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# A Novel Approach Using Transcomplementing Adenoviral Vectors for Gene Therapy of Adrenocortical Cancer

**Abstract**

Current therapies for adrenocortical carcinomas do not improve the life expectancy of patients. In this study, we tested whether a gene-transfer therapy based upon a suicide gene/prodrug system would be effective in an animal model of the disease. We employed E4- and EIA/B-depleted, *herpes simplex* virus-thymidine kinase-expressing adenoviral mutants that transcomplement each other within tumor cells, thereby improving transgene delivery and efficacy by viral replication *in situ.* Transcomplementation of vectors increased the fraction of transduced of tumor cells. This increase was accompanied by greater tumor volume reduction compared to non-transcomplementing approaches. Survival time improved with non-replicating vectors plus GCV compared to controls. However, transcomplementation/replication of vectors led to a further significant increment in anti-tumor activity and survival time ($p < 0.02$). In treated animals, we observed a high number of apoptotic nuclei both adjacent to and distant from injection sites and sites of viral oncolysis. Ultrastructural analyses exhibited nuclear inclusion bodies characteristic of virus production *in situ,* and provided further evidence that this therapy induced apoptotic cell death within tumor cells. We conclude that the efficacy of suicide gene therapy is significantly amplified by viral replication and, in combination with GCV, significantly reduces tumor burden and increases survival time.

Key words
Adrenal Gland • Cancer • Gene Therapy • Adenovirus -Transcomplementing • Bicistronic

## Introduction

Adrenocortical carcinomas are severe malignancies. The prognosis is poor, as metastases in lung and liver develop rapidly; 5-year survival rates range between 15 and 35% [1]. Two age peaks of higher incidence within the 1st and 4th decades of life have been reported [2,31. Molecular, paracrine, immunologic, and hormonal features of these tumors have been studied extensively, and prognostic signs have been developed [4]. Impaired cellular communication between tumor and effector cells of the immune system may be related to immune surveillance due to the decreased MHC 3 class II expression [5]. Interestingly, soluble mediators of the immune system itself have been shown to promote adrenocortical tumors by the action of aberrantly expressed receptors [6]. However, despite efforts to shed light upon the molecular and immunologic mechanisms leading to neoplastic degeneration in the adrenal gland, no effective treatments have been developed. Few chemotherapeutic options for patients

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bearing unresectable or metastasizing tumors are available. Standard therapies have severe side effects, and evidence of their success is anecdotal [7-9].

Animal modeling of adrenocortical carcinoma with new treatment modalities could accelerate the development of therapies for this disease, and may have broader application; however, no appropriate model is currently available to test possible treatment strategies. CE mice have been reported to develop adrenocortical hyperplasia with progression to carcinoma after ovariectomy [10]. This is highly suggestive of an involvement of regulatory hormones; indeed, estradiol replacement prevented development of transplanted carcinomas in mice [11]. However, hormone-dependent adrenal carcinomas have not been described in humans with or without adrenal hyperplasia. To develop a more pertinent animal model of this disease, we generated SW-13-derived human adrenocortical carcinoma xenografts in nude mice. The SW-13 cell line was initiated from an hormonally inactive small-cell carcinoma of the human adrenal cortex [12] and had previously been used to test compounds with some chemotherapeutic capacity [13-15].

Gene-transfer therapy could improve treatment of adrenocortical carcinomas. A principal limitation of gene therapy is inefficient gene delivery [16]. Therefore, biological amplification of anti-tumor effects against both transduced and non-transduced tumor cells has been sought in the recruitment of host immune effector cells [17], production of antiangiogenic factors [18-21], or the use of suicide genes. The prototypical suicide gene, *herpes simplex virus* (hsv)-thymidine kinase (TK), activates a relatively non-toxic prodrug (such as Ganciclovir) to a cytotoxic compound [22,23]. In addition to direct toxicity in the transduced cell, the expression of hsv-TK causes cell death in non-transduced tumor cells *via* a "bystander effect," which transfers the activated prodrug from hsv-TK expressing cells to adjacent, metabolically coupled cells [24-27].

Anti-tumor strategies have recently been modified by including viral replication as a means of increasing intratumoral transgene delivery [28] and as an induction of direct viral cytotoxicity [29,30]. In animal models using *adenoviruses* with attenuated replication potential, increased survival has been demonstrated [31]; a further improvement was reported when partial replication competence was combined with suicide gene expression [30]. However, adenoviral vectors expressing *herpes simplex* virus-thymidine kinase may damage proliferating non-cancerous tissues, and can cause severe liver necrosis, including death in animal models after systemic application [32,33]. Such toxicities may be even more prominent in systems using replication competent vectors expressing hsv-TK.

As a new therapeutic approach for adrenocortical cancer, we modified the replicating vector strategy to utilize replication-dependent vector distribution within the tumor, but sought to reduce systemic vector spread, potentially minimizing side effects. To achieve this, we employed two replication-deficient adenoviral vectors, E4-deleted \\5.dll014 [34] and an ElA/B-deieted vector carrying a *herpes simplex* vin/s-thymidine kinase gene cloned into a vector derived from H5.d/309 (AVC2.TK). The two separate adenoviral variants are individually replication-deficient due to defects in essential viral genes (in *cis-)* but replication-competent when combined in cells *via* transcomplementation. This system was tested to establish whether replication results in increased vector spread within tumors following co-transduction and whether anti-tumor efficacy in adrenocortical tumor xenografts is improved by this approach. The *in vivo* tumor model was characterized by radiographic and histological studies. Adenoviral vectors have been injected into xenograft tumors directly to achieve efficient transduction of tumor cells by both vectors. We then used FACS analysis, immunohistochemistry, *in situ* end-labeling, ultrastructural analyses to monitor gene transfer, virus particle production, apoptotic cell death, and virus/transgene-mediated tumor lysis. Our findings show that co-transduction of adrenocortical tumor cells enables transcomplementation and results in replication of vectors. This local production of vector leads to a further increase in the fraction of transduced tumor cells and enhances transgene distribution, hereby improving efficacy of suicide gene/prodrug strategy.

## Materials and Methods

### Cells

293 cells»(CRL-1573). Yl cells (CCL-79), AtT20 ceils (CCL-89), and SW-13 cells (CCL-105) (12) were obtained from ATCC (Gaithersburg, MD). WI62 cells were kindly provided by G. Ketner (Johns Hopkins U., Baltimore, MD). Rat 9L cells were kindly provided by E. Oldfield (NlNDS, Bethesda, MD). All cell lines were grown in DMEM medium supplemented with l0% FCS, 2 mM L-glutamine, lOOU/ml penicillin and lOOug/ml streptomycin (Life Technologies, Grand Island, NY) at 37 C in l0% $C0_2$.

### Adenoviral vectors

AVC2.null is identical to AVC2TK except for the absence of the hsv-TK transgene, and was generated by the technique of Graham [35]. Briefly, the adenoviral shuttle plasmid pAVC 2 [36] was introduced into pJM17 [37] by Ca-phosphate co-precipitation technique in 293 cells [35]. Recombinant El-deleted AVC2TK was generated by the technique of Okada [38]. Hsv-TK function was confirmed by transduction of rat 9L cells at a multiplicity of infection (MOI) of 50 PFU/cell, exposure to ganciclovir, and measurement of $^3$H-thymidine incorporation. AVG L is an El-deleted virus expressing green fluorescent protein under the control of the EFl-a promoter (kindly provided by J. Wahlfors, University of Kuopio, Finland). The E4 mutant *dll014* (kindly provided by G. Ketner, Johns Hopkins University) was grown on WI62 cells and isolated by standard technique. Viruses were plaque-purified twice, amplified using the standard technique, and analyzed by restriction digestion of proteinase K-digested viral DNA. All viral stocks were plaque-titered in triplicate by serial dilution and agar overlay on their respective transcomplementing cell lines according to standard protocols.

### *In vitro* transduction and FACS analyses

For *in vitro* transduction, SW-13 ceils, AtT20- and Yl-cells were plated in 6-well plates at a density of 300 000 ceils per well. Cells were transduced with MOIs of 1 to 100 PFU/cell. After 80 h. cells were trypsinized, harvested by centrifugation and resuspended in ice-cold HBSS containing 5% FCS and 0.1 M EDTA (Sigma, St. Louis, MO). Cells were subjected to FACS analysis using a Becton Dickinson FACSort analyzer and 10000 ungated events were collected for analysis.

**Mice and tumor generation, treatment groups**

6-8 week-old femal e nude mice were obtained fro m DCT (Frederick, MD) . Mic e wer e injecte d subcutaneousl y int o th e righ t flank with 10 x $10^6$ cells in 200pi HBSS (Biofluids, Gaithersburg, MD) containin g 20 % Matrige l (Collaborativ e Biomedica l Products, Waltham, MA). Weekly monitoring of tumor size was carried out by measurement diameters in three dimensions at right angles with calipers [volume = 1 xwxh]. *In vivo* transduction was performed whe n tumor s grew to a mea n volum e o f 300 $mm^3$. Treated tumors were injected with 2 * $10^9$ PFU of adenoviral vector in a volume of 100 pi using 25 g needles. In treatment groups, GCV (Roche Bioscience, Palo Alto, CA) was injected intraperitoneally twice daily for 5 days at a dose of lOOmg/kg/d. Animals were injected with 1 x $io^9$ PFU AVC2TK plus 1 x $lo^9$ PFU AV.d//0J4, groups [TK/d/I0J4/$GCV_{80}$] ( n = 8) and [TK/d/70J4/$GCV_{30}$] ( n = 7) or 2 x $10^9$ PFU AVC2.TK, group [TK/GCV) (n = 9) to compare transcomplementing vs . replication-defectiv e vecto r approache s o f therapy. Ganciclovi r was injecte d intraperitoneall y after 80 h i n group [TK/dl/0/4/$GCV_{80}$] an d additionall y afte r 30 h i n grou p [TK/d//0/4/$GCV_{30}$] to compare early and late blocking of viral replication. Two control groups [TK/dUOM] (n = 9) and [TK] (n = 8) were transduced as groups [TK/d/?0/4/$GCV_{80}$] and [TK/GCV], but did not receive GCV. *\dll014]* (n = 9) was transduced with 2 x $io^9$ PFU AV.d/*1014* alone, [GCV] (n = 9) was only treated with GCV and (untreated) (n = 9) received n o treatment. Animal s were euthanized when tumo r size exceeded 150 0 $mm^3$. All anima l experiments wer e conducte d i n accordanc e wit h NI H guideline s fo r the care and treatment of laboratory animals.

**Planar imaging/positron emission tomography**

The accumulation o f F-l 8 fluorodeoxyglucos e (FDG) i n th e implanted adrena l tumor s wa s imaged tomographicall y i n severa l mice by planar imaging/positron emissio n tomograph (PiPET), a device develope d specificall y fo r imagin g smal l animals . FD G was administere d b y tai l vei n injectio n (15 0 microcuries / 0.1 ml). At 3 0 minutes post-injection, the animal was fixed to a vertical rotation device between the two PiPET detectors and rotated continuousl y for 30mi n durin g acquisitio n o f all possibl e coincidence line s connecting th e two detectors. Two sequential data collection s wer e performe d wit h th e firs t collectio n bein g used t o visualiz e the subcostal region s o f the animal s followe d by a second, which visualized th e more rostral areas of the animals. Thes e tw o dat a set s wer e reconstructe d wit h filtere d back-projection (ram p filter ) and , afte r variou s corrections , merged t o form a continuous tomographic representation o f the distribution of FDG in the whole animal.

**Electron microscopy**

Small tissue pieces of adrenal tumor were fixed in 4% paraformaldehyde - 1 % glutaraldehyde in 0.1 mol/1 phosphate buffer, pH 7.3, for 3 h, postfixed in 2% Os04 in 0.1 mol/1 cacodylate, pH 7.3, dehydrated in ethanol, and embedded in epoxy resin. 70 nm sections were stained wit h uranyl acetat e and lead citrat e and examined at 80 kV under a Phillips electron microscope 301 (Phillips. Rahway, NJ).

***In situ* labeling of DNA fragments**

The staining of apoptotic nuclei was achieved by non-radioactive *in situ* end-labeling. The free 3'-ends o f cellular DN A served as template an d Wer e elongate d an d labele d wit h digoxigenin - marked deoxyUTP. The digoxigenin was subsequentl y immunodetected wit h anti-DI G peroxidase , visualize d wit h 3-amino-9 - ethylcarbazole (AEC) and counterstained with methylene green. In additiona l experiments , immunodetectio n an d visualizatio n were carried out with anti-digoxigenin-rhodamine conjugate according to the supplier s protoco l (ApopTag®-Kit, Intergen Company, Purchase, NY).

**Statistical analyses**

All *in vitro* experiments were performed in quadruplicate. Statistical analyse s wer e performe d wit h Prism2/GraphPa d softwar e using the Mann-Whitney test for evaluation of tumor volume experiments and the Kaplan-Meier method for survival curves. Results were considered statistically significant where $p < 0.05$.

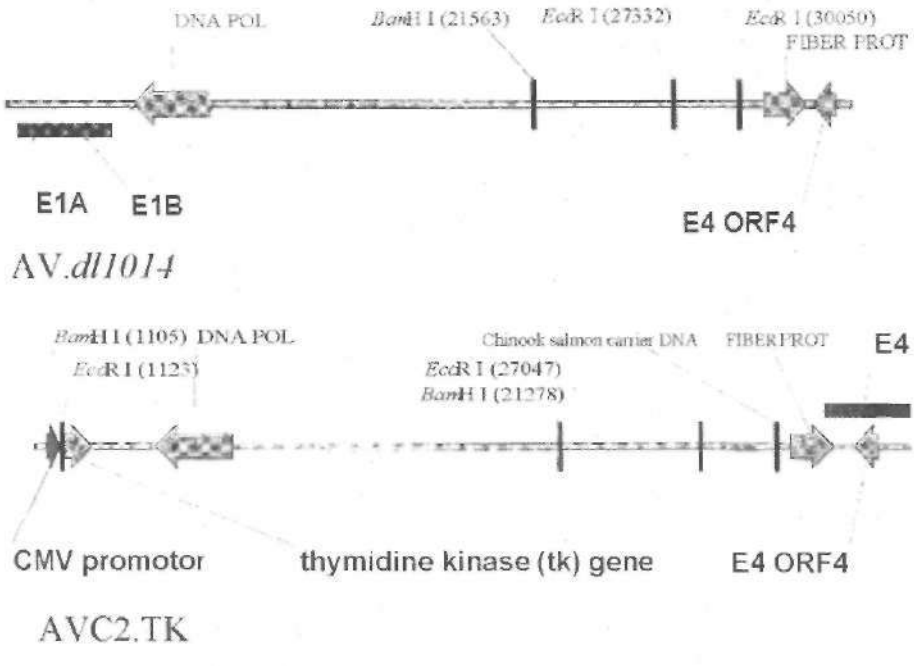



Fig. 1 Vecto r maps of AVC2.TK and AV.dl1014. AVC2.TK has a deletion in early region 1 , in which the CMV promotor and herpes simplex type I thymidine kinase have been inserted. AV.dll014 has a deletion in early region 4 with only open reading frame 4 left (F.4orf4). Both deletions render the adenoviral vectors replication incompetent, however, if El and E4 products will be expressed in a single cell from both vectors, replication can occur due to transcomplementation. Horizontal black bars in the figure depict early region 1 or 4 by which gene products have to be supplemented to the second vector.

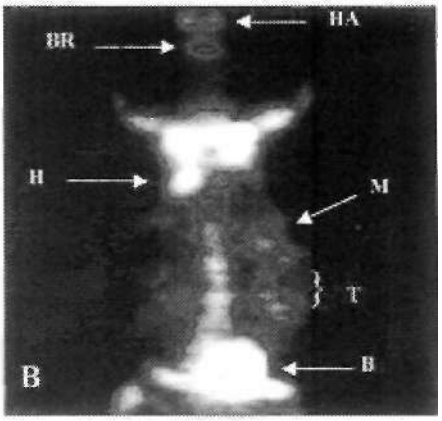




Fig. 2 Huma n adrenocortical xenograft tumors in nude mice, A Femal e nude mice were injected subcutaneously into the right flank with 10 *10$^6$ SW-13 cells. Prominent tumor nodules formed intra- and/or subcutaneously and afte r 6-7 weeks metastases occurred. B Whole-body volume reprojection image of an adrenal tumor-bearing mouse after injection of $^{18}$F-FDG. Primary tumor (T) and metastasis (M)are visible. Two data collections were required to visualize the whole animal (H = heart, BR = brain, HA = Harderian glands, B = bladder).

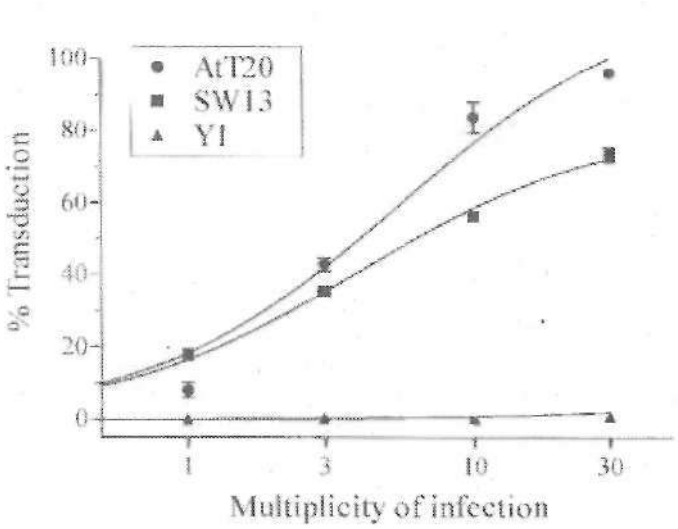
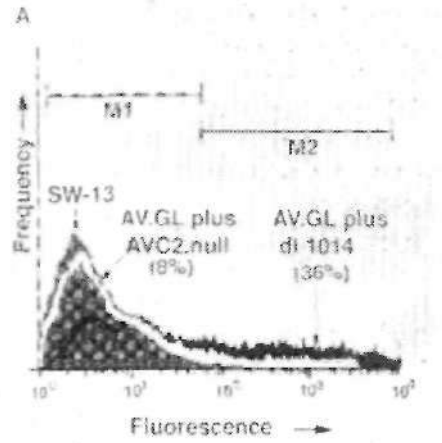



Fig. 3 Transductio n of tumor cells *in vitro.* Cells plated in 6-well plates at a density of 3 * 10"$^J$ cells per well were transduced with AV.CL at MOI of 1 to 100 PFU/cell, harvested after 80 h and subjected to FACS analysis. Maximu m transductio n fo r huma n adrenocortica l SW-1 3 an d mouse pituitar y AtT20-cell s wa s achieved a t MOI s o f 30PfU/cell . A t higher MOIs no further increase in transduction rate could be detected and virus dependent cytotoxity increased (data not shown).



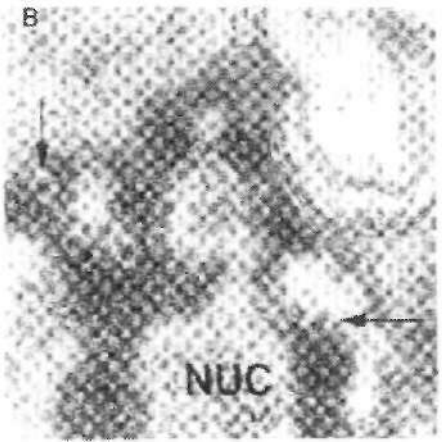




Fig. 4 *I n vivo* transduction of human adrenocortical cell xenografts. A Tumor s were transduced *in vivo* with either 2 * 10$^9$ PF U AVC2.-null, 1 *x* 10$^{"1}$ PFU AV.GLp/us 1 x 10$^9$ PFU AVC2.-null o r 1*10 $^9$ PF U AV.C L *plus* 1x10 ° PF U H5.*d!10l4* and transgen e expression was assessed by FACS analyses. Tumors transduced with transcomplementin g vector s exhibite d an u p t o 4-fol d highe r rat e o f transduction , median transductio n efficienc y wa s 9.8 % with non-transcornplementin g viruse s an d 36.7% fo r transcomplementin g transduce d tumors (p<0,05) . B Transmissio n electro n micrographs demonstrating nuclear.viral par-ticles in *situ.* Transcomplementing transduced tumors wer e examine d fo r newl y assemble d viral particle s 9 6 h afte r transduction . *D e novo* assemblin g o f viral particle s insid e th e nucleus is a characteristic feature of virus production and indicative for replication. The figure depicts characteristi c adenoviral particle s with circula r electro n dens e structur e an d halo as nuclea r inclusions i n a SW-1 3 tumor cell.

## Results

### Characterization o f **tumor model**

SW-13-derived tumor nodules were established i n the flanks of nude mic e b y subcutaneou s and/o r intraderma l injectio n o f 10 * 10$^6$ cells. The adrenocortical tumor model used in this study was a progressive and lethal disease with no evidence of spontaneous regression . Animal s wit h ulceratin g tumor s o r tumor s greater than 150 0 mm$^3$ were euthanized, and are referred t o in the assessment of the survival rate as "succumbed to tumor burden". Tumors reached an average volume of 300 mm$^3$ by 5 weeks after inoculation. By 7 to 9 weeks, metastases could be detected by visual examinatio n (Fig. 2 A) o r gross dissectio n and metastatic tumor deposits could b e readily localized b y PET scan with

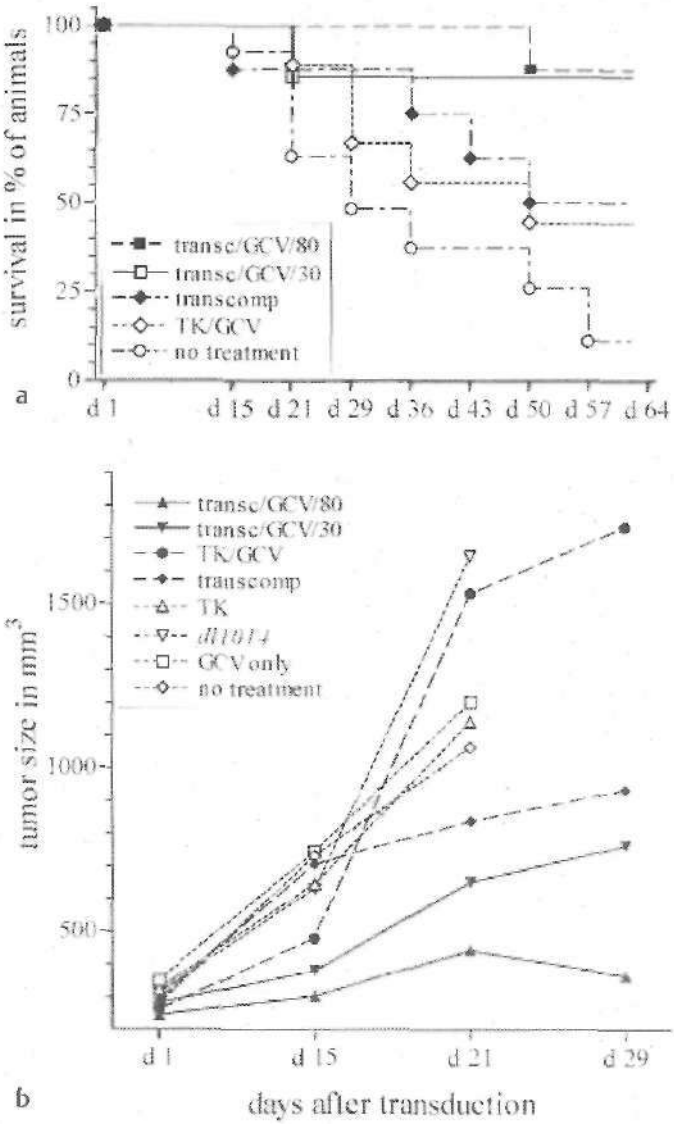



Fig. 5 Treatmen t of human adrenocortical SW-1 3 xenograf t tumors in nud e mice . Tumor s wit h a mea n volum e o f3.0 0 mm$^3$ (vol - ume = l *x* w x h) were transduced with 2 * 10$^9$ PFU of either trans-complementing o r non-replicatin g adenovira l vectors . Ganciclovi r ("IQOmg/kg/d) was given in divided doses twice daily for 5 days by intraperitoneal injection . Contro l animal s receive d eithe r viral vecto r without GCV or GCV alone. A The additive effects of TK, transcomplementation and GC V administration markedl y improved survival compared to [TK/GCV] (p<0.02). Median survival time was 50 d (p = 0.07) forfJK/GCV], and 60.5 d (p.= 0.002) for animals transduced with transcomplementing vectors but without ganciclovir (transcomp). B Tumo r size was monitored weekly . Growt h inhibitio n and tumo r volum e reduction in animal s receiving transcompiementating vectors plus GCV at 80h[transc/GCV/80] post-transductio n was superior to all other approaches, including non-replicating AVC2.TK *plus* GCV treatment (after 29 d; p » 0.001). Transcomplementin g transduction wit h viral replication retarded by administration of GCV at 30 h post-transductio n demonstrated a noticeable , bu t statisticall y insignifican t improvemen t comparable to AVC2.TK pZusH5.dll 014 without GCV.

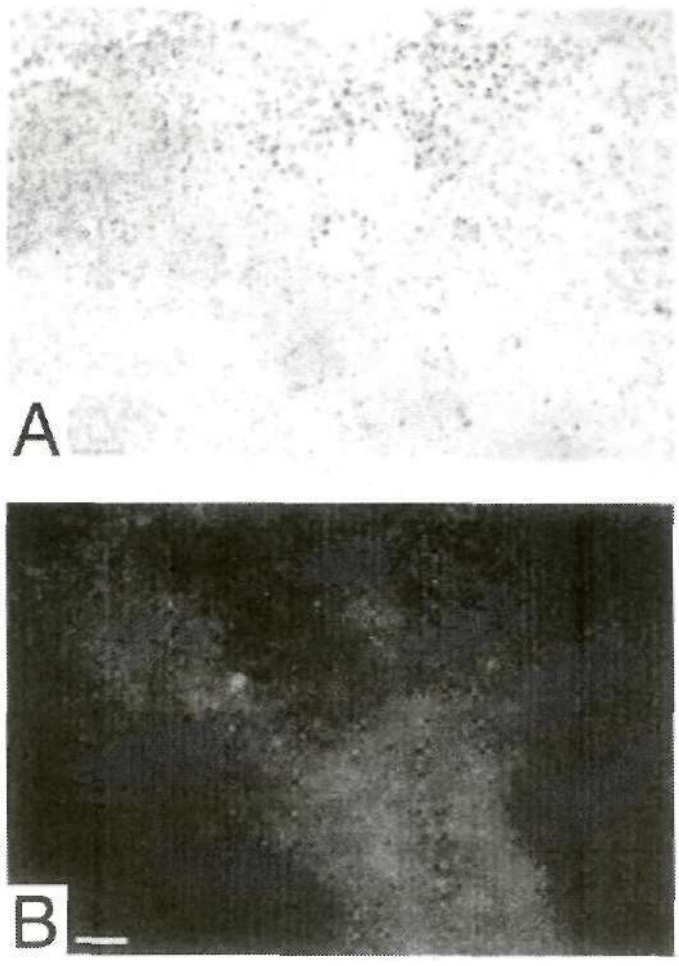



Fig. 6 Fre e 3'-end s of DN A of apoptotic nuclei were labeled wit h digoxigenin-marked deoxyUTp , subsequentl y immunodetecte d wit h anti-DIG-peroxidase and visualized with AEC . Fig.6 A shows an AV.GL transduced SW-13 tumor with single apoptotic nuclei and cell clusters •with brown stained nuclei throughout the specimen. In Fig. 6B apoptotic foci wer e frequentl y foun d adjacen t t o site s of injectio n and t o areas within th e tumo r demonstratin g viral transductio n a s demonstrated by flourescence induction of the transgene (bar = 50 Lim).

$^{18}$F-FDG (Fig.2B) . Histologica l studie s reveale d multilobulate d tumors wit h an adrena l glandula r texture (Fig . 6). The y developed a pseudo-capsule and were highl y vascularized. Histolog y of th e primar y an d metatstati c tumo r deposit s demonstrate d characteristic signs of their common origin; bot h demonstrated compact cells with pleomorphic nuclei and an abundance of mitotic figures. Tumor cell masses could frequentl y be found on the outer side of the pseudo-capsule and occasionall y within penetrating vessels. On the ultrastructural level, tumors exhibited mitochondria with tubulovesicular structure and ample smooth endoplasmic reticulu m typica l o f adrenocortical cells . The tumo r cells di d no t expres s ga p junctions b y ultrastructura l analysi s (see Figs. 4B and 7) or immunohistochemistry (data not shown).

*In vitro* transduction with AVGL

SW-13 cells were transduced *in vitro* with AVGL at MOIs of 1 to 30PFU/cell. Transductio n efficienc y wa s compare d t o mous e adrenocortical Y1 cell s and the pituitary cell line AET20. A high rate of transduction in human SW-13 cells occurred at MOIs of 10 to 30 PFU/cell, whereas murine cell lines showed a vastly lower transduction rate (Fig.3). At MOIs of 100PFU/cell . no further increase i n transductio n rat e coul d b e detected , and virus-dependent cytotoxicity increased .

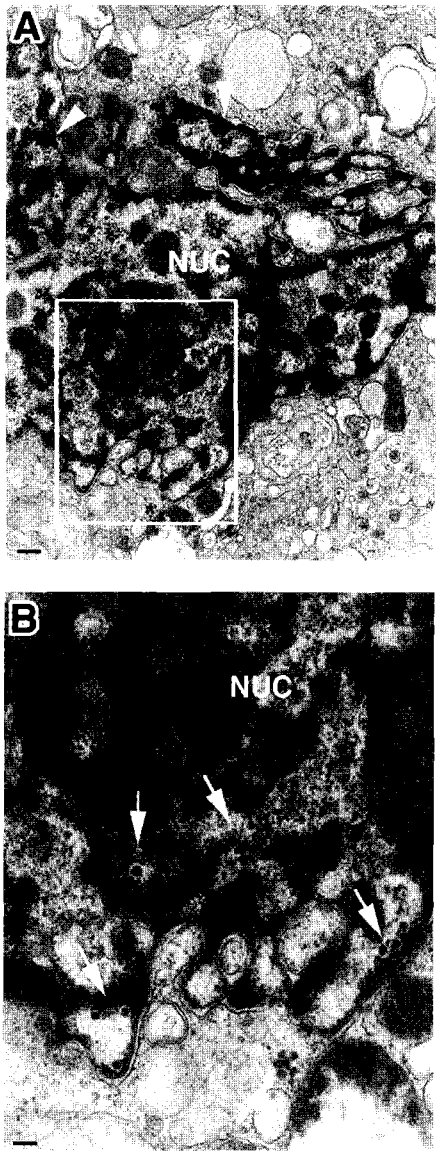



Fig. **7** Ultrastructural analysis of cell death in tumors Xenografts transduced with transcomplementing adenoviral vectors AVC2.TK *plus* H5.*dl1014*. Tumors exhibited mitochondria with tubulo-cristular structure typical of adrenocortical cells. **A** Cells showed apoptotic signs such as vacuolization of the nucleus (N) and cytoplasm, but only a slight condensation of chromatin and fragmentation of nuclei. The mitochondria (M) exhibited in this figure remained morphologically unaffected, indicating a grossly intact metabolism in these cells (bar = 1 μm). **B** Higher magnification of the inset from Fig. **7A** demonstrating intranuclear viral particles (arrows) indicative of viral replication (bar = 0.1 μm).

### *In vivo* transduction and transcomplementation

Tumors (n = 10) were transduced *in vivo* with either $2 \times 10^9$ PFU AVC2.null, $1 \times 10^9$ PFU AV.GL plus $1 \times 10^9$ PFU AVC2.null, or $1 \times 10^9$ PFU AV.GL plus $1 \times 10^9$ PFU AV.*dl1014*. Tumors were harvested, digested with triple enzyme solution [39], and transgene expression was assessed by FACS analyses 72 and 96 h after injection. Tumors transduced with transcomplementing vectors exhibited up to 4 times the rates of transduction. Median transduction efficiency was 9.8% with non-transcomplementing viruses and 36.7% for tumors transduced with vectors that transcomplement ($p < 0.05$; Fig. **4A**). The percentage of transduced cells remained constant after three days, suggesting that a steady state between viral *de novo* transduction and cell loss *via* viral cell lysis is reached in this time frame.

### Treatment of adrenocortical cell tumors

Tumors with an average volume of 300 $mm^3$ at the time of treatment were injected with $2 \times 10^9$ PFU of adenoviral vector. The effect of the treatment was monitored by weekly measurements of tumor size and assessment of survival.

A suppression in the growth of SW-13 derived tumors could be observed in all animals transduced with vectors expressing *hsv*-TK and treated with GCV (groups [TK/*dl1014*/$GCV_{80}$], [TK/*dl1014*/$GCV_{30}$] and [TK/GCV]) within 15 days after onset of treatment. Shrinkage of the tumors in group [TK/*dl1014*/$GCV_{80}$] continued up to 30 days after GCV treatment (Fig. **5A**). Interestingly, there was a gradation of effectiveness in growth inhibition and volume shrinkage. The gradation was dependent on time allowed for completion of virus replication and ganciclovir administration; growth inhibition after transcomplementing transduction with GCV treatment 80 h post-transduction was superior to transcomplementing transduction and GCV treatment 30 h post-transduction as well as tumors transduced with AVC2.TK plus GCV treatment and significant after 29 d ($p = 0.001$; Fig. **5A**). The tumors grew continuously in the untransduced control groups [GCV] and [untreated] and in the AVC2.TK and AV.*dl1014* transduced control groups [TK] and [*dl1014*].

Survival was poor in the control groups with an average survival rate below 37% after 4 weeks and below 25% after 6 weeks, with a median survival time of 29 d. Median survival in single groups was: [GCV] = 22 d, [untreated] = 22 d, and [*dl1014*] = 29 d. In contrast, mice with transduced tumors and GCV treatment had a notably prolonged survival when compared to controls. The median survival time was 50 d ($p = 0.07$) for AVC2.TK with GCV treatment (group [TK/GCV]), and 60.5 d ($p = 0.002$) for animals transduced with transcomplementing vectors but without GCV (group [TK/*dl1014*]). Importantly, administration of GCV to transcomplementing transduced animals further improved survival significantly (Fig. **5B**, $p < 0.02$), showing additive effects of TK, transcomplementing and GCV administration. There was no statistical difference between transcomplementing transduced animals with early (after 30 h) or delayed (after 80 h) administration of GCV, groups [TK/*dl1014*/$GCV_{30}$] and [TK/*dl1014*/$GCV_{80}$]. The survival was 100% in the transcomplementing transduced group with GCV treatment 80 h after transduction after 6 weeks and declined minimally to 85% at the termination of the experiments, providing an estimated median survival time of 1.2 years by regression analyses.

### Analyses of cell death in adrenocortical-small cell carcinoma after transduction

Tumors were examined 96 hours after transduction to explore the mechanism of tumor-cell death induced by this therapy. The cleavage of nuclear DNA with a characteristic pattern of fragmentation is a hallmark of programmed cell death. The free 3'-ends of these fragments are available for labeling and thus allow detection of apoptotic nuclei within a tissue specimen. In trans-

duced SW-13 tumors, we observed single apoptotic nuclei as well as groups and cell clusters with stained nuclei throughout the specimens (Fig. **6A**). Clusters were frequently found adjacent to sites of injection and virus replication (Fig. **6B**).

To further examine this prominence of apoptosis, we investigated transduced and non-transduced tumors by electron microscopy. Ultrastructural analyses showed signs of apoptosis such as condensation of chromatin and cytoplasm, but also vacuolization of the nucleus and cytoplasm. Cytosolic organelles such as mitochondria remained morphologically unaffected, indicating a grossly intact metabolism in these cells. Most often, slight condensation of chromatin and fragmentation of nuclei with blebbing and a vacuolated structure were observed, especially when the nucleus contained inclusion bodies as a sign of viral replication in the transcomplementing transduced groups (Figs. **7A** and **B**).

## Discussion

In this study, we tested virally-mediated suicide gene therapy in a human xenograft model of adrenocortical carcinoma in nude mice. Transfer of the suicide gene *hsv*-TK with *in situ* transcomplementation of vectors and the subsequent administration of the antiviral drug GCV was a powerful tool against SW-13 derived adrenocortical tumors. The anti-tumor effects of viral cytolysis and transgene-mediated tumor cell ablation yielded more effective inhibition of tumor growth and significantly increased the survival rate in this model.

In the presented study, we used two separate viral species with either E4 (H5.*dl1014*) or E1A/B deletions (AVC2.TK; Fig. **1**) that are replication deficient in cis- but replication-competent in co-transduced cells. Transcomplementation in one cell creates a permissive environment for amplification and local spread of both vectors. The selection of H5.*dl1014* to transcomplement E1 defects of AVC2.TK was based on its unique properties related to the solely expression of E4orf4 of the E4 gene products. The E4orf4 protein itself is cytotoxic when expressed in multiple transformed cell lines [40]. Induction of a p53-independent pathway is implicated in the apoptotic death caused by E4orf4 expression in the cell lines that were tested [41]. The ability of *dl1014* to kill transduced tumor cells beyond the focus of transcomplementation enabled replication may greatly increase its utility as the vector that supplies the E1-transcomplementing function. Although we have not seen statistically significant anti-tumor activity of *dl1014* when used as a monovalent vector, no amplification or spread of the virus is possible in the absence of E4-transcomplementation, and toxicity induced in 5 – 10% of the cells within injected tumors is undetectable by measuring tumor volumes. However, *in situ* end-labeling of nuclei reveals increased intratumoral apoptosis at sites both adjacent and distant to areas of viral replication following tumor transduction (Fig. **6**). Transgene expression, direct viral cytotoxity or stimulation of normal apoptotic pathways that appear to be otherwise diminished might have contributed to this phenomenon [49].

In our model, *tumor lysis* by transcomplementation alone increases survival time to 60 days, consistent with recent reports on beneficial oncolytic effects through replicating adenoviral vectors without suicide gene [42]. The expression of both E1 and E4 gene products are essential for viral replication; there are wide areas of tumor tissue subjected to cell lysis when the transcomplementing approach is used (Fig. **6**). In addition, ultrastructural analyses demonstrate nuclei with many inclusion bodies and altered morphologies in treated tissues, evidence of viral replication and associated cytopathic effects.

Viral replication in the transcomplementing system improves *transgene distribution*. Ninety-six hours after vector injection, there is a cell transduction rate 4 times above that seen in transductions using non-transcomplementing vectors. In this way, superior transgene spread relative to replication-deficient vectors *in vivo* was achieved as visualized through AV.GL-expression (Fig. **4**). The efficacy of the *hsv*-TK transgene and/or E4orf4 increased in a manner dependent upon viral replication in our system and, therefore, significantly improves estimated median survival in an additive manner when combined with GCV. Replication-attenuated adenoviral vectors have been tested in systemic and local application strategies for biological amplification of anti-tumor effects [30,43], and additive benefits after GCV treatment have been reported [44]. It is noteworthy, however, that replication-attenuated vectors with E1B deletions may require dysfunctional p53 mechanisms for viral replication to occur [29]. As such, this approach may not be suitable for treatment of approximately two-thirds of adrenocortical carcinomas, which possess intact p53.

Gap junctions enable distribution (local *bystander effect*) of toxic GCV metabolites into neighboring cells, leading to wider cell ablation, but many human malignancies form few if any gap junctions [45,46]. In fact, the original tumor from which SW-13 was derived was reported not to express gap junctions; in our ultrastructural and immunohistochemical analyses, gap junctions could not be observed. This is consistent with recent reports on decreased numbers of *intercellular* junctions in adrenocortical tumors *in vivo* [47]. Although gap-junction expression can be improved through several compounds [48], a substantial benefit may be achieved by the spread of the suicide gene(s) rather than toxic metabolites, using our transcomplementing system in tumors lacking significant gap junctions. Further on, healthy untransformed cells are more likely to become affected by the bystander effect since they adequately express gap junctions. However, there is no information available concerning an inadvertent bystander effect in healthy cells, and the impact of this probably toxic side effect has yet to be determined.

In our nude mouse model, efficacy is dependent upon the additive effects of direct viral cytotoxity and TK/GCV induced cell death in a fashion independent of normal immune effectors. Nevertheless, tumor lysis induced by specific gene transfer therapies has been suggested for improving the host's immune response in some models [17] and allows speculation of further increments in efficacy in immunocompetent organisms. Interestingly, communication between T-lymphocytes and adrenocortical cells has been reported [50]. Normal adrenocortical cells express MHC class II molecules, but this is diminished in adrenocortical tumors [5], and this disruption may be implicated in the escape of adrenocortical tumors from immune surveillance. Indeed, we have found that upregulation of MHC class I molecules occurs during apoptosis in rat glioma cells expressing *hsv*-TK after GCV administration and that this is associated with im-

proved anti-tumor responses which can retard progression of, or ablate, distant tumor deposits (W.J.R., personal observation). Up-regulation of MHC molecule expression induced by the therapy we describe may then improve host anti-tumor immunity in models where intact immune functions are present. Virus-mediated cell lysis will cause a release of mediators, which then activates the immune system and surrounding infected cells and – eventually – the viral cycle as well.

Inadvertent dissemination or systemic application of vectors expressing *hsv*-TK and even inactivated virus particles lacking transgene expression may cause severe liver damage, inflammation and necrosis, and death in experimental animals after exposure to GCV has been reported [32,33]. Similarly, liver toxicity through non-replicating vector administration has been seen in primates and humans [51]. However, in the model investigated, release of virus from transduced tumors is accompanied by a dilutional effect that stochastically diminishes the likelihood of generating the co-transduced conditions necessary for viral replication. Furthermore, H5.*dl1014* expresses only the orf4 peptide of early region 4 and, therefore, cells infected *in vitro* with H5.*dl1014* demonstrate only minimal viral DNA replication, which is unique among E4 mutants [34,52]. When cells are infected in the absence of E4orf6 or E4orf3, E4orf4 downregulates the activity of E1A proteins. This leads to reduced expression of viral genes dependent upon E1A R289 phosphorylation and causing the observed replication deficient phenotype [53–55]. Separate studies will be necessary to establish what dose-limiting toxicities exist in this model and if modifications can lessen any such toxicity. In addition, a restricted opportunity for the generation and release of pseudo-wild type revertants must be carefully considered and addressed when the transcomplementing approach should be used in a clinical setting. However, the chance of recombination-mediated reversion to wild type adenovirus is low [56].

In summary, our findings demonstrate that this novel transcomplementing system of adenoviral vectors significantly enhances *hsv*-TK/GCV suicide gene therapy for cancer. Blockade of tumor growth improves and survival time is greatly prolonged. The mechanisms underlying tumor growth retardation and/or ablation involve increased replication-dependent cytoloysis and more efficient transgene delivery, which provide improved local bystander effects and are characterized by a high frequency of apoptotic cell death in transcomplementing transduced tumors. Transcomplementing vectors are a promising system, displaying enhanced local transduction rates but low systemic toxicity. When used as vehicle for delivery of the *hsv*-TK transgene, significant therapeutic benefits can be observed in a model of adrenocortical cancer. This approach may be refined to become a new treatment option for patients with advanced adrenal cancer.

## Abbreviations

GCV: Ganciclovir; *hsv*-TK: *herpes simplex virus* type one thymidine kinase; MOI: multiplicities of infection; MHC: major histocompatibility complex.

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