt (Table II). However, compared to slices derived from normal pancreas tissue transduction of cancer slices by wt Ad was clearly reduced. A 5-fold higher dose of wt Ad ($5x10^8$ per slice) was required to provide transduction levels in cancer slices comparable to those in normal tissue ($1.0x10^8$ genomic copies per slice). Our observation that cancer tissue in this *ex vivo* system is more refractory to adenoviral infection than normal tissue suggests that the poor transduction of PC *in vivo* may in part be due to loss of adenovirus to surrounding normal tissue.

Receptor expression in pancreatic tissue. The observed variations in targeting indexes between tissue slices is most likely caused by differences in receptor expression. Therefore, we decided to determine the expression of the receptors that mediate entry of unmodified vector (CAR) and YSA redirected vector (EphA2). However, expression of both receptors could not be studied in all specimens due to limited availability of tissue. We observed differential CAR expression among

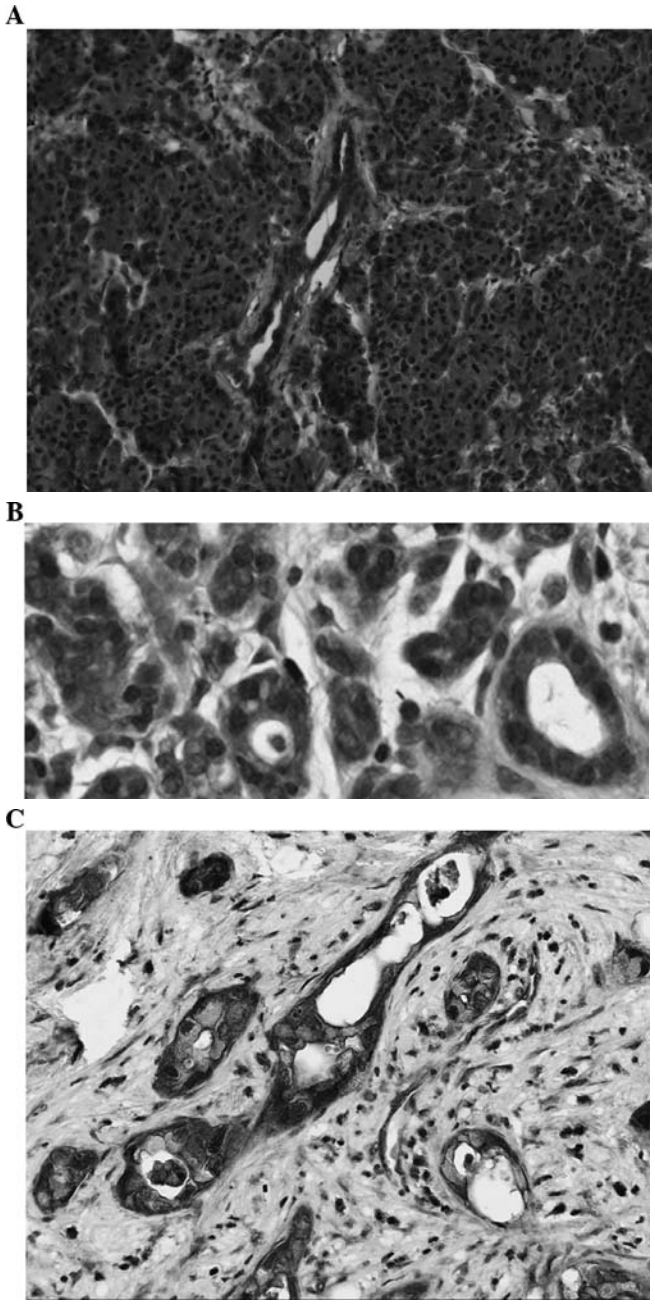

Figure 4. Expression of the EphA2 receptor in normal human pancreatic tissue (A and B, original magnification, x100) and in human pancreatic cancer (C, original magnification, x200) as detected by immunohistochemistry.

pancreatic cancers (Table II). CAR was completely absent in two poor to moderately differentiated pancreatic cancers (T.5 and T.7). The majority (5 out of 9) of sections revealed a mixed staining pattern with areas that completely lacked CAR expression to areas with weak positive staining. Strongest staining was observed in a moderately differentiated tumour (T.3), whereas poorly differentiated areas within a tumour frequently lost CAR expression. Blood vessels and inflammatory cells were not labelled. CAR localized to the apical membrane and at the domain of cell-to-cell contact of acinar and ductal cells in normal pancreas, while islet cells appear to be negative.

The expression of the EphA2 receptor also differed largely between specimens. EphA2 expression was detected in the

islets of Langerhans, exocrine- and ductal cells, fibroblasts, nerves, and muscle tissue in non-malignant pancreas (Fig. 4A). EphA2 staining was mostly observed as a cytoplasmic staining (Fig. 4B), although in ductal cells focally membranous staining was seen. Expression of EphA2 varied not only between pancreatic cancer specimens but also within the specimens (Table II). Some tumours contained regions without any EphA2 signal while other regions had a very clear signal. Similarly to normal tissue, the pattern of EphA2 staining was mostly diffusely cytoplasmic only in T.1 and T.3, thus in 2 out of 11 (18%) cases, clear staining of the membrane was observed (Fig. 4C). Reduced membraneous staining for EphA2 seemed to correlate with loss of differentiation in T.3. In some cases malignant tissue was accompanied by a desmoplastic reaction or chronic fibrosing pancreatitis. Fibroblasts and macrophages present in these areas also showed cytoplasmic staining. In conclusion, the localization and intensity of EphA2 staining varied not only between patient specimens but also within pancreatic cancers specimens.

Discussion

Lack of effective treatment for pancreatic cancer urges the need for new therapies such as adenovirus mediated gene therapy. Although pre-clinical studies showed efficacy of these approaches both *in vitro* as well as in animal models *in vivo*, subsequent clinical trials, however, lacked efficacy. Several studies demonstrated that low adenoviral transduction of cancer cells limited the effect of this treatments. The aim of this study was to improve viral entry of pancreatic cancer cells by incorporating peptides into the HI loop that had been demonstrated to target receptors highly expressed on pancreatic cancer. We studied the tropism of the modified adenoviral vectors upon insertion of peptides in the fiber knob. We report the successful incorporation into the HI loop of YSA, a peptide ligand targeting the EphA2 receptor, and K237, a peptide targeting the VEGFRII. Subsequently we showed that both peptides enhanced the transduction of a number of human PC cancer cell lines that abundantly express the targeted receptor. Additional competition studies confirmed that the YSA peptide indeed redirects adenovirus from CAR to EphA2 receptor mediated entry. In contrast, lack of competition inhibition suggested that instead of VEGFRII mediated entry, Ad-K237 enters in a non-specific manner. Since receptor expression and accessibility on established cell lines frequently differs from that of cancers *in vivo*, we went on testing our redirected adenoviral vectors in an *ex vivo* system. Slices of pancreatic cancer and normal pancreas were transduced with the redirected vectors and the adenoviral vector containing the natural tropism as a control. We demonstrated that Ad-YSA revealed increased transdution to *ex vivo* pancreatic cancer slices compared to Ad-wt, and that it targets the EphA2 receptor. Since tissue structure remains intact transduction efficiencies observed in this system may more reliably predict the effects of tropism modification on cancer transduction in patients *in vivo* (45).

Other groups have also reported on the exposure of short peptides in the adenovirus fiber knob region to overcome the limiting CAR expression on cancers *in vivo*. Several groups for instance have used peptides that were identified by phage library bio-panning. Apart from some successful studies (46-48) a considerable number of identified peptides did not appear to be feasible for expression in the HI loop. In part this is due to the structural hurdles of knob manipulation such as inhibition of fiber trimerization and/or changes in the peptide structure. A recently developed random peptide display vector-screening system does allow *in vivo* selection of pancreatic cancer specific peptides such as SYE for incorporation into the fiber knob (49). Up to now, most peptide based retargeting studies were performed with the RGD motif. *In vivo*, this peptide enhances gene delivery to pancreatic cancer (22) and melanoma (22,50,51) but also to kidney, spleen and heart (52), which shows its specificity for cancer cells is limited. The limited specificity in part is due to the presence of the binding sites for the native receptors, integrins and CAR, on the modified vector. This problem can be overcome by ablation of these binding sites (53) or by using a bi-specific adaptor molecule (54).

In this study we focused on several peptide sequences which bind to up-regulated membrane antigens known to be over-expressed in PC. Except for the p30 peptides, all selected peptides could be inserted into the adenoviral knob without interfering replication of adenovirus. Repeated transfections of the constructs containing the insertion of the p30 peptide and p30-1 in the HI loop failed to show the typical plaque formation observed upon adenoviral replication. Since sequencing did not reveal mutations it seems that insertion of these peptides interferes with fiber knob trimerization. One explanation could be the fact that this peptide has a poor water solubility due to the presence of several hydrophobic amino acid residues (34). Neurotensin (55) is the ligand peptide for neurotensin receptor-1. Although only the last six amino acids are important for its biological activity, we chose to express the full length peptide to preserve its natural conformation. Ad-NT had a relatively high genomic copies to plaque forming units ratio, suggesting that this peptide may lower the transduction efficiency of the virus. In addition, incorporation of the neurotensin peptide did not increase transduction of NTR-1 expressing cells.

Similar to that seen for the p30 peptides, it seems that the presence of aromatic amino acids in the neurotensin peptide hampers adenoviral biology.

EphA2 has become a prime target in the field of cancer research. Most solid tumours over-express the protein on the cell surface where it accumulates in its unphosphorylated inactive form. EphA2 fails to bind its down-regulating EphrinA1 ligand, although over expressed on the cell surface in cancer cells. Since activation of EphA2 impairs growth, survival, migration and invasion of tumour cells the insensitivity of this receptor seems to increase malignancy (56). In addition, several studies strongly support a role for EphA2 in pathological vascular development such as tumour angiogenesis. Several strategies have been employed successfully to target EphA2 including immunotherapy, soluble EphA2 receptors, antisense oligonucleotide-based targeting, and siRNA targeting (57). We showed that the YSA peptide, which is a high affinity mimetic agonist for EphA2, retained its functional characteristics in the HI loop of fiber knob by directing non-replicating adenovirus from CAR to EphA2 on tumour cells *in vitro*. It is unknown if the bioactive properties

of the peptide, i.e. stimulation of EphA2 tyrosine phosphorylation and retaining of signalling are still present. In line with the reported affinities of both EphA2 binding peptides (37) the YSA peptide proved to be more potent than the SWL peptide and may thus be a valuable tool to target EphA2 expressing tumour cells and tumour endothelium at sites of angiogenesis.

The poor translation from pre-clinical gene therapy studies to subsequent clinical trials indicated that both *in vitro* systems and animal models do not reflect the nature of cancers in patients. To overcome this problem for adenoviral gene therapy *ex vivo* thin tissue slice technology has been developed. In this system adenoviral transduction is studied in freshly obtained benign and tumour specimens that, depending on the organ, can be kept alive for up to three days (58). *Ex vivo* tissue slices represent a close reflection of *in vivo* organ complexity, compared to monolayer cell lines that accumulate mutations. We have developed a slice system for pancreas; using supplement-enriched medium we are able to culture pancreatic tissue for up to six days with preservation of most morphological characteristics (44). The conversion of WST-1 is maximal at three days and subsequently slightly decreases. Based on this good viability after three days of culture we propose that this system is suitable for transduction of adenoviral vectors. Analysis with GFP indicated that adenoviral infection of the slices is limited to the superficial layers. As reported for slices from other organs such as liver and esophageal cancer, physical barriers such as ECM seem to prevent tissue penetration of adenovirus particles.

We chose to perform co-infection with a non-modified adenoviral vector expressing dsRED. Adding this control allowed to exclude less viable slices. Importantly, adding an internal control made it possible to correct for effects on adenoviral transduction due to differences in composition, size and viability of the slices since targeting efficiencies can be determined within each slice. Ad-YSA showed a low targeting index: <2.4 compared to normal pancreas tissue. In pancreatic cancer the targeting index with Ad-YSA was enhanced although in some tumour specimens targeting was absent or very low. With Ad-RGD similar differences in the targeting index were found. The targeting index depends on the transduction efficiency of the control virus, determined mainly by CAR and integrins, and of the modified viral vectors, determined by presence of the EphA2 receptor. Therefore, a low targeting index may not only be due to a low expression of the EphA2 receptor but may also result from a high CAR expression. Previous studies suggested that low receptor expression is one of the factors causing a poor efficacy of non-modified adenovirus *in vivo*. However, since our data showed a large difference in targeting efficiency we decided to determine expression of the EphA2 receptor and CAR. We have observed that pancreatic cancers do express CAR with a tendency of higher expression in well-differentiated tumour areas. Our finding confirms a recent report showing a significant correlation between CAR expression and histological grade, with moderately to poorly differentiated pancreatic tumours most frequently demonstrating loss or reduction of CAR expression (59). Other studies showed that additional cellular factors are involved in adenovirus entry such as integrins and the surface bound heparan sulfate proteoglycans syndecan-1 and glypican-1 that are over-expressed in pancreatic cancer (22,60,61). The presence of all receptors involved in adenoviral entry agrees with our finding that all pancreatic cancers slices could be transduced with wild-type adenovirus. These observations suggest that pancreatic cancer cells are not entirely refractory to adenovirus transduction. However, we also showed that compared to normal pancreas tissue adenoviral transduction efficiency is 5-fold less in cancer specimens. Therefore, it seems that in addition to features such as widespread fibrosis, loss of virus to surrounding normal tissue may hamper adenovirus efficacy in patients. Since all requirements for entry of non-modified adenovirus seems to be present on these cells the differences in targeting index were expected to be due to differences in the EphA2 and VEGRFII receptors. Indeed we observed that the EphA2 receptor was differentially expressed among and within tumour specimens, with primarily cytoplasmic staining. This is unexpected to some extent since the receptor resides in the membrane. However, previous studies from other groups observed a similar staining pattern (62,63). It seems that an important amount of the receptor has an intracellular localization. Our data with the Ad-YSA and the competition experiments, however, clearly indicate that the receptor is accessible. A possible explanation may be that the receptor shuttles between the membrane and the cytoplasm. A recent study reported that in comparison to normal pancreas the expression of the EphA2 is increased in infiltrating cancer and metastasis especially in poorly differentiated carcinoma (64).

In our study we also found high expression of the EphA2 receptor but did not observe a tight correlation between the EphA2 status and targeting index. This lack of correlation may in part result from heterogeneity in cancer specimens (65). The small size of most tumour specimens did not allow us to determine and to quantify the presence of all receptors relevant for adenoviral entry that may explain the observed differences in targeting index. The targeting indexes for RGD retargeting, that redirects adenovirus from CAR to integrins, also show a large variation. This suggests that the expression of these two native receptors in slices is indeed heterogeneous and may cause the lack of correlation between EphA2 expression and Ad-YSA transduction.

Overall, we demonstrated in this study that Ad-YSA targets the EphA2 receptor. Furthermore, we show that this results in increased transduction of established human pancreatic cancer cell lines as well as freshly obtained pancreatic cancer slices. Thus, insertion of the YSA motif results in an increase in transduction efficiency comparable to that seen for the RGD motif. Since the YSA peptide targets a receptor which is more specific for pancreatic cancer, it may also prove to be more specific in an *in vivo* model. Additional *in vivo* studies to compare RGD to YSA therefore should be performed to determine the potential benefit of this novel vector.

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