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Multimodal approach using oncolytic adenovirus, cetuximab, chemotherapy and radiotherapy in HNSCC low passage tumour cell cultures

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is a common and often devastating disease without curative treatment when advanced or recurrent. The aim of this study was to assess whether capsid-modified oncolytic adenoviruses have therapeutic efficacy in HNSCC low passage tumour cell cultures and if it could be further improved by combination with cetuximab, radiotherapy and chemotherapy. We investigated which adenoviral capsid modifications allow best gene transfer and cell killing of HNSCC substrates. Gene transfer was assessed using replication-deficient adenoviruses expressing luciferase. Cell killing was studied *in vitro* and *in vivo* using oncolytic adenoviruses, which kill tumour cell by viral replication. The most effective capsid-modified oncolytic adenoviruses were combined with HNSCC standard therapies and their efficacy was assessed *in vitro* as well as *in vivo*. Cell killing was assessed *in vitro* by MTS assay and *in vivo* by HNSCC subcutaneous tumour growth follow-up in nude mice. Cetuximab treatment was found to enrich CD133+ and CD44+ tumour-initiating type cells in tumours grown in mice. Capsid-modified viruses showed increased transduction and oncolysis of HNSCC substrates in comparison to Ad5-based agents. Polylysine (pK7)-modified oncolytic virus resulted in significant tumour growth reduction *in vivo*. Combination of chemotherapy (cisplatin and 5-fluorouracil), radiotherapy and cetuximab with oncolytic adenovirus therapy resulted in further increases in cell killing effect *in vitro* and complete eradication of tumours *in vivo*. Our pre-clinical data suggest that it is feasible and efficacious to combine oncolytic adenoviruses with HNSCC standard therapies into a multimodality treatment regimen for clinical testing in HNSCC patients.

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1. Introduction

Each year, more than 615,000 new head and neck cancer cases are diagnosed worldwide. Approximately 90% of them are squamous cell carcinomas (HNSCC). Currently, the standard of care for HNSCC combines surgery, radiotherapy and chemoradiotherapy. Despite some progress, the overall 5-year survival rate is below 50%; unchanged for nearly three decades. Surgery can be mutilating and often has significant effects on swallowing and speech. The addition of chemotherapy to radiotherapy has been useful in the context of organ preservation, but has resulted in limited improvement in survival rates.[1] Cisplatin and 5-FU remain the standard chemotherapy agents for HNSCC. Mortality associated with disease and morbidity associated with its treatments have encouraged the pursuit of alternative therapeutic strategies.

The main biochemical mechanism of action of cisplatin involves the binding of the drug to DNA in the cell nucleus and subsequent interference with normal transcription and/or DNA replication mechanisms.[2] However, there are also other possible targets.[3,4] 5-Fluorouracil (5-FU) is a pyrimidine analog that requires cellular uptake and metabolic activation in order to exert cytotoxicity.[5,6] Ionising radiation targets primarily DNA molecules and produces an array of lesions that include single-strand breaks, base alterations, oxidative damage and double-strand breaks.[7]

Monoclonal EGFR inhibiting antibodies have improved the efficacy of conventional chemotherapy in both pre-clinical and clinical studies. Although such therapies may lead to partial response or disease stabilisation in some patients, many patients do not benefit from EGFR inhibitors therapy. Even those who do, eventually develop resistance.[8] Great interest therefore exists in elucidating resistance mechanisms. The molecular mechanisms of resistance can be attributed to several general processes: (a) resistance due to the activation of alternative tyrosine kinase receptors that bypass the EGFR pathway (e.g. c-Met and IGF-1R), (b) resistance due to increased angiogenesis, (c) resistance based on constitutive activation of downstream mediators (e.g. PTEN, K-ras and others) and (d) the existence of specific EGFR mutations.[9] Therefore, combination treatments may be useful for avoiding development of EGFR inhibitor-resistant disease.

Tumour-initiating cells ('cancer stem cells') are defined as cells that have the capacity to self-renew and to cause the heterogeneous lineages of cells that comprise the tumour.[10] They have been suggested to represent a distinct subpopulation of cells in many human tumours including HNSCC,[11] while more differentiated and less tumourigenic cells constitute the bulk of tumour cells.[10] Tumour-initiating HNSCC cells have been proposed to present a distinct phenotype identifiable by surface markers CD44 and CD133.[11] An important implication of this concept is that cancer stem cells are possibly more resistant to treatment with drugs or radiation, which can lead to tumour regrowth and relapse.[12]

Oncolytic adenoviruses have been explored in adenoviral gene therapy for enhanced tumour transduction and amplification of effect.[13] These viruses have a cytolytic nature, i.e. the replicative life cycle of the virus results in host cell death. Infection by most adenovirus serotypes is mediated by the knob region of the fibre binding to CAR. There is a growing body of evidence that a decrease in CAR expression is frequently associated with tumour aggressiveness and that many advanced tumours feature variable and often low expression of CAR.[14] Thus, it would be advantageous to transductionally target adenovirus to non-CAR receptors for increased tumour transduction and/or reduced infection of non-target tissues. A particularly powerful approach is transductionally targeted oncolytic viruses.[15]

The oncolytic adenoviruses used in this study feature loss-of-function mutations in the virus genome, which are trans-complemented by features of cancer but not normal cells. They have a 24-bp deletion in the constant region 2 domain of the adenoviral E1A gene.[16] Therefore, the E1A protein is unable to bind the retinoblastoma (Rb) tumour-suppressor protein for release of E2F and subsequent effective viral replication in non-cycling normal cells. Heretofore, the virus replicates selectively in cells deficient in the Rb/p16 pathway, including most if not all cancer cells.[17] Tumour specificity of these adenoviral mutants has been previously demonstrated.[13,15,16]

Even though all the modalities used in this study have antitumour efficacy even as single agents, the complexity of advanced tumour masses may present challenges for curative treatment. It is likely that the therapeutic efficacy of any approach can be further improved by combining it with other modalities, which may also be useful because of non-overlapping side-effects and the potential for additive or synergistic efficacy. Therefore, we investigated the combination of oncolytic adenoviruses with cetuximab, radiotherapy and chemotherapy in HNSCC low passage tumour cell cultures.

2. Materials and methods

2.1. *Low passage tumour cell cultures*

Human head and neck squamous cell carcinoma (HNSCC) low passage tumour cell cultures UT-SCC 8 (supraglottic larynx), UT-SCC 9 (glottic larynx), UT-SCC 10 (mobile tongue) and UT-SCC 29 (glottic larynx)[18] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (PromoCell GmbH, Heidelberg, Germany), 1% non-essential amino acids (Gibco, Invitrogen, Carlsbad, California), 2 mmol/L glutamine, 100 U/mL penicillin and 100 Ag/mL streptomycin (all from Sigma, St. Louis, MO). The UT-SCC cells were used in low passage, typically passages 15–30.

2.2. *Tumour immunofluorescence studies*

Tumour cryosections were deposited onto glass slides, fixed in 4% paraformaldehyde (room temperature, 10 min), permeabilised with 0.1% Tween in PBS and blocked for 30 min with goat immunomix (5% normal goat serum, 0.2% BSA and 0.05% sodium azide in PBS) at room temperature. Indirect immunofluorescent labelling was used to identify CD133-expressing cells using rabbit anti-CD133 1:40 polyclonal antibody (Santa Cruz Biotechnology Inc.). Slides were washed (0.5% BSA in PBS) and incubated in the dark at room temperature for 1.5 h with

Alexa Fluor® 405-conjugated goat anti-rabbit IgG monoclonal antibody. Direct immunofluorescent labelling technique was used to identify CD44-expressing cells using APC-conjugated mouse anti-CD44 1:5 monoclonal antibody (BD Pharmingen). Slides were washed (0.5% BSA in PBS). Tumour sections were post-fixed with 4% paraformaldehyde (RT, 10 min), washed in PBS and mounted with Vectashield without DAPI. Intensity ratios were calculated using Image J 1.39a (Wayne Rasband, National institutes of Health, USA).

2.3. Adenoviruses

Viruses were propagated as reported[19] and their main features are described in Table 1.

2.4. Marker gene transfer assays and cell killing in vitro

Cells were infected with marker gene expressing, replication-deficient viruses for 30 min, then washed and incubated with complete growth medium at 37 °C. 24 h later, luciferase expression was analysed (Luciferase Assay System, Promega, Madison, WI). *In vitro* cytotoxicity assays with HNSCC cells (1.5×10^4) were performed on 96-well plates. After 1 h, infection medium was replaced with medium containing 5% FCS, which was changed every other day. Eight to 11 d later, cell viability was analysed by MTS assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega).

2.5. Synergy between oncolytic adenoviruses and chemotherapy on HNSCC low passage tumour cell cultures

HNSCC low passage tumour cell cultures were seeded at 1.5×10^4 cells/well on 96-well plates. Next day, cells were either infected with virus or treated with cisplatin and fluorouracil (5-FU) or with a combination of both. Virus and chemotherapeutics were diluted in growth media with 2% FCS and cells were infected for 1 h at 37 °C. After infection, cells were washed and incubated in 5% FCS containing cisplatin and 5-FU. Cell viability was measured 6 d after infection (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Promega).

2.6. Radiotherapy

Irradiation was performed with a linear accelerator (model: Clinac 600 C/D, Varian Medical Systems, Palo Alto, USA) using a 6 MV photon beam and dose rate 400 MU/min (~4 GY/min). *In vitro*, cells were irradiated on cell culture plates through a 1 cm thick plastic phantom bottom and 1 cm thick layer of water in the phantom. Mice remained free in standard plastic cages that were placed in the middle of the radiation field.

2.7. Synergy between oncolytic adenovirus and radiotherapy on HNSCC low passage tumour cell cultures

HNSCC low passage tumour cell cultures were treated in triplicate with oncolytic adenoviruses or radiation or combination of both, cell viability was measured 9 d after infection with MTS assay as described above. Virus infection was done 24 h after radiation treatment.[20]

2.8. Triple and quadruple combinations with virus, cetuximab, chemotherapy or radiotherapy on HNSCC low passage tumour cell cultures

HNSCC low passage tumour cell cultures were treated with oncolytic adenovirus or/and radiation or/and cisplatin and 5-

Table 1 – Adenoviruses used in this study.

Virus name	E1	Reporter gene	Fibre	Fibre Main receptors	Ratio[a]	Reference
Ad5luc1	Deleted	Luciferase	Wild-type Ad5	CAR	28	[45]
Ad5lucRGD	Deleted	Luciferase	RGD motif in HI loop	αvβ integrins and CAR	53	[46]
Ad5/3luc1	Deleted	Luciferase	Serotype 3 knob	Unknown (receptor X)	11	[45]
Ad5.pK7(GL)	Deleted	GFP + luciferase	7 lysine residues at COOH terminus	HSPGs and CAR	14	[47]
Ad5.RGD.pK7(GL)	Deleted	GFP + luciferase	RGD motif in HI loop and 7 lysine residues at COOH terminus	αvβ integrins and HSPGs and CAR	38	[47]
Ad5(GL)	Deleted	GFP + luciferase	Wild-type Ad5	CAR	11	[47]
Ad5/3-Δ24	24-bp deletion[b]		Serotype 3 knob	Unknown (receptor X)	6	[48]
Ad5.pK7-Δ24	24-bp deletion[b]		7 lysine residues at COOH terminus	HSPGs and CAR	96	[19]
Ad5-Δ24RGD	24-bp deletion[b]		RGD motif in HI loop	αvβ integrins and CAR	11	[49]
Ad5-Δ24E3	24-bp deletion[b]		Wild-type Ad5	CAR	8	[48]
Ad300wt	Wild-type		Wild-type	CAR	3	American Type Culture Collection

a Ratio of viral particles to plaque-forming units, a quality control measure and indicator of viral packaging efficacy.
b A 24-bp deletion in constant region 2 of E1A region of adenovirus genome mediates selectivity to p16/Rb pathway mutant tumour cells.

FU, cell viability was measured 10 d after infection with MTS assay as described above. Virus infection was done simultaneously with cisplatin + 5-FU combination treatment and/or cetuximab treatment and/or 24 h after radiation treatment.

2.9. Animal experiments

All animal experiments were approved by Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Human HNSCC explant xenografts were established by injecting 5×10^6 UT-SCC 8 cells mixed with matrigel (BD Pharmingen, Franklin Lakes, NJ) into the flanks of 5–6-week-old female NMRI/nude mice (Taconic, Ejby, Denmark). After 30 d, tumours ($n = 10$/group) were injected for 3 consecutive days with 3×10^8 VP (days 0, 1 and 2). After 22 d, viruses were injected intratumourally (1×10^8 VP) on days 1, 4, 8 and 11. Cetuximab (750 μg) and/or chemotherapy (25 μg cisplatin+ 250 μg 5-FU) was given intraperitoneally on days 0, 3, 7 and 10, similarly to previous reports.[21,22] Whole body radiation (1 Gy) was given on days 0, 3, 7 and 10.[20] On day 97, tumours were collected and immunofluorescence staining studies were performed. The formula (length × width^2 × 0.5) was used to calculate tumour volume.

2.10. Statistical analysis

To compare differences between groups, two-tailed Student's *t*-test was used and a p value of <0.05 was considered significant. Chou and Talalay's median-effect method[23] was used to calculate combination index (CI) values under assumption of mutually non-exclusive drug interactions using S-PLUS 6.0 (Insightful Corporation, Seattle, WA). In CI analysis <1 indicates synergism, 1 = additivity and CI > 1 indicates antagonism. One-sample *t*-test was performed to determine whether the mean CI from separate experiments at multiple effects levels was significantly different from a value of 1.0. For all analyses, a p value of <0.05 was deemed statistically significant. *P* values of the *in vivo* experiment were calculated by Mann–Whitney test (SPSS 15.0, SPSS Inc, Chicago, IL).

3. Results

3.1. High frequency of CD133+ and CD44+ cells in HNSCC tumours recurring after cetuximab treatment

Previous data suggest that current treatment regimens used for HNSCC may selectively kill differentiated cancer cells, producing tumour regression, while sparing tumour-initiating cells, which can lead to tumour relapse.[1,24] In order to assess if cancer stem cells might be involved in the mechanism of resistance to cetuximab, we treated tumours with cetuximab and followed them until they relapsed 2–3 months later. Tumours cryosections were stained for CD44, CD133 and EGFR before (Fig 1A) and after cetuximab (Fig. 1B). The ratio of the CD44/CD133 signal intensity to EGFR signal intensity increased 16-fold as a result of cetuximab treatment (from 4.6 ± 0.33 to 72 ± 66.0). Therefore, in relapsing tumours, there were much more CD44+ and CD133+ cells in comparison to EGFR+ cells, suggesting that cetuximab had killed most EGFR+ cells but not CD44+ and CD133+ cells.

3.2. Adenoviral capsid modifications promote increased gene transfer to HNSCC low passage tumour cell cultures

In order to assess which capsid modifications allow the highest transductional efficacy in HNSCC low passage tumour cell cultures, infection was performed with a panel of capsid-modified replication-deficient adenoviruses (Fig. 1C and D and Supplementary Fig. S1). Gene transfer with integrin binding Ad5lucRGD was increased to a maximum of 3-, 8- and 2-fold in UT-SCC 9, 10 and 29, respectively, when compared to Ad5luc1 featuring wild-type serotype 5 capsid. Ad5/3luc1, a chimera Ad5-based adenovirus bearing the fibre of Ad3 to target the Ad3 receptor, showed a gene transfer increase a maximum of 12-, 5-, 53- and 7-fold compared to the respective isogenic unmodified Ad5 control virus in UT-SCC 8, 9, 10 and 29, respectively. Ad5.pK7 (GL) features a 7 polylysine tail at the COOH terminus that targets the virus to heparan sulphate proteoglycans (HSPGs), which resulted in up to 51-, 54-, 172- and 251-fold increases (UT-SCC 8, 9, 10 and 29, respectively) in gene transfer in comparison to the respective isogenic unmodified Ad5 control virus. Finally, Ad5.pK7.RGD(GL) increased the gene transfer up to 2-, 45- and 13-fold compared to the respective isogenic unmodified Ad5 control virus in UT-SCC 9, 10 and 29, respectively. Therefore, polylysine modification of the fibre emerged as the optimal capsid modification for gene delivery to HNSCC low passage tumour cell cultures.

3.3. Capsid-modified oncolytic adenoviruses are effective in killing HNSCC low passage tumour cell cultures in vitro

We infected HNSCC low passage tumour cell cultures with a panel of transductionally targeted oncolytic adenoviruses (Fig. 2A and B and Supplementary Fig. 2). The oncolytic potency of Ad5.pK7-Δ24 and Ad5/3-Δ24 was significantly improved in comparison to wild-type Ad5. Ad5.pK7-Δ24 exhibited the highest oncolytic effect in UT-SCC 9, 10 and 29 (Supplementary Fig. 2A and B and Fig. 2B) whereas Ad5/3-Δ24 had the strongest oncolytic effect in UT-SCC 8 (Fig. 2A). In general, the potency of capsid-modified oncolytic adenoviruses correlated well with gene delivery data.

3.4. Efficacy of capsid-modified oncolytic adenoviruses on HNSCC explants in vivo

Ad5.pK7-Δ24 resulted in significant anti-tumour effect in comparison to mock-treated mice (Fig. 2C). Ad5/3-Δ24 did not decrease the growth rate of tumours.

3.5. Combination effect of oncolytic adenovirus and cetuximab on HNSCC low passage tumour cell cultures in vitro

The combination of Ad5.pK7-Δ24 and cetuximab resulted in conflicting data in different low passage tumour cell cultures (Fig. 3 and Supplementary Fig. 3). The combination index mean (CI) in UT-SCC 8 suggested antagonism (CI = 14.2, $p = 0.411$), in UT-SCC 9 suggested synergy (CI = 0.830, $p = 0.207$), in UT-SCC 10 there was antagonism (CI = 2.026,

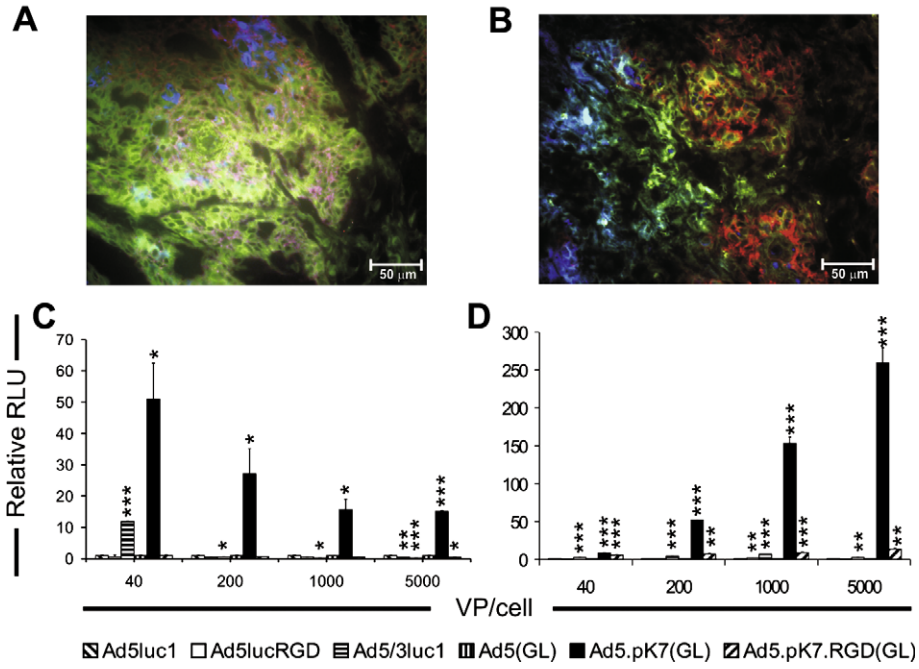

Fig. 1 – Immunofluorescence cryosections of human HNSCC xenografts. UT-SCC8 tumours were established in nude mice, and treated intraperitoneally with (A) growth medium only or (B) cetuximab (750 μg) on days 0, 3, 7 and 10. On day 97, tumours were collected and immunofluorescence staining studies were performed. CD133-positive cells (blue), CD44-positive cells (red) and cetuximab-positive cells (green) are shown. Magnification used of 40×, bar 50 μm. (C and D) Transductional targeting for increasing gene transfer into HNSCC low passage tumour cell cultures UT-SCC 8 (C) and UT-SCC 29 (D). Cells were infected with transductionally modified replication-deficient viruses at the indicated viral particles/cell (VP/cell). Luciferase activity was measured as relative light units (RLU) 24 h after infection. Results represent RLU compared to the isogenic control virus with an unmodified serotype 5 capsid, which was given the value of 1. Mean background transgene activity was subtracted from the data. Columns, mean of triplicates; bars, ±SE (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus Ad5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$p < 0.001$) and in UT-SCC 29 was synergism with a CI of 0.276 ($p < 0.01$).

Similar results were seen for Ad5/3-Δ24 and cetuximab (Supplementary Fig. 3). The CI mean in UT-SCC 8 was synergistic at 0.226 ($p < 0.05$), in UT-SCC 9 also synergistic (CI = 0.137; $p < 0.001$), while in UT-SCC 10 there was antagonism (CI = 1.3×10^{14}; $p < 0.001$) and in UT-SCC 29 there was antagonism (CI = 2.9×10^{25}; $p < 0.001$) (Supplementary Fig. 3). Overall, there did not seem to be clear synergism between oncolytic adenovirus and cetuximab *in vitro*.

3.6. *Oncolytic adenovirus in combination with the cisplatin and 5-flurouracil*

Cisplatin and 5-FU were used according to the standard 10-fold concentration ratio popular in routine clinical use.[25] Cisplatin + 5-FU concentrations used in combination treatment were chosen based on dose–response curves for drug alone (data not shown).

Ad5.pK7-Δ24 with the highest dose of cisplatin + 5FU revealed a complete cell killing effect in all HNSCC low passage tumour cell cultures tested (Fig. 4A and B and Supplementary Fig. 4C and F). The CI suggested antagonism for UT-SCC 8 (CI = 1.164, $p = 0.172$), UT-SCC 9 (CI = 1.281, $p = 0.294$). Synergism was seen for UT-SCC 10 (CI = 0.705, $p = 0.426$) and UT-SCC 29 (0.736, $p = 0.32$). Similar data were seen for Ad5/3-Δ24 with cisplatin + 5FU (Supplementary Fig. 4A, C, F and I). The CI was synergistic for UT-SCC 8 (CI = 0.719, $p < 0.05$), UT-SCC 10 (CI = 0.619, $p = 0.192$) and UT-SCC 29 (0.715, $p = 0.456$). Antagonism was seen for UT-SCC 9 (CI = 1.151, $p = 0.643$). These data suggest variation in the chemosensitivity between individual tumours, as seen clinically. Ad5luc1 was used as a replication-deficient control and did not cause oncolysis or potentiation of chemotherapy treatment (Supplementary Fig. 4B, E, H and J).

3.7. *Synergy between oncolytic adenovirus and radiotherapy in HNSCC low passage tumour cell cultures in vitro*

Ad5.pK7-Δ24 and radiotherapy combination revealed a maximum cell killing effect of 94.3%, 94.5%, 95% and 100%, respectively, in UT-SCC8, UT-SCC9, UT-SCC10 and UT-SCC29 *in vitro* (Fig. 4C and D and Supplementary Fig. 5C and F). The CI mean for UT-SCC 8 suggested synergy (CI = 0.884, $p = 0.706$) and in UT-SCC 29 there was synergy (CI = 0.599, $p < 0.01$). Combination of Ad5/3-Δ24 and radiotherapy revealed a maximum cell killing effect of 99.4%, 96.3%, 95.75% and 100%, respectively, in UT-SCC8, UT-SCC9, UT-SCC10 and UT-SCC29 (Supplementary Fig. 5A, D, G and I). Furthermore the CI suggested synergy at

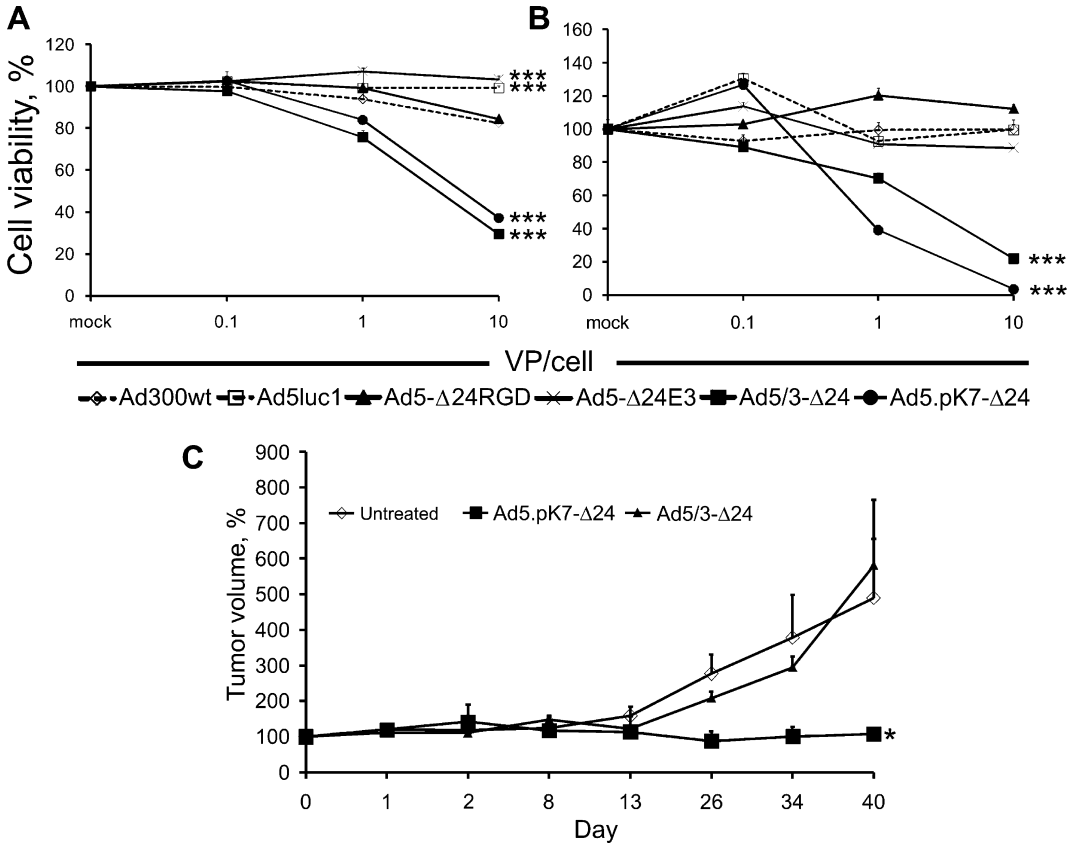

Fig. 2 – Oncolysis of HNSCC low passage tumour cell cultures UT-SCC 8 (A) and UT-SCC 29 (B) following infection with oncolytic adenoviruses. Cells were infected with viruses at the indicated viral particles/cell (VP/cell) and analysed for viability using MTS assay. Each data point represents the mean ±SE ($p < 0.01$; ***$p < 0.001$ versus Ad300wt) of triplicate determinations. (C), effect of oncolytic adenovirus on human HNSCC explant xenografts in nude mice. UT-SCC8 tumours were injected daily for 3 d with 3×10^8 VP (days 0, 1 and 2). Tumour volume at day 0 was set 100% and tumour size is presented relative to the initial tumour size. Data are represented as means ± SE of 4–6 tumours/group. Statistical significance between untreated and Ad5.pK7-Δ24 treated tumours was observed with the Mann–Whitney test ($p < 0.05$).**

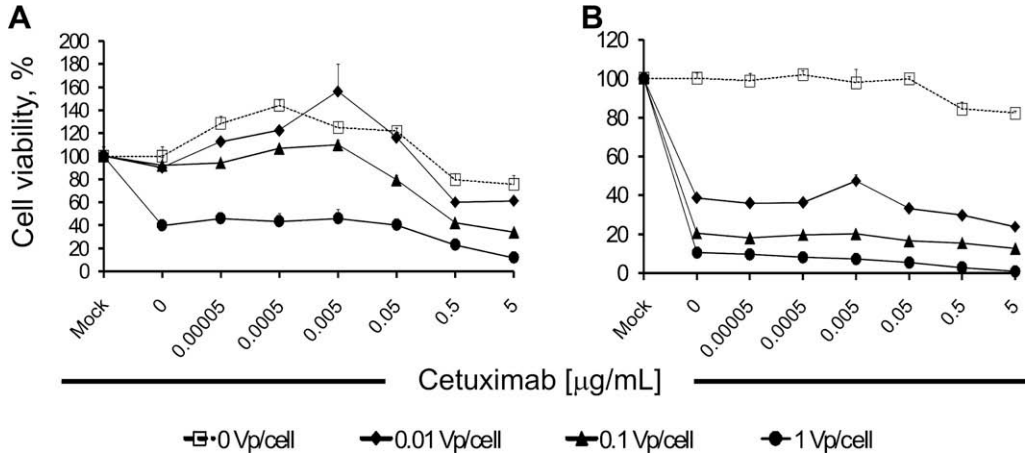

Fig. 3 – Synergy between the oncolytic adenovirus Ad5.pK7-Δ24 and Cetuximab. HNSCC low passage tumour cell cultures UT-SCC8 (A) and UT-SCC 29 (B) were treated with oncolytic adenovirus or cetuximab or combination of both at given concentrations. Cell viability was measured with MTS assay. Virus infection was done simultaneously with the monoclonal antibody treatment. The OD450 values of uninfected and untreated cells were set as 100%. Data are represented as means ± SE of triplicate experiments. The combination of cetuximab with Ad5.pK7-Δ24 suggested antagonism (CI = 14.2, $p = 0.411$) and was synergistic (CI = 0.276, $p < 0.01$), respectively, for UT-SCC 8 and UT-SCC 29.

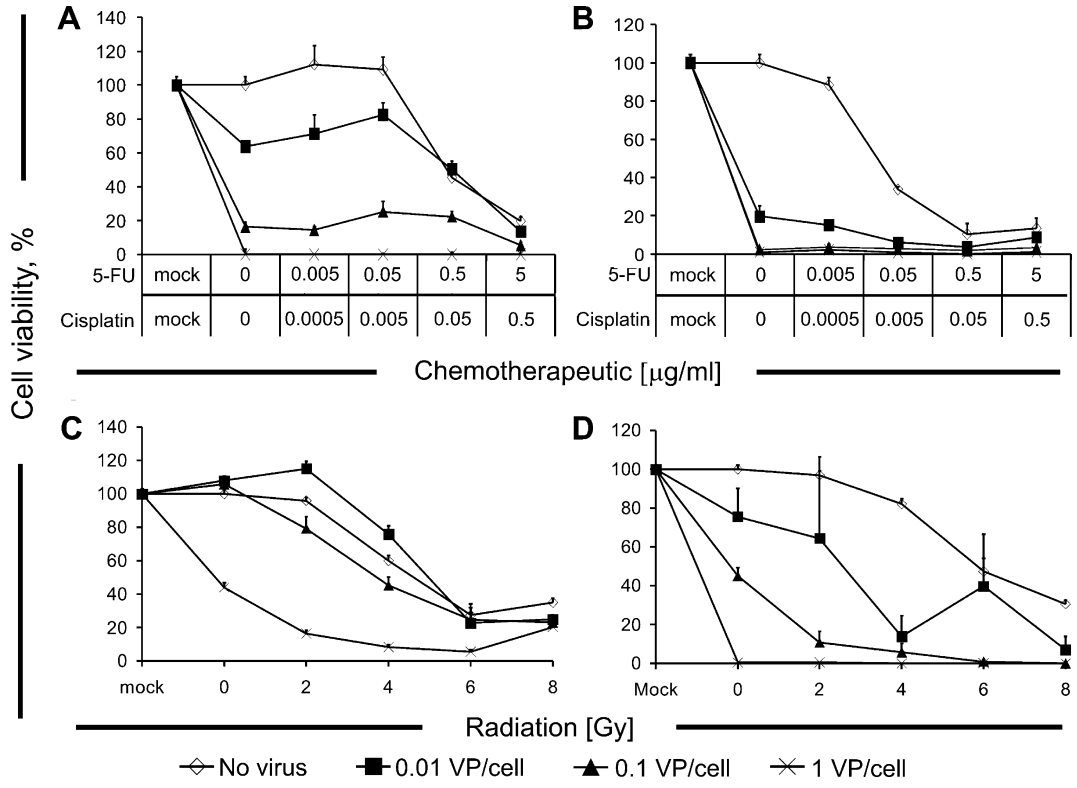

Fig. 4 – Combination between oncolytic adenovirus and chemotherapy (A and B) or radiotherapy (C and D). HNSCC low passage tumour cell cultures UT-SCC 8 (A and C) and UT-SCC 29 (B and D) were treated with oncolytic adenovirus Ad5.pK7-Δ24 or cisplatin + 5-flurouracil (5-FU) or combination of both. Cell viability was measured with MTS assay. Virus infection was done simultaneously with chemotherapeutic treatment. The OD450 values of uninfected and untreated cells were set as 100%. Data are represented as means ± SE of triplicate experiments. The combination of chemotherapy with Ad5.pK7-Δ24 suggested antagonism for UT-SCC 8 (CI = 1.164, $p = 0.172$) and synergism for UT-SCC 29 (CI = 0.736, $p = 0.32$). HNSCC low passage tumour cell cultures were treated with oncolytic adenovirus Ad5.pK7-Δ24 or radiation or combination of both, cell viability was measured with MTS assay. Virus infection was done 24 h after irradiation. The OD450 values of uninfected and untreated cells were set as 100%. Data are represented as means ± SE of triplicate experiments. The combination of radiotherapy with Ad5.pK7-Δ24 suggested synergy (CI = 0.884, $p = 0.706$) and was synergistic (CI = 0.599, $p < 0.001$), respectively, for UT-SCC 8 and UT-SCC 29.

0.783 ($p = 0.537$) in UT-SCC8 and in UT-SCC 29 there was synergy with a CI mean of 0.667 ($p < 0.05$).

3.8. *Triple and quadruple combinations with virus, EGFR inhibitors, chemotherapy and/or radiotherapy on HNSCC low passage tumour cell cultures in vitro*

Triple and quadruple combinations of Ad5.pK7-Δ24 with radiation and/or cisplatin + 5-FU and/or cetuximab had a maximum cell killing of 96% and 99%, respectively, in UT-SCC 8 and UT-SCC 29 (Fig. 5A and Supplementary Fig. 6A and B). We observed a decrease in the needed concentration of Ad5.pK7-Δ24 to achieve 50% cell killing (LC50) when combined with standard therapies (Fig. 5B and Supplementary Fig. 7A and B). The lowest LC50 (0.0098 VP/Cell, mean of all cell lines) was obtained with the quadruple combination (Fig. 5B).

Triple and quadruple combinations of Ad5/3-Δ24 with radiation and/or cisplatin+5-FU and/or cetuximab had a maximum cell killing of 94% and 99%, respectively, in UT-SCC 8 and UT-SCC 29 (Supplementary Fig. 6C and D). Similarly to what was observed with the Ad5.pK7-Δ24 virus, we observed a decrease in the LC50 of Ad5/3-Δ24 when combined with the standard therapies (Supplementary Fig. 7A and B). Interestingly, the antagonism observed in the combination of cetuximab and Ad5.pK7-Δ24 was not observed when radiation was added. The synergism between radiation with cetuximab and/or radiation with oncolytic virus seems to override the variable combination effects of cetuximab with virus.

3.9. *Efficacy of the quadruple combinations in vivo*

Cetuximab alone and the quadruple combination showed a significant antitumour effect compared to mock-treated mice (both $p < 0.01$; Fig. 5C). Follow-up of tumour size was continued until day 97. No toxicity was seen in any of the groups, although further studies may be required to more carefully analyse this. Tumours in the quadruple combination group were completely eradicated while tumours in the cetuximab group relapsed ($p < 0.01$ between the two groups; Fig. 5D).

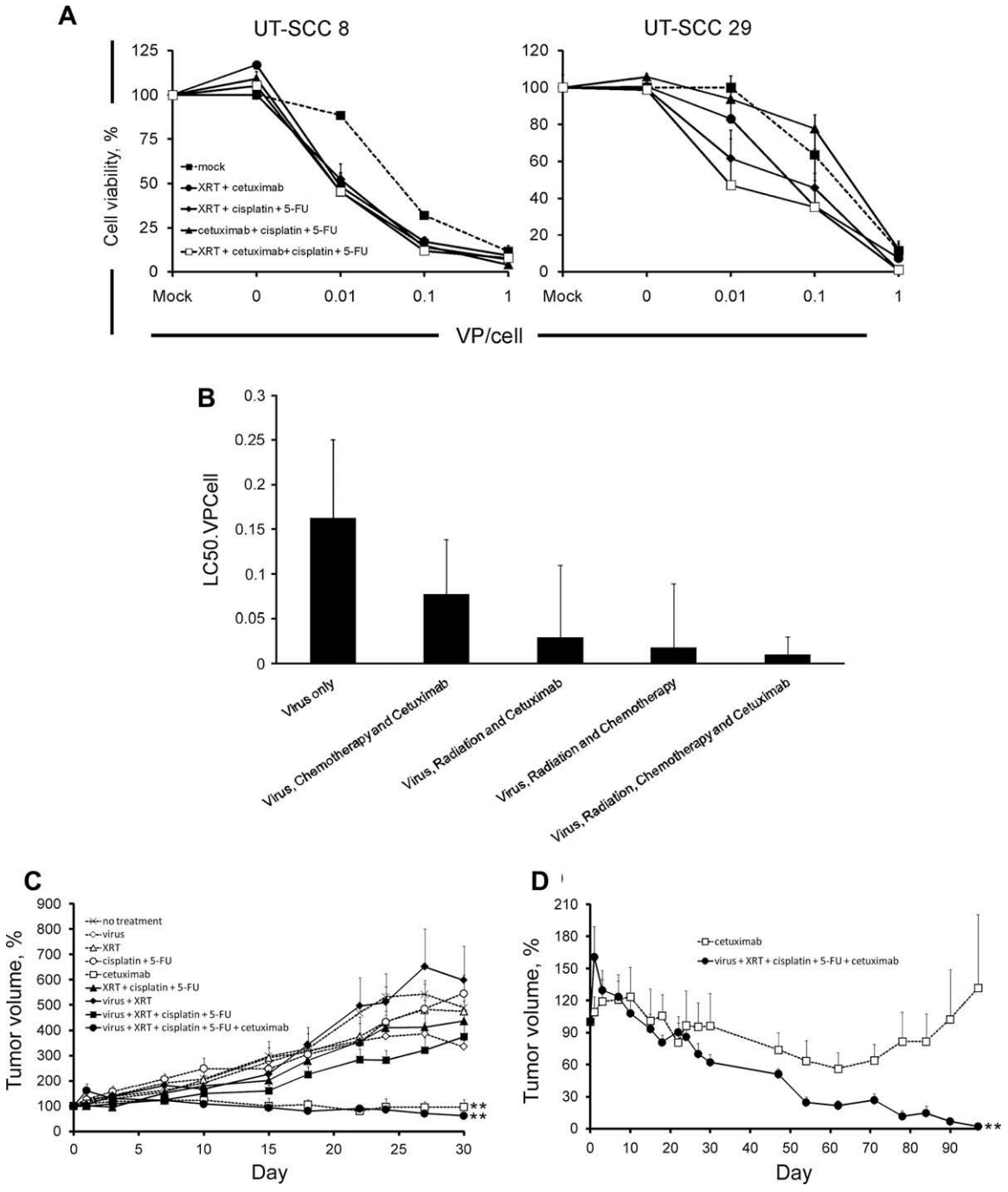

Fig. 5 – Triple and quadruple combinations with virus, cetuximab, chemotherapy or radiotherapy (A). HNSCC low passage tumour cell cultures were treated with oncolytic adenovirus Ad5.pK7-Δ24 and/or cetuximab (0.0005 μg/ml) and/or cisplatin (0.0005 μg/ml), 5-FU (0.005 μg/ml) and/or radiation (2 Gy). Virus infection was done simultaneously with chemotherapy and/or cetuximab treatment and/or 24 h after radiation treatment. The OD450 values of uninfected and untreated cells were set as 100%. Data are represented as means ± SE of triplicate experiments. (B) Lethal Concentration 50%, average of UT-SCC 8 and UT-SCC 29 for Ad5.pK7-Δ24 in VP/Cell alone or in combination with HNSCC standard therapies. (C and D) Multimodality treatment of human HNSCC explant xenografts. UT-SCC8 tumours (n = 10/group) were established in nude mice, and treated intratumourally with 1×10^8 VP of oncolytic adenovirus Ad5.pK7-Δ24 on days 1, 4, 8 and 11. Cetuximab (750 μg) or chemotherapy (25 μg cisplatin + 250 μg 5-FU) was given intraperitoneally on days 0, 3, 7 and 10. Whole body radiation (1 Gy) was given on days 0, 3, 7 and 10. Tumour volume at day 0 was set 100% and tumour size is presented relative to the initial tumour size. Data present means ± SE, $^{**}p < 0.01$ (Mann–Whitney test) versus no treatment group. Most groups had to be terminated on day 30 due to tumour size (C). However, in two groups good efficacy was seen and the experiment could be continued until day 97 (D). Tumours were completely eradicated in the quadruple treatment group. $^{**}p < 0.01$ (Mann–Whitney test) versus cetuximab group.

4. Discussion

The therapies that are currently used for the treatment of recurrent HNSCC yield median survivals of less than 1 year. Also, the morbidity associated with the treatments has encouraged the pursuit of alternative therapeutic strategies. Nowadays there is an increased use of epidermal growth factor receptor (EGFR) inhibitors (e.g. cetuximab), which have shown utility in combination with chemotherapy, radiotherapy and chemoradiotherapy.[26,17] However, increasing evidence suggests that patients who initially respond to EGFR inhibitors may subsequently become refractory.[8] Therefore, it is important to study the mechanisms of resistance to the treatments in order to further developments and improvements. Also, it is worthwhile to note that EGFR inhibition is usually not effective enough to give clinical benefit to HNSCC patients, and utility of the approach is most convincing in combination regimens.[27]

One reason why HNSCC tumours recur after treatment may relate to tumour-initiating cells.[10,12] In line with such reports, we also found a higher proportion of CD133+ and CD44+ cells, in comparison to EGFR positive cells, in cetuximab refractory tumours. This prompted us to study if a combination regimen could be utilised to increase therapeutic efficacy of cetuximab and HNSCC standard therapies.

We and others have previously studied adenoviral gene therapy agents that enter tumour cells through CAR-independent mechanisms.[19] This might be useful for avoiding inefficient transduction due to variable or low CAR expression, which has been reported to be common in many tumour types.[28] In order to select which capsid modifications allow increased gene transfer and cell killing of human HNSCCU, we first tested four different HNSCC low passage tumour cell cultures representing three different tissues for the transduction efficacy of the capsid modifications. Low passage tumour cell cultures might resemble patient tumours more closely than conventional cell lines.[18]

Our findings demonstrate that 5/3, pK7, RGD and pK7.RGD capsid modifications resulted in increased levels of gene delivery when compared with Ad5-based viruses, and pK7 resulted in the highest increases in gene delivery. Further *in vitro* cell killing studies revealed 5/3 and pK7 as the most promising candidates, and subsequent *in vivo* studies showed that only pK7 resulted in significant tumour growth inhibition. The high levels of heparan sulphate proteoglycan (HSPG) reported in HNSCC might explain the high efficacy of pK7.[29] The modest results obtained *in vivo* for the 5/3 modification might be explained by virus biology specific aspects, e.g. bioavailability, or receptor expression/availability differences *in vitro* versus *in vivo*.

With regard to combination effects between oncolytic adenovirus and cetuximab, chemotherapy or radiotherapy, *in vitro* data suggested positive effects between the virus and the latter two modalities, when pair-wise analysis was performed. As far as we know, the combination of oncolytic adenoviruses and cetuximab has not been studied before. In contrast, the utility of combining oncolytic virus with various chemotherapeutics, including cisplatin and 5-FU, is well established[30] and our synergistic results are well in accord with previous findings. Also, we found synergy with radiation, as reported.[31]

Antitumour efficacy *in vivo* is more complex than tumour cell killing *in vitro*. Therefore, we were not completely surprised that the rather modest results observed *in vitro* for the quadruple combination showed much improvement in mice. Although cetuximab alone was quite effective, as reported[32], only the quadruple combination achieved complete tumour regression *in vivo*. It is well established that cetuximab has a number of different antitumour mechanisms, including direct inhibition of EGFR tyrosine kinase activity, the inhibition of cell cycle progression, and the increase and activation of pro-apoptotic molecules.[33] Only these mechanisms can be reliably studied *in vitro*, but the Fc tail may also be able to direct complement and promote antibody-dependent cell-mediated cytotoxicity against the tumour.[27] Further, indirect effects on angiogenesis, invasion and metastasis may play a role in the *in vivo* effects of cetuximab.[33]

The mechanisms of augmented therapeutic effects obtained by combining oncolytic adenoviruses, EGFR inhibitors, chemotherapy and radiotherapy require further studies. Nevertheless, our data allow several hypotheses to be put forth. Cultured cells treated with ionising radiation show increased levels of phosphorylated EGFR[34] with subsequent EGFR import to the nucleus, where it activates the DNA-protein kinase (DNAPK) leading to DNA repair and cell survival.[35] Tumour cells treated with various cytotoxic drugs (e.g. cisplatin and 5-FU[36]) also promote EGFR phosphorylation in order to enable cell survival. Cetuximab inhibits radiation-induced activation of DNAPK, as well as EGFR nuclear import, DNA repair and survival from radiation induced damage.[37] The adenoviral protein E4orf6 enables prolonged auto-phosphorylation of DNAPK after ionising radiation. This inhibits damage repair and reduces cancer cell survival.[38] It has also been shown that adenoviral E1A gene products can inhibit HER-2/c-erbB-2 expression[39] for further inhibition of the erbB signalling pathway already partially blocked by cetuximab. Cetuximab and adenovirus use different ways to inhibit the activation of the erbB survival pathway. This might make it more difficult for tumour cells to overcome pathway inhibition for gaining resistance to chemo and radiation regiments.

Given our initial findings that tumours relapsing after cetuximab are enriched in CD133 and CD44+ cells (Fig. 1), it is tempting to hypothesise that the quadruple combination was able to kill these cells. This seems to be confirmed by the finding that tumours were completely eradicated by the quadruple combination (Fig. 5D) and also *in vitro* all cells could be killed (Figs. 4 and 5). Tumour-initiating cells may be resistant to radiotherapy, chemotherapy and kinase inhibitors.[10,12] Therefore, the oncolytic virus present in the quadruple combination may have been important with regard to complete tumour eradication. In particular, previous reports suggest that a serotype 5-based virus may not be effective in killing tumour-initiating cells, while capsid-modified viruses may be more useful in this regard.[40,41] Although oncolytic adenoviruses are promising developmental anticancer agents in their own right, our data are in accord with previous reports[42] suggesting that they are not effective enough on their own to achieve complete eradication of advanced tumours.

Combination therapies can be more toxic than single therapies. Therefore, it is key to perform careful studies in order to understand if additional efficacy is gained at the price of severe side-effects. However, tumour progression often causes the most severe symptoms and increased tumour control may thus result in fewer symptoms overall. Lack of external signs of toxicity in the quadruple treatment group in the *in vivo* studies is promising but further studies are needed.

In summary, the combination of Ad5.pK7-Δ24 with radiotherapy, chemotherapy and cetuximab appears promising for the treatment of HNSCC. However, clinical trials are needed to confirm the data in humans. Recent breakthroughs in clinical gene therapy have demonstrated that small incremental improvements have the potential for making large differences to patients. Often, these advances have been achieved through combination therapies.[43,44]

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.11.005.

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