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Oncolytic alphavirus SFV-VA7 efficiently eradicates subcutaneous and orthotopic human prostate tumours in mice

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Background: Despite recent therapeutic and diagnostic advances, prostate cancer remains the second leading cause of cancer-related deaths among men in the Western world. Oncolytic viruses that replicate selectively in tumour cells represent a novel treatment candidate for these malignancies.

Methods: We analysed infectivity of avirulent Semliki Fire virus SFV-VA7 in human prostate cancer cell lines VCaP, LNCaP and 22Rv1 and in nonmalignant prostate epithelial cell line RWPE-1. Therapeutic potency of SFV-VA7 was evaluated in subcutaneous and orthotopic mouse LNCaP xenograft models.

Results: SFV-VA7 infected and killed the tested human prostate cancer cell lines irrespective of their hormone response status, while the nonmalignant prostate epithelial cell line RWPE-1 proved highly virus resistant. Notably, a single peritoneal dose of SFV-VA7 was sufficient to eradicate all subcutaneous and orthotopic LNCaP tumours.

Conclusions: Our results indicate that SFV-VA7 is a novel, promising therapeutic virus against prostate cancer warranting further testing in early clinical trials.

Prostate cancer is by far the most common malignancy in men, and after lung- and bronchial cancer, the second leading cause of cancer-related male deaths in the developed world (Siegel *et al*, 2016). Approximately 1 out of 7 men will be diagnosed with prostate cancer during his lifetime (ACS cancer statistics, 2016). Despite improvements in early detection, advanced disease is still incurable (Siegel *et al*, 2016). Oncolytic virotherapy with tumour-specific viruses represents a novel treatment option for such urological malignancies. Indeed, vaccinia-, adeno- and reoviruses have shown promise against prostate cancer in early-phase clinical trials (Delwar *et al*, 2016). Oncolytic Semliki Forest virus SFV-VA7

has demonstrated significant promise in a variety of murine cancer models, including human melanoma (Vähä-Koskela *et al*, 2006), osteosarcoma (Ketola *et al*, 2008), lung carcinoma (Määttä *et al*, 2008), glioma (Heikkilä *et al*, 2010) and syngeneic colorectal carcinoma (Ruotsalainen *et al*, 2015), but its potential against prostate cancer has thus far remained unexplored.

Here we have studied antiviral type I interferon (IFN-I) responses of VCaP, LNCaP and 22Rv1 prostate cancer cell lines and their susceptibility to SFV-VA7-mediated oncolysis in comparison to nonmalignant RWPE-1 prostate endothelial cells. We show that prostate cancer cell lines were readily infected and

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killed by SFV-VA7, whereas nonmalignant prostate cells displayed resistance to infection. Of the tested cell lines, malignant VCaP and nonmalignant RWPE-1 showed functional IFN- β response indicated by STAT1 phosphorylation. Peritoneal administration of SFV-VA7 eradicated 100% of the large, established subcutaneous and orthotopic tumours without observed toxicities.

MATERIALS AND METHODS

Cell lines and viruses. RWPE-1 (ATCC) cells were cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor (GIBCO). 22Rv1 (ATCC) and LNCaP cells were cultured in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Autogen Bioclear, Calne, UK) and 1% L-glutamine (Sigma-Aldrich). VCaP cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% FBS and 1% L-glutamine. All the used culture media were supplemented with 1% penicillin, streptomycin (Sigma-Aldrich). Production of replicative SFV VA7-EGFP and VA7-Rluc have been described earlier (Vähä-Koskela *et al*, 2003; Heikkilä *et al*, 2010).

Measuring of virus production. A total of 80 000 RWPE-1, 22Rv1 or VCaP cells were seeded on 12-well plate and infected 3 days later with VA7-EGFP (MOI = 0.01) in 1 ml volume. Cells were washed with PBS 2 h post infection (pi) and fresh medium was added. Medium (200 μ l) from two parallel wells was collected 48 and 72 h pi. for plaque titration (Ruotsalainen *et al*, 2015). Cell infections were carried out with SFV-VA7 viruses expressing either

EGFP or Rluc transgene, which do not influence the infection (Heikkilä *et al*, 2010).

Cell viability assay. A total of 40 000 RWPE-1, 22Rv1, VCaP or LNCaP cells were seeded on 96-well plate and infected with VA7-EGFP using MOI = 0.01–10. Cell viability was measured from 3 to 4 parallel wells 4 days pi. with Cell proliferation Kit I (Roche Applied Science, Penzberg, Germany). LNCaP viability was measured with Calcein AM assay.

Western blot. To induce STAT1 phosphorylation, cells were treated with 1000 units per ml of human recombinant IFN- β (Nordic Biosite, Täby, Sweden) and incubated at +37 °C for 20 min. Expression of viral proteins and infection-induced STAT1 phosphorylation was detected from VA7-EGFP-infected cells MOI = 10, 18 h pi. Western blotting was performed as described before (Martikainen *et al*, 2015) using the following antibodies: Rabbit polyclonal antibody against nsP3 (kindly provided by Tero Ahola, Helsinki) or viral structural proteins (produced in house), rabbit anti-STAT1 (61011; BD Transduction Laboratories, Franklin Lakes, NJ, USA), rabbit anti-Tyr701-phosphorylated STAT1 (D4A7; Cell Signaling Technology, Danvers, MA, USA) and mouse anti- β -actin (C4, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-rabbit-Cy5 and anti-mouse-Cy3 (ECL Plex, GE Healthcare, Chicago, IL, USA) were used as secondary antibodies. Imaging was performed with a Typhoon scanner (GE Healthcare).

Immunohistochemistry. Brain and tumour tissue samples were collected from mice 72 h after intraperitoneal injection of 1×10^6 PFU VA7-EGFP virus or PBS. Paraformaldehyde-fixed and paraffin-embedded tissue sections were stained using the Vectastain ABC kit (rabbit IgG; Vector Laboratories, Burlingame, CA,

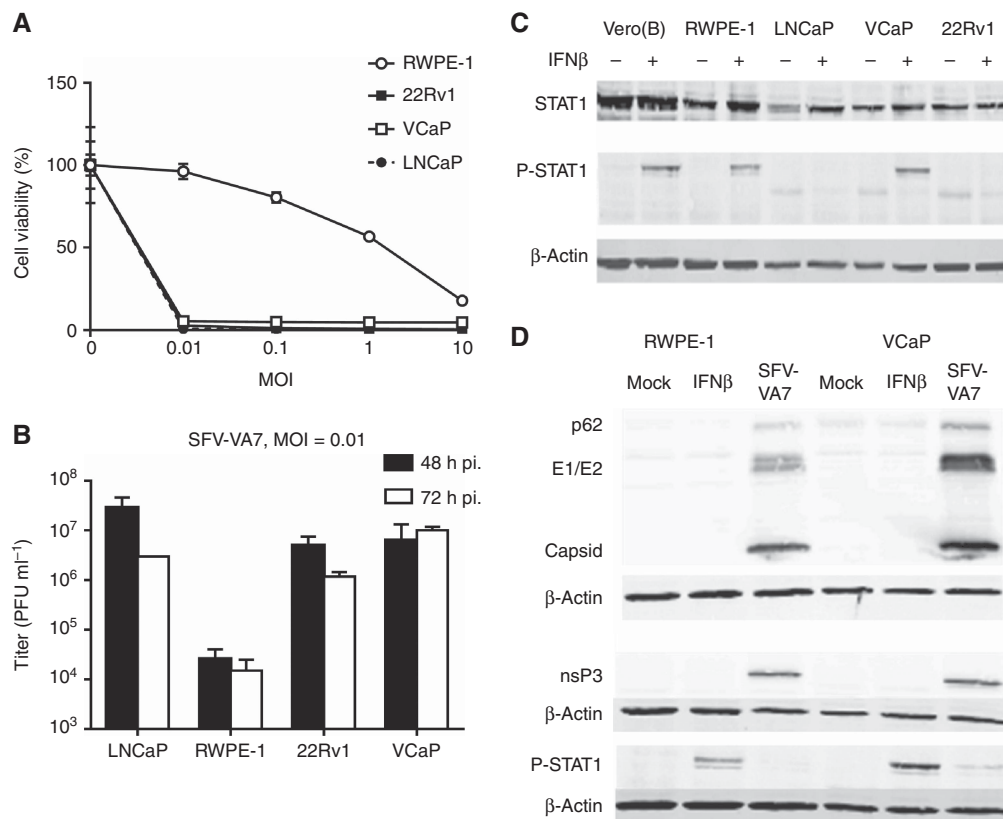


Figure 1. SFV-VA7 infects and kills prostate cancer cell lines VCaP, LNCaP, 22Rv1, while replicating poorly in nonmalignant RWPE-1 prostate cells. **(A)** Cell viability (mean + s.d.) measured 72 h after infection with different MOI of SFV-VA7. The data from two experiments is presented. **(B)** virus titer (mean + s.d.) in cell culture medium measured at 48 h and 72 h post infection with SFV-VA7 MOI 0.01. The data from two experiments presented. **(C)** Western analysis of STAT1, P-STAT1 and β -actin in cells treated with recombinant IFN- β . **(D)** Western analysis of viral proteins and P-STAT1 in IFN-responsive VCaP and RWPE-1 cells infected with SFV-VA7 (MOI = 10, 18 h pi).

USA) and a polyclonal rabbit antibody reactive against SFV structural proteins (produced in-house). 3,3'-diaminobenzidine (SigmaFast DAB; Sigma-Aldrich) was used for colour reaction.

In vivo experiments. CAnN.Cg-Foxn1nu/Crl Balb/c nude mice (Charles River) were used for the animal studies. Work was conducted following Finnish Board of Animal Experimentation guidelines, licence number ESAVI/3257/04.10.07/2014.

For subcutaneous tumours, 5×10^6 LNCaP cells were injected in 100 μ l volume of PBS/Matrigel (50 out of 50) into left flanks of mice. Tumour growth was stimulated with implanted testosterone pellet (10 mg, Innovative Research of America, Sarasota, FL, USA). Upon palpable tumours (100–300 mm³ as measured with caliper ((length \times width²)/2)), mice received a single intraperitoneal injection of 1×10^6 PFU VA7-GFP in 100 μ l PBS. PBS was used as the vehicle treatment. Mice were killed when tumour size reached 1000 mm².

Orthotopic prostate tumours were induced as previously described (Tuomela *et al*, 2009). Briefly, a small incision was made above pubic symphysis and the bladder and seminal vesicles were carefully moved to expose the prostate. 5×10^5 LNCaP cells in 20 μ l volume of PBS/Matrigel were injected into the prostate. Prostate-specific antigen (PSA) secretion into serum was measured with PSA detection kit (Roche, Applied Science). Mice received a single intraperitoneal injection of 1×10^6 PFU VA7-GFP in 100 μ l PBS, when PSA was detectable.

RESULTS

VA7-EGFP infects and kills human prostate cancer cell lines but not nonmalignant prostate epithelial cells. To test the feasibility of SFV-VA7 against prostate cancer, we first analysed its infectivity *in vitro*. Interestingly, even low multiplicity of infection (MOI) of SFV-VA7 efficiently infected and killed all tested VCaP, 22Rv1 and LNCaP prostate cancer cell lines (Figure 1A), whereas the

nonmalignant RWPE-1 cells were significantly more resistant and displayed more than 100-fold reduction in supernatant viral titers (Figure 1B).

LNCaP and 22Rv1 prostate cancer cells have dysfunctional JAK/STAT signaling pathway. We have observed previously that poorly IFN-I responsive tumours represent susceptible targets to SFV-VA7 (Ruotsalainen *et al*, 2012, 2015). Thus, we next studied IFN-I responsiveness of the malignant and nonmalignant prostate cells. As expected, IFN-I treatment induced robust STAT1 phosphorylation in RWPE-1 and positive control cell line Vero(B). In contrast, out of the three studied prostate cancer cell lines LNCaP, VCaP and 22Rv1 only VCaP displayed STAT1 phosphorylation upon IFN- β treatment (Figure 1C). Whereas both RWPE-1 and VCaP cells were type I IFN responsive, only the nonmalignant cells were resistant to oncolytic infection. Thus, we proceeded to further analyse SFV-VA7 replication in these cells. Interestingly, SFV-VA7 infection itself did not induce STAT1 phosphorylation in RWPE-1 cells and only weakly in VCaP cells (Figure 1D). In line with the poor productive infection (Figure 1A), RWPE-1 cells also contained markedly less viral envelope proteins after infection, but interestingly highly similar levels of non-structural protein 3 (nsp3) (Figure 1D).

SFV-VA7 eradicates subcutaneous and orthotopic LNCaP tumours *in vivo*. We next analysed the oncolytic potency of SFV-VA7 in the LNCaP prostate cancer model *in vivo*. LNCaP was chosen due to its unresponsiveness to type I IFN, which was found to be the predominant phenotype in the tested malignant cells. In addition, according to literature, LNCaP is the most widely used cell line tested in oncolytic virotherapy *in vivo* and hence the obtained results were deemed readily comparable to those reported from earlier studies. Strikingly, progressively growing subcutaneous tumours in nude mice started shrinking after intraperitoneal injection of SFV-VA7 and disappeared in 24 days after treatment (Figure 2A and B). Importantly, high virus loads could be detected in the tumours 72 h after infection both by immunohistochemistry

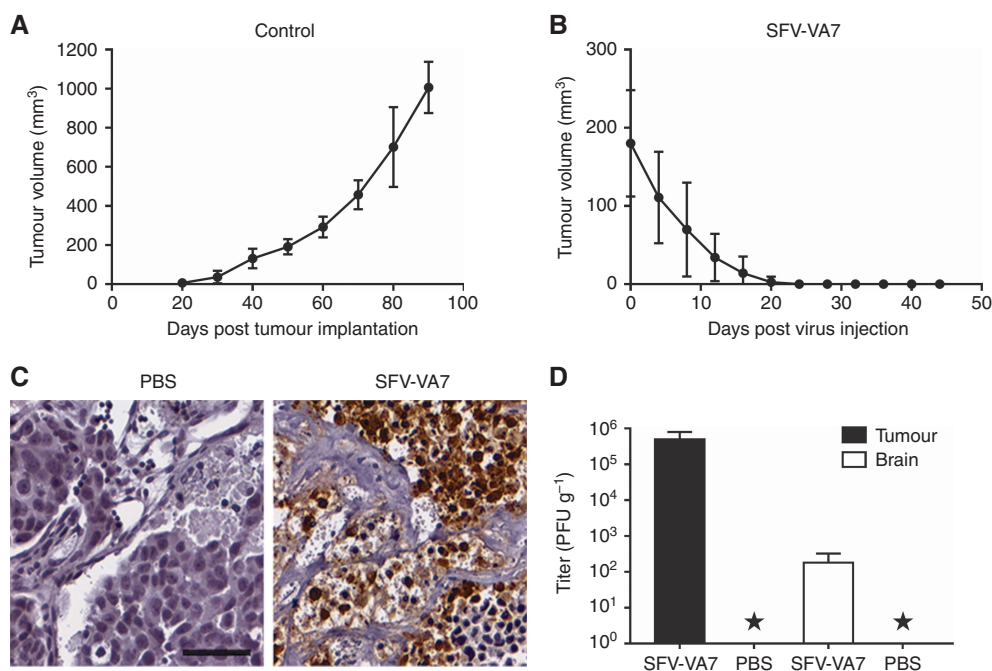


Figure 2. Intraperitoneally administered SFV-VA7 homes into subcutaneous LNCaP tumours and eradicates them. Tumour volumes (mean + range) measured in (A) untreated mice ($n=5$) and (B) mice treated with single intraperitoneal dose of SFV-VA7 ($n=8$, 1×10^6 PFU). (C) Representative immunohistochemical staining of LNCaP tumour samples collected 72 h pi. Virus antigen stained brown. Scale bar 200 μ m. (D) Titration of SFV-VA7 from collected tumour and brain samples 72 h pi. Star: not detected.

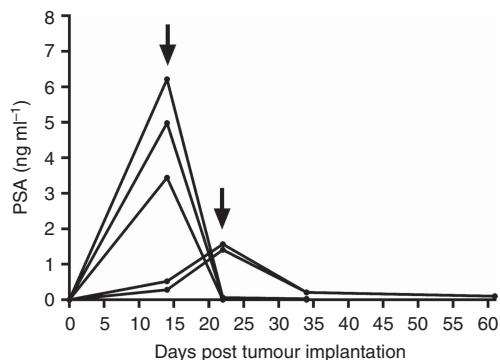


Figure 3. Intraperitoneally administered SFV-VA7 clears orthotopic LNCaP tumours. Human PSA concentrations (ng ml^{-1}) were measured from mouse serum. VA7-EGFP (1×10^6 PFU) was administered i.p. upon detectable PSA levels (black arrows). Each line represents individual LNCaP-tumour-bearing mouse.

(Figure 2C) and plaque titration (Figure 2D). Despite the known neurotropism of SFV, viral loads in brain were low and the infected mice did not display clinical symptoms (Figure 2D).

Finally, we tested SFV-VA7 therapy efficacy in a clinically relevant setting by treating orthotopic LNCaP -tumours in nude mice. Tumour burden was quantified by measuring serum levels of secreted human prostate-specific antigen (PSA). Experiment end point was set to 20 ng ml^{-1} of PSA, which was reached without treatment in ~ 30 days after first detectable PSA correlating with notable tumour mass in the prostate. Compellingly, all peritoneally infected mice experienced rapid and stable reduction in the serum PSA (Figure 3). SFV-VA7-treated mice were killed 90 days after LNCaP cell inoculation, and *post mortem* examination revealed no evidence of residual tumour mass.

DISCUSSION

Intratumoural administration of oncolytic virus has been shown to be effective against prostate cancer xenografts (Schenk *et al*, 2014; Mansfield *et al*, 2016). Systemic administration would be preferable in targeting advanced disease but has usually shown clearly limited efficacy (Berry *et al*, 2008; Li *et al*, 2013). Furthermore, preclinical oncolytic virotherapy research in prostate cancer has relied heavily on subcutaneous tumour models due to the high technical demands of orthotopic inoculation. In one of the few studies available, conditionally replicating adenovirus AxdAdB-3 was reported to modestly improve the survival of SCID mice bearing orthotopic DU145 tumours after intratumoural administration (Satoh *et al*, 2007). In another study, macrophage-based delivery of an oncolytic adenovirus in combination with docetaxel and radiation therapy showed significantly increased survival in orthotopic LNCaP model (Muthana *et al*, 2013).

Here we report that all tested prostate cancer cell lines (LNCaP, VCaP and 22Rv1) were efficiently infected and killed *in vitro* by SFV-VA7, but nonmalignant RWPE-1 cells proved resistant. While the mechanism of RWPE-1 resistance to SFV-VA7 infection warrants further study, high viral nsp3 protein levels after infection indicate that the inhibition takes place after viral entry and early expression of non-structural proteins.

A single peritoneal administration of SFV-VA7 resulted in a 100% cure rate both in subcutaneous and orthotopic LNCaP tumour models, thus suggesting that SFV-VA7 is a novel and highly promising option for treatment of prostate cancer and supporting early clinical testing. The fact that the used mice were immunosuppressed poses a potential caveat in clinical translation.

However, earlier we have shown that SFV-VA7 could destroy type I IFN unresponsive mouse colorectal tumours with equal efficiency in immunocompetent and immunosuppressed hosts (Ruotsalainen *et al*, 2015). Notably, both hormone responsive LNCaP and hormone unresponsive 22Rv1 displayed poor IFN-I responsiveness and were highly susceptible to SFV-VA7 lysis. This result is in line with earlier reports of IFN-I defects in a proportion of human prostate tumours (Danziger *et al*, 2016) and implies that SFV-VA7 could possibly be utilised as a salvage therapy in castration resistant prostate cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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