Oxidative Stress-Regulated Lentiviral TK/GCV Gene Therapy for Lung Cancer Treatment

Hanna M. Leinonen1, Anna-Kaisa Ruotsalainen1, Ann-Marie Määttä2, Heidi M. Laitinen1, Suvi M. Kuosmanen1, Emilia Kansanen1, Jere T. Pikkarainen1,3, Jari P. Lappalainen1, Haritha Samaranayake1,3, Hanna P. Lesch3, Minna U. Kaikkonen1,3, Seppo Yli-Herttuala1,2, and Anna-Liisa Levonen1

Abstract

Nuclear factor erythroid-2 related factor 2 (Nrf2) is a transcription factor that regulates protection against a wide variety of toxic insults to cells, including cytotoxic cancer chemotherapeutic drugs. Many lung cancer cells harbor a mutation in either Nrf2 or its inhibitor Keap1 resulting in permanent activation of Nrf2 and chemoresistance. In this study, we sought to examine whether this attribute could be exploited in cancer suicide gene therapy by using a lentiviral (LV) vector expressing herpes simplex virus thymidine kinase (HSV-TK/GCV) under the regulation of antioxidant response element (ARE), a cis-acting enhancer sequence that binds Nrf2. In human lung adenocarcinoma cells in which Nrf2 is constitutively overexpressed, ARE activity was found to be high under basal conditions. In this setting, ARE-HSV-TK was more effective than a vector in which HSV-TK expression was driven by a constitutively active promoter. In a mouse xenograft model of lung cancer, suicide gene therapy with LV-ARE-TK/GCV was effective compared with LV-PGK-TK/GCV in reducing tumor size. We conclude that ARE-regulated HSV-TK/GCV therapy offers a promising approach for suicide cancer gene therapy in cells with high constitutive ARE activity, permitting a greater degree of therapeutic targeting to those cells.

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Introduction

Oxidative stress, an imbalance between the production and disposal of reactive oxygen species (ROS), plays an important role in carcinogenesis due to mutations in DNA, proteins, and lipids caused by ROS (1). Importantly, many carcinogens act as reactive electrophilic intermediates. On the other hand, the effect of various anticancer drugs is based on ROS-mediated apoptosis (2). Many cancers have developed resistance against anticancer drugs, and in drug-resistant cancer cells the expression of many self-defence genes, such as antioxidant and phase II detoxifying enzymes as well as drug efflux proteins is increased (3–6).

The Kelch-like ECH-associated protein 1 (Keap1)–nuclear factor erythroid-2 related factor 2 (Nrf2), NFE2L2 pathway is the major sensor of endogenous and environmental oxidant and electrophile stress. Nrf2 is a member of the Cap’n’Collar basic leucine zipper (bZIP) family of transcription factors, and it recognizes the antioxidant response element (ARE) sequence in the regulatory regions of target genes. In basal conditions, Nrf2 is located in the cytoplasm and is repressed by the Keap1 dimer, which facilitates proteasomal degradation of Nrf2 through the Cullin3-Rbx1 ubiquitin ligase complex. On exposure to inducing agents, the interaction of Keap1 with Nrf2 is interrupted so that Keap1 no longer is able to direct Nrf2 to proteasomal degradation, and therefore Nrf2 stabilizes and translocates to the nucleus. In the nucleus, Nrf2 binds to ARE as a heterodimer with small Maf proteins, and drives the expression of large number of ARE-regulated genes (7, 8). These genes encode, for example, antioxidant and phase II detoxification enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase (GCL), and heme oxygenase-1 (HMO; ref. 9). Nrf2 has also been shown to regulate the expression of some efflux proteins, such as multidrug resistance proteins (10, 11).

Nrf2 has long been recognized as the key mediator of cancer chemoprevention (12, 13). Several chemopreventive agents, such as dithiolethiones and isothiocyanates, have been shown to exert their actions via Nrf2 activation, and some of these agents, such as oltipraz and sulforaphane, have entered clinical trials. In addition, Nrf2-deficient mice are more susceptible to chemical-induced carcinogenesis (7, 14). However, it has been recently discovered that Nrf2 not only protects against cancer development but can also protect cancer cells against chemotherapeutic agents thereby providing advantage for cancer growth (15). Of note, it has been reported that Nrf2 is...
overexpressed in several lung cancer cell lines and patient samples, and this is mainly due to mutations in NFE2L2 or KEAP1 genes. This has led to a notion that enhanced ARE activity is in part responsible for the development of chemoresistance and that inhibition of the Nrf2 signaling pathway could be beneficial in certain cancers (16–19).

Suicide gene therapy is widely used in cancer gene therapy. One of the most common suicide gene therapy systems is based on thymidine kinase (TK) gene of the herpes simplex virus (HSV). When thymidine kinase is transferred into cancer cells and cells are treated with a prodrug ganciclovir (GCV), ganciclovir is metabolized to ganciclovir-monophosphate. Monophosphorylated ganciclovir is further phosphorylated by cellular kinases into a triphosphate form, which is toxic to cells, leading to death of not only tumor cells containing transferred thymidine kinase gene but also the neighboring cells due to the bystander effect (20). To enhance the tumor selectivity of the system, several natural or synthetic enhancer-promoters have been used to drive thymidine kinase expression. For example, systems inducible by hypoxia, radiation, and oxidative stress have been developed and successfully applied to tumor xenograft models (21–23).

The notion that ARE activity is constitutively high in certain cancer cell types, prompted us to study whether this attribute could be exploited in HSV-TK/GCV gene therapy by using the ARE element to drive the transgene expression. Here, we show that ARE-regulated HSV-TK/GCV therapy is effective both in vitro and in vivo in A549 lung cancer cells, which have constitutive ARE activity due to mutations in Keap1 protein.

Materials and Methods

Cell Culture

All the cell lines used were purchased from The American Type Culture Collection between years 2001 and 2010 and were systematically validated for the expression of specific marker genes, such as NFE2L2 and KEAP1. Human lung adenocarcinoma epithelial A549 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Sigma) supplemented with 10% (v/v) FBS (Sigma) and 1% penicillin/streptomycin (Sigma). Human bronchiolar pulmonary adenocarcinoma NCI-H441 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 1% sodium-pyruvate (Sigma), 1% HEPES (Sigma), and 1% penicillin/streptomycin. Bronchiolar Beas2B cells were maintained in Bronchial Epithelial Cell Growth Medium (Lonza) and were cultured in precoated [0.01 mg/mL fibronectin (Sigma), 0.03 mg/mL bovine collagen type I (Cultrex), and 0.01 mg/mL bovine serum albumin (Sigma) in HBSS (Sigma)] plates.

DNA sample collection and sequencing

Beas2B and NCI-H441 cells were lysed to nuclear extraction buffer (10 mmol/L Tris pH 7.4, 10 mmol/L NaCl, 5 mmol/L MgCl2, 0.1% NP-40) and centrifuged for 5 minutes, 1,500 x g, at +4°C to collect the nuclei. The nuclei were lysed to SDS lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris–HCl pH 8.1) and the samples were sonicated for 5 minutes (30–30 seconds cycles) using Bioruptor UCD-200 instrument (Diagenode) and centrifuged full speed for 10 minutes at more than 4°C. DNA was extracted using a DNA extraction kit (JETquick, GEN-OMED) according to the kit protocol.

All coding exons for KEAP1 and exon2 for NFE2L2 were amplified by PCR using Phusion High-Fidelity DNA Polymerase (M0530S, New England Bio Labs) and appropriate primers from 2 recent publications (18, 24). Primers were synthesized by TAG Copenhagen. PCR products were purified and analyzed by sequencing at the DNA Sequencing Facility of the A.I. Virtanen Institute, University of Eastern Finland (Kuopio, Finland).

RNA isolation and quantitative real-time PCR

Total RNA was extracted with TRI Reagent (Sigma-Aldrich). One microgram of the RNA was used for cDNA synthesis by High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems). The relative expression levels were measured by quantitative real-time PCR (qRT-PCR) using relative standard curve method (StepOnePlus Real-Time PCR systems, Applied Biosystems) with specific Assays-on-Demand target mixtures for Nrf2 (Hs00232352_m1, Applied Biosystems) and Keap1 (Hs0022227_m1, Applied Biosystems).

Western blotting

Cells were lysed and protein concentrations were measured with the BCA Kit (Pierce). Equal amounts of protein were used for electrophoresis. Proteins were transferred to nitrocellulose membrane, blocked o/n in more than 4°C with 5% nonfat dry milk in TBS-Tween, and incubated for 2 hours in room temperature or o/n in −4°C with the primary rabbit polyclonal Nrf2 antibody (sc-13032, Santa Cruz Biotechnology), with the rabbit polyclonal Keap1 antibody (cat. # 1050, Proteintech Europe) or with the rabbit polyclonal β-actin antibody (#4967L, Cell Signaling Technology). Blots were detected by incubating the blots with horseradish peroxidase–conjugated secondary antibody (Thermo Scientific) and by using ECL Plus Western Blotting Detection System (GE Healthcare) with Typhoon 9400 (GE Healthcare). Images were processed with ImageQuant TL (GE Healthcare).

Lentiviral constructs

The cloning of third generation lentiviral vectors, which express the luciferase reporter gene under minimal SV–40 promoter (LV-control) or minimal SV–40 promoter together with 4 AREs from human GCL modifier subunit (LV-ARE-luc) have been described elsewhere (25). Lentiviral ARE-TK (LV-ARE-TK) was cloned similarly but the luciferase gene was replaced with HSV-TK (Fig. 3A). For cloning thymidine kinase under constitutively active human phosphorylase kinase (hPGK) promoter expressing lentiviral vector (LV-PGK-TK), thymidine kinase gene from pUC-TGL-TK (26) was cloned to pENTR-221 (Invitrogen) and further from this plasmid to the lentiviral backbone using Gateway Technology (Invitrogen). The lentiviral vector containing no transgene was used as a control vector (empty virus) in MTT assays and in vivo (25).

Third generation lentiviruses were produced (27) and the titer was assessed by HI p24 ELISA (PerkinElmer). To test the transduction efficiency, the construct expressing GFP under hPGK promoter was used (28).
Luciferase assays

For the luciferase assay, 20,000 to 30,000 cells were plated on black 96-well view plates (PerkinElmer). The transduction efficiency was assessed by transducing cells with GFP expressing lentivirus and fluorescence-activated cell sorting (FACS) analysis as in ref. (28). Multiplicities of infection (MOI) that yielded similar number of GFP-positive cells in FACS analysis as in ref. (28). Multiplicities of infection were injected in 50 μL volume subcutaneously into the flanks of 7-weeks old male NMRI nu/nu mice (Charles River). Two constructs. Forty-eight hours later, cells were treated with LV-control or LV-ARE-luc constructs. Eighty-four hours later, luciferase assay was conducted with Britelite Reporter Assay System (PerkinElmer) according to the manufacturers' instructions. Results were normalized to protein measured with the BCA kit. All data are depicted relative to luciferase activity of LV-control.

Lentiviral HSV-TK/GCV therapy in vitro

A total of 20,000 to 30,000 cells were plated on 96-well plates, and cells were transduced with empty virus, LV-ARE-TK or LV-PGK-TK. The next day, medium was changed and 24 hours later, cells were treated with 10 to 100 μmol/L ganciclovir. Ganciclovir treatment was repeated 2 days later. Five days after the first ganciclovir treatment, the cell survival was measured using CellTiter 96 Aqueous One Solution Cell Proliferation-assay (Promega) according to the manufacturers' instructions. When DEM was used in experiments, it was given 24 hours after transduction in 50 μmol/L concentration together with ganciclovir and was repeated with the ganciclovir treatment. Similarly, doxorubicin was given 24 hours after the first ganciclovir treatment using 1 to 500 nmol/L concentration, and again together with ganciclovir 48 hours later.

Lentiviral HSV-TK/GCV therapy in vivo

A549 cells were seeded on 10-cm plates and transduced with empty virus, LV-ARE-TK or LV-PGK-TK using MOI 10 and grown until near confluent. Cells were washed once with PBS (Sigma), trypsinized (Sigma), and pelleted by centrifugation. Cell pellets were washed with Optimem (Gibco) and finally suspended in a small volume of Optimem. Three million cells were injected in 50 μL volume subcutaneously into the flanks of 7-weeks old male NMRI nu/nu mice (Charles River). Two tumors were injected per mouse. After 1 week, when the tumor sizes were approximately 15 to 25 mm³, mice were given ganciclovir (Roche) 50 mg/kg/day intraperitoneally (i.p.) twice a day for 2 weeks. Control groups were injected with 0.9% NaCl. Tumor sizes were measured twice a week, and tumor volumes were calculated as 4/3 × π × (a/2 × b/2 × c/2) in which a, b, and c are tumor width, length, and height, respectively. After ganciclovir treatment, mice were followed for additional 7 days, and were then sacrificed. All mice were kept in the National Laboratory Animal Centre (Kuopio, Finland), and the experimental procedure was approved by the National Animal Experiment Board (Finland).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism Software. GraphPad Software, Inc.). Statistical significance of in vitro data was evaluated by paired t test and for in vivo data 2-way ANOVA post hoc was used. A P < 0.05 was considered statistically significant. Results are expressed as mean ± SEM.

Results

Status of Keap1 and Nrf2 in Beas2B, NCI-H441 and A549 cells

Recently, it has been shown that the transcription factor Nrf2 is overexpressed in several lung cancer cell lines (16–19). In A549 cells, a Gly—Cys amino acid change at the first Kelch domain of Keap1 (amino acid position 333) has been identified (18), inhibiting Keap1 function and rendering Nrf2 overactive. The sequence of NFE2L2 has been shown to be wild-type in A549 cells (24). The Keap1 gene in Beas2B has been found to be wild-type (18), but the sequence of NFE2L2 in these cells and both genes in NCI-H441 are not known. All identified cancer mutations for NFE2L2 are clustered in exon 2 in sites that are critical for the binding of Nrf2 to Keap1 (29). Therefore, only exon 2 was sequenced in Beas2B and NCI-H441 cells and was found to be wild-type in both cell lines. The locations for Keap1 mutations are more evenly distributed throughout the gene, and the whole Keap1 gene was sequenced in NCI-H441. In NCI-H441, Keap1 exon 4 contained a synonymous substitution in codon 471, which had no impact on the amino acid sequence [CTC (Leu) → CTG (Leu)].

Keap1 expression has been suggested to be regulated via epigenetic mechanisms in human lung cancer cells and tissues (30, 31). We therefore also assessed the mRNA and protein expression of both Nrf2 and Keap1 in all 3 cell lines (Fig. 1A–E). There were no statistically significant differences in Nrf2 mRNA expression between different cell lines (Fig. 1A), whereas Keap1 mRNA was significantly lower in NCI-H441 cells in comparison with Beas2B and A549 cells (Fig. 1B). In line with previous reports (18, 32), Nrf2 protein is increased in comparison with Beas2B and NCI-H441 cells (Fig. 1C and E), whereas the lowest Keap1 protein levels were found in A549 cells (Fig. 1D and F).

Basal and inducible ARE activities in Beas2B, NCI-H441, and A549 cells

We then sought to evaluate the basal ARE activities in Beas2B, A549, and NCI-H441, by transducing cells with the LV-control or with the LV-ARE-luc reporter construct (Fig. 2A; ref. 25). A549 cell line had the highest ARE activity, 26-fold higher than the LV-control, whereas NCI-H441 cell line had the lowest activity having only 2-fold increase in luciferase activity in comparison with LV-control (Fig. 2B). The Beas2B control cells had 5-fold ARE activity compared with LV-control.

Next, we examined whether it would be possible to further increase the ARE activity in A549, NCI-H441, and Beas2B cells by using Nrf2 activators tBHQ and DEM. The inducible ARE activity was measured by transducing cells with either LV-control or LV-ARE-luc reporter construct, and by treating the
cells with different concentrations of DEM and tBHQ (Fig. 2C–E). In A549 cells, in which the basal ARE activity was very high, it was not increased by Nrf2 activators any further (Fig. 2E). In contrast, in both Beas2B and NCI-H441 cells, in which the ARE activity was low in basal conditions, the activity was increased by both DEM and tBHQ (Fig. 2C and D).

We also examined the effect of ganciclovir and doxorubicin, which were used in subsequent experiments, on ARE activity in A549 and NCI-H441 cells. It was found that neither ganciclovir nor doxorubicin had an effect on ARE activity in either cell line (Supplementary Fig. S1).

**ARE-regulated HSV-TK/GCV therapy is effective in A549 and NCI-H441 cell lines in vitro**

Inasmuch as in A549 cells, the constitutive ARE activity was high, and in NCI-H441 cells, the ARE activity was highly inducible by Nrf2 activators, we next assessed whether ARE-regulated vectors could be used to target suicide gene therapy into lung cancer cells. To study this, we used an established prodrug activating HSV-TK/GCV gene therapy approach that has been successfully used to treat non–small cell lung cancer in vitro and in vivo (33, 34). To this end, we cloned thymidine kinase into LV-ARE-luc by displacing luciferase reporter gene with the HSV-TK gene (Fig. 3A), and the vector was used to study the effect of ARE-regulated HSV-TK/GCV therapy in vitro in A549 and in NCI-H441 cell lines. Cells were transduced with an empty lentivirus containing no transgene (empty virus) or LV-ARE-TK. Two days after transduction cells were incubated with different concentrations of ganciclovir for 5 days, and the cell survival was measured with the MTT assay. There was a concentration-dependent reduction of viability in LV-ARE-TK–transduced A549 cells (Fig. 3B). With 100 μmol/L ganciclovir, less than 10% of cells were viable at the end of the study. Ganciclovir treatment did not affect the viability of nontransduced cells or cells transduced with empty virus (Fig. 3B).

In NCI-H441 cells, on the other hand, in which the basal ARE activity is lower, LV-ARE-TK/GCV therapy was not as effective as in A549 cells. Even after 100 μmol/L ganciclovir treatment, almost 40% of cells were still viable (Fig. 3C).

**ARE-regulated HSV-TK/GCV suicide therapy is more efficient than constitutive HSV-TK/GCV therapy in A549 cells but not in NCI-H441 cells in vitro**

To compare the efficacy of ARE-regulated HSV-TK/GCV therapy with constitutive HSV-TK/GCV therapy, A549 cells or NCI-H441 cells were transduced either with LV-ARE-TK or with lentiviral vector expressing HSV-TK under the constitutively active hPGK promoter and treated as previously described. In A549 cells, LV-ARE-TK was equally effective with low ganciclovir concentrations and significantly more effective with higher concentrations than LV-PGK-TK (Fig. 3D). In contrast, constitutive LV-PGK-TK/GCV therapy was significantly more effective than ARE-regulated TK/GCV therapy with all ganciclovir concentrations used in NCI-H441 cells (Fig. 3E).
The combination of LV-ARE-TK/GCV therapy with DEM in NCI-H441 cells or doxorubicin in A549 cells enhanced the effect of LV-ARE-TK/GCV therapy

Because in NCI-H441 cells the ARE activity was highly inducible with DEM and tBHQ, we next examined whether 50 μmol/L DEM could be used to increase the effect of ARE-regulated HSV-TK/GCV therapy. There was a small but significant (with 10 μmol/L ganciclovir \( P = 0.0451 \), with 50 μmol/L ganciclovir \( P = 0.0197 \)) increase in cytotoxicity when 10 or 50 μmol/L ganciclovir was used together with 50 μmol/L DEM as compared with ganciclovir treatment alone, but no further effect was seen when DEM was used together with 100 μmol/L ganciclovir (Fig. 4A).

In addition, we evaluated the efficacy of doxorubicin chemotherapy combined with ARE-regulated TK/GCV suicide therapy in A549 cells (Fig. 4B). Although doxorubicin has been shown to increase cellular ROS formation (35), it did not increase the ARE activity, assessed by the luciferase assay (Supplementary Fig. S1C). Doxorubicin at concentrations 250 and 500 nmol/L, that alone were not toxic to the cells, significantly enhanced the efficacy of HSV-TK/GCV therapy.

The effect of ARE-regulated HSV-TK/GCV therapy \textit{in vivo}

Because ARE-regulated HSV-TK/GCV suicide gene therapy was efficient in A549 cells \textit{in vitro}, we next examined its efficacy \textit{in vivo} using subcutaneous tumor model in NMR nu/nu mice. A549 cells were transduced with empty virus, LV-PGK-TK or LV-ARE-TK, collected and injected subcutaneously into the flanks of nude NMR nu/nu mice, 2 tumors per mouse. After 1 week, when tumors were approximately 20 mm\(^3\), ganciclovir was injected i.p. twice a day 50 mg/kg/day for 2 weeks. Mice were followed for another week and then sacrificed. In both control groups (nontransduced cells and empty virus–transduced cells) tumors grew faster than in other groups in which thymidine kinase was expressed (Fig. 5A and B). Interestingly, LV-ARE-TK alone without ganciclovir caused a significant reduction of tumor growth in comparison with LV-PGK-TK–transduced tumors (Fig. 5B), presumably because of the high expression of transgene that occupies the transcriptional–translational machinery, thus causing cytostasis. However, the tumors were statistically significantly smaller in size in both LV-PGK-TK and LV-ARE-TK in mice receiving ganciclovir in comparison with respective saline controls from day 17 onwards, and by day 28, there were no visible tumors left. In all other groups, there were clearly visible tumors at the end of the study.

Discussion

Accumulating evidence suggests that high constitutive expression of Nrf2 and subsequent upregulation of phase II...
enzymes and other prosurvival genes plays a pivotal role in cancer chemoresistance (36). The growing list of cancer types in which the Keap1–Nrf2 system has been shown to be hyperactive includes lung (24), gall bladder (37), pancreatic (38), hepatocellular (39), ovarian (40), and prostate (41) cancers. This is either due to mutations in KEAPI or NFE2L2 genes, accumulation of proteins that disrupt the Keap1–Nrf2 interaction, such as p62, or Keap1 promoter methylation resulting in reduced Keap1 expression (reviewed in ref. 29). In A549 lung cancer cells, both Keap1 promoter hypermethylation (30, 42) and a point mutation in the Nrf2 interacting domain in KEAPI (43) have been found. In this study, we sought to examine whether dysregulation of the Keap1–Nrf2 system could be exploited to therapeutic advantage by using the Nrf2-driven ARE-element to drive the expression of the HSV-TK suicide gene. The data presented herein show the feasibility and efficacy of such approach in cancer gene therapy.

Ideally, cancer gene therapy should affect only the cancerous tissue having minimal or no effect on healthy and dividing cells. Transcriptional targeting is one of the approaches in which the selectivity is improved by using promoter elements that are primarily active in cancer cells. With respect to gene therapy using the HSV-TK/GCV suicide gene approach, tumor microenvironment-specific promoters that have been used for this purpose include hypoxia response elements derived from the VEGF gene (44) and the glucose responsive hexokinase II promoter (45). In addition, redox-responsive DNA motifs have been used to regulate the HSV-TK/GCV suicide gene therapy in cancer cells that have higher ROS levels than healthy cells (23). ROS are produced also in normally dividing cells as a by-product of cellular metabolism, but their production as well as the activity of ROS-responsive promoters is increased in certain malignant cells, by 2- to 3-fold in comparison with healthy cells assessed by the luciferase reporter assay (23). Similarly, Nrf2 is broadly expressed also in healthy cells, but dysregulation of its activity results in a substantially higher basal activity in A549 cells in comparison with Beas2B and NCI-H441 cells (Fig. 1B), sufficient to confer specificity to this treatment. Remarkably, ARE-driven HSV-TK was more effective than constitutively active PGK promoter in reducing viability in HSV-TK/GCV gene therapy (Fig. 3D) in A549 cells in vitro, showing the efficacy of this approach. In vivo, both PGK-TK and ARE-TK were equally effective, as the response to treatment was complete in both groups treated with ganciclovir, with complete disappearance of tumors in both groups by day 28.

In addition to the use of ARE in the regulation of transgene expression in cells with high basal ARE activity, ARE-driven vectors could be potentially used as an inducible vector system regulated by Nrf2 activators. With respect to cancer, it is interesting to note that certain anticancer agents (46) and

Figure 3. The effect of ARE-regulated HSV-TK/GCV suicide gene therapy. A, a lentiviral vector containing HSV-TK gene, 4 AREs, and minimal SV40 promoter was cloned. HSV-TK regulated by a constitutively active hPGK promoter was also used in this study. B and C, A549 and NCI-H441 cells were transduced with empty lentiviral vector containing no transgene (empty virus) or LV-ARE-TK vector using MOI 10. After 48 hours, ganciclovir was added to cell culture medium and cells were incubated in the presence of ganciclovir for 5 days. Cell viability was measured with the MTT assay and is depicted as percentage viability versus no virus control without ganciclovir; mean ± SEM, n = 12–18. D and E, A549 and NCI-H441 cells were transduced with empty virus, LV-ARE-TK or LV-PGK-TK vector using MOI 10. After 48 hours, cells were treated with ganciclovir and 5 days later, cell viability was measured using the MTT assay. The data are depicted relative to nontransduced cells without ganciclovir; mean ± SEM, n = 12–18. *, P < 0.05; **, P < 0.01; *** , P < 0.001.
activity in our setting (Supplementary Fig. S1C and S1D) nor effect on cell viability in control cells (Fig. 4B), substantially enhanced the cytotoxic effect of ARE-HSV-TK (Fig. 4B). As single agent cancer therapies often result in drug resistance and are therefore less likely to be curative than combination strategies targeting multiple pathways (48, 49), the synergistic effect of the combination of doxorubicin with the ARE-HSV-TK is therefore encouraging and suggests that this combination could be used to enhance therapeutic efficacy.

In conclusion, we have shown that ARE-regulated HSV-TK/GCV therapy is a powerful option for cancer treatment in cells with high constitutive Nrf2 expression. Furthermore, ionizing radiation (47) cause a modest but measureable increase in ARE activity, thus providing a possibility that chemotherapeutic agents or radiotherapy could also be used to boost the efficacy of ARE-HSV-TK/GCV therapy. However, in NCI-H441 cells in which the basal ARE activity is low but highly inducible by commonly used ARE-activators DEM and tBHQ, as assessed by the luciferase reporter assay (Fig. 2C), DEM treatment did not substantially enhance toxicity of the ARE-HSV-TK/GCV therapy (Fig. 4A). Although it is possible that factors, such as the selection of the inducing agent and timing of administration were not optimal to maximize the efficacy of ARE-driven HSV-TK expression, it is unlikely that the inducible ARE activation could be effectively used in vivo. However, doxorubicin, which on its own had no impact on ARE

Figure 4. Enhancement of the effect of ARE-regulated HSV-TK/GCV suicide gene therapy by DEM and doxorubicin. A, NCI-H441 cells were transduced with empty virus or LV-ARE-TK vector using MOI 10. Forty-eight hours after transduction, cells were treated with ganciclovir and the next day, 50 μmol/L DEM was added to cell culture medium. After 5-day, ganciclovir treatment cell viability was measured using the MTT assay. B, A549 cells were transduced with empty virus or LV-ARE-TK vector using MOI 10. Forty-eight hours after transduction, cells were treated with ganciclovir (25 μmol/L) following the addition of doxorubicin (doxo) treatment the next day. Doxo/GCV treatment was renewed every other day. Cell viability was measured with MTT assay 5 days after the beginning of ganciclovir treatment. The data are depicted relative to nontransduced cells without ganciclovir, mean ± SEM, n = 6–8. * P < 0.05; ** P < 0.01; *** P < 0.001.

Figure 5. The effect of ARE-regulated HSV-TK/GCV suicide gene therapy in vivo. A549 cells were transduced with empty virus, LV-ARE-TK or LV-PGK-TK using MOI 10. Cells were collected when near confluent and 3 million cells were injected subcutaneously into 38 NMRI nu/nu mice (2 tumors/mouse). The groups were as follows: no virus (8 mice, n = 12), empty virus (8 mice, n = 12), LV-PGK-TK virus without ganciclovir (8 mice, n = 16), LV-PGK-TK virus + GCV (50 mg/kg/day; 6 mice, n = 12), LV-ARE-TK virus without ganciclovir (6 mice, n = 12), and LV-ARE-TK + GCV (50 mg/kg/day; 6 mice, n = 12). A week after tumor implantation, ganciclovir (or saline) was given i.p. twice a day for 14 days, and a week later mice were sacrificed. Tumor sizes were measured twice a week and tumor volumes were calculated using the following formula: tumor volume = 4/3π(a/2 × b/2 × c/2), in which a, b, and c are tumor length, width, and height, respectively. Results are presented as the tumor volume mm³ ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001 in comparison with nontreated LV-ARE-TK or LV-PGK-TK.
transcriptional targeting with the ARE element, either alone or in combination with other targeting methods, may improve the safety of HSV-TK/GCV therapy.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors' Contributions**

**Concept and design:** H.M. Leinonen, H.P. Lesch, M.U. Kaikkonen, S. Ylä-Herttuala, A.-L. Levenon

**Development of methodology:** H.M. Leinonen, J.T. Pikkarainen, H. Samaranayake, H.P. Lesch, M.U. Kaikkonen, A.-L. Levenon

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** H.M. Leinonen, A.-K. Ruotsalainen, M.U. Kaikkonen, S. Ylä-Herttuala, A.-L. Levenon

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H.M. Leinonen, A.-K. Ruotsalainen, A.-M. Maatia, S. M. Kuosmanen, E. Kansanen

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