The Oncolytic Adenovirus VCN-01 as Therapeutic Approach Against Pediatric Osteosarcoma

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Abstract

Purpose: Osteosarcoma is the most common malignant bone tumor in children and adolescents. Despite aggressive chemotherapy, more than 30% of patients do not respond and develop bone or lung metastasis. Oncolytic adenoviruses engineered to specifically destroy cancer cells are a feasible option for osteosarcoma treatment. VCN-01 is a replication-competent adenovirus specifically engineered to replicate in tumors with a defective RB pathway, presents an enhanced infectivity through a modified fiber and an improved distribution through the expression of a soluble hyaluronidase. The aim of this study is to elucidate whether the use of VCN-01 would be an effective therapeutic strategy for pediatric osteosarcoma.

Experimental Design: We used osteosarcoma cell lines established from patients with metastatic disease (531MII, 678R, 588M, and 595M) and a commercial cell line (143B). MTT assays were carried out to evaluate the cytotoxicity of VCN-01. Hexon assays were used to evaluate the replication of the virus. Western blot analysis was performed to assess the expression levels of viral proteins and autophagic markers. The antitumor effect of VCN-01 was evaluated in orthotopic and metastatic osteosarcoma murine animal models.

Results: This study found that VCN-01, a new generation genetically modified oncolytic adenovirus, administered locally or systemically, had a potent antitumor effect in vitro and in vivo in mouse models of intratibial and lung metastatic osteosarcoma. Moreover, VCN-01 administration showed a safe toxicity profile.

Conclusions: These results uncover VCN-01 as a promising strategy for osteosarcoma, setting the bases to propel a phase I/II trial for kids with this disease. Clin Cancer Res; 22(9): 2217–25. ©2015 AACR.

Introduction

Osteosarcoma is the most common primary malignant bone tumor (1). It appears principally in the metaphysis of the long bones of children and adolescents (2), often during the period of rapid skeletal growth of the first two decades of life (3). Osteosarcoma is a highly invasive tumor that frequently metastasizes to the lungs. Pathogenesis involves a wide range of different molecular pathways, but of fundamental relevance is the inactivation of tumor suppressor genes, particularly p53 (4) and the retinoblastoma susceptibility gene (RB1; refs. 5, 6); further support for this model has recently come from whole-genome sequencing (WGS, ref. 7).

Nowadays, the standard treatment for osteosarcoma is neoadjuvant chemotherapy followed by conservative surgery (in which, as far as possible, function is preserved). Followed by postoperative chemotherapy. Despite aggressive chemotherapy protocols, 30% to 40% of patients do not respond and relapse, developing bone or lung metastasis (8, 9). For these patients, survival remains poor, with an overall five-year survival rate of about 20% (10). Thus, new treatments are needed. Oncolytic adenoviruses engineered to specifically destroy cancer cells are a feasible option. VCN-01 is an oncolytic adenovirus that harbors a 24-base pair region, and the native E1A promoter has been modified by the insertion of eight E2F-binding sites organized in four palindromes and one Sp1-binding site. These modifications selectively restrict the replication of VCN-01 to cells with a defective pRB pathway (11, 12). In addition, the VCN-01 adenovirus displays improved infectivity and bioavailability as a result of the inclusion of an RGDK motif in the heparin sulphateglycosaminoglycans (HSG)–binding domain KRTK of the fiber shaft (13); this modification improves antitumor potency compared with the adenovirus modified with an RGD motif in the fiber HI loop (14).

A third modification engineered into the VCN-01 genome is a novel element, an expression cassette with the human PH20 gene (12). This cassette enables expression of a soluble hyaluronidase, active under physiologic conditions (pH 7), that can degrade extracellular matrix hyaluronic acid. Hyaluronic acid is a negatively charged, high molecular weight polysaccharide that forms part of and confers specific properties to the extracellular matrix (15). There is an association between hyaluronic acid and malignancy, and this molecule is overproduced in...
many cancers (16, 17). In some tumors, high levels of hyaluronic acid have been found to be related with low survival rates and also with development of chemoresistance (18, 19).

Osteosarcoma is characterized by the production of malignant osteoid by tumor cells. It has been hypothesized that the degradation of hyaluronic acid can destabilize such osteoids thereby improving viral efficacy. Supporting this notion, several studies have shown that drugs capable of disturbing hyaluronan-rich pericellular matrix cells result in less accumulation of hyaluronan, and that, consequently, these drugs have a potent antisarcoma effect while maintaining a safe toxicity profile in relevant sarcoma animal models. Our results suggest a strong preclinical rationale for propelling a phase I/II study with VCN-01 for pediatric osteosarcoma.

**Translational Relevance**

Osteosarcoma is the most common primary malignant tumor of bone in children and adolescents. Despite significant improvements in treatment of the primary tumor, a significant proportion of osteosarcoma patients eventually develop lung metastases and succumb to their disease even after multistage conventional chemotherapy and surgical excision. Thus, there is a need to develop new and safe approaches for the treatment of osteosarcoma. Oncolytic adenoviruses have already proven effective and safe in the clinic. VCN-01 is a replication-competent adenovirus specifically engineered to replicate in tumors with a defective RB pathway, presents enhanced infectivity through a modified fiber and improved distribution through the expression of a soluble hyaluronidase. Our results show that VCN-01 showed a potent antisarcoma effect while maintaining a safe toxicity profile in relevant sarcoma animal models. Our results suggest a strong preclinical rationale for propelling a phase I/II study with VCN-01 for pediatric osteosarcoma.

**Materials and Methods**

**Cell lines and culture conditions**

Primary osteosarcoma-derived cell lines 531MII, 588M, 595M, and 678R were developed at the Clínica Universidad de Navarra (Pamplona, Spain) as previously described (15). In addition, we used the 143B cell line, obtained from the ATCC. All the cell lines were maintained in minimum essential medium supplemented with 10% FBS in a humidified atmosphere containing 5% CO₂ at 37°C. All the cell lines were tested and authenticated at the CIMA Genomic Core Facility (Pamplona, Spain) using short tandem repeats DNA profiling.

**Adenovirus construction and infection**

The construction of VCN-01 and method of viral infection followed procedures described elsewhere (21, 22).

**Cell viability assays**

531MII, 588M, 595M, and 678R cells were seeded at a density of 1 × 10⁴ cells per well in 96-well plates, and one day later, cells were infected with VCN-01 at MOIs (multiplicity of infection) of 5, 10, 20, 40, and 100. Cell viability was assessed five days later using the MTT assay (Sigma-Aldrich) as previously described (23).

Dose-response curves were analyzed using GraphPad Software, which fits the dose–response curves to Chou–Talalay lines (24); IC₅₀ is the median-effect dose (the dose causing 50% of cells to be affected, i.e., in this case IC₅₀ is the amount of virus that results in 50% cell death or, equivalently, 50% survival). In each experiment, cells were plated in triplicate and each experiment was performed three times.

**Viral replication assays**

Osteosarcoma cells were seeded at a density of 1 × 10⁴ cells/well in 6-well plates and infected 20 hours later with 1 MOI of VCN-01. Three days later, cells were collected, and the final amount of virus was determined by means of a method based on anti-hexon staining in HEK293 cells (25).

**Immunoblotting**

For immunoblotting assays, samples subjected to the same treatment as described earlier and containing identical amounts of protein, were subjected to SDS-PAGE. Membranes were incubated with the following antibodies: E1A, (Santa Cruz Biotechnology), fiber (NeoMarkers), and GRB2 (BD Transduction Laboratories). The membranes were developed according to Amersham enhanced chemiluminescence protocol.

**qRT-PCR analysis of gene expression**

Total RNA was isolated and cDNA was synthesized using the high-capacity cDNA Archive Kit (Applied Biosystems). Total RNA was extracted from paraffin-embedded sections using RecoverAll Total Nucleic Acid Isolation (Ambion by Life Technologies). Reverse transcriptase reactions contained 1 μg of RNA, 1× RT buffer, 4 μl of dNTPs (100 mmol/L), 1× random primers, 5 μl of MultiScribe Reverse Transcriptase (50 U/μl), and 0.005 μl of RNase inhibitor (0.20 U/μl). The reactions were incubated in a GeneAmp PCR System 2400 (Applied Biosystems) for 10 minutes at 25°C and then 2 hours at 37°C. Each cDNA sample was analyzed in triplicate by fast quantitative-PCR analysis (Applied Biosystems 7900 Sequence Detection System) using Fast SYBR Green PCR Master Mix (Applied Biosystems) and specific primers for fiber (forward, 5’-CAAGCGCTTGGATTTATG-3’; reverse, 5’-GTTAAGGAGATGTTGCAAAT-3’), PH20 (forward, 5’TACA-CACTCCGTGCTCCTGG-3’; reverse, 5’-CCTAGTCTCAGAGGAGCC-3’), and the housekeeping gene GAPDH (forward, 5’-AGGCACATCGTCAGACAC-3’; reverse, 5’-GCCCAATACGACGACACT-3’). For thermal cycling, fast real-time conditions were 20 seconds at 95°C for one cycle, 1 second at 95°C, and 20 seconds at 60°C for 40 cycles. To determine relative levels of gene expression, the comparative threshold cycle (Ct) method was used, and data were presented as 2–ΔΔCt.

**Animal studies**

Ethical approval for animal studies was granted by the Animal Ethical Committee of the University of Navarra (CEEA; Comité Eético de Experimentación Animal under the protocol number CEEA/065-13 and CEEA/075-13).
For the orthotopic intratibial model, 531MII osteosarcoma cells (5 x 10⁶) were engrafted by injection through the tibial plateau in the primary spongiosa of both tibias of female nude mice (Taconic Farms, Inc.). Seven days after injection, animals were randomized to 3 groups (control with PBS and two groups of VCN-01 treated with two different doses). PBS or VCN-01 [10⁶ plaque-forming unit (pfu)/animal and 10⁸ pfu/animal] were administered into the tibia of the animals on days 7 and 14 after cell implantation. Tumor development was monitored every week until the end of the experiment (day 90 after cell implantation), and mice were then sacrificed. When a tumor became visible, its size was measured weekly, and tumor volume was calculated (26).

For the lung-metastatic model, 531MII osteosarcoma cells (2 x 10⁶ cells) were injected through the tail vein. Seven days later, animals were arbitrarily randomized to 3 groups (control, PBS-treated, or VCN-01 with the same doses as above), and the VCN-01 virus was intravenously administered in the tail vein.

All animals were weighed every two weeks throughout the experiment to evaluate toxicity. Animals were weighed weekly during the period of VCN-01 administration.

PET analyses

As part of the orthotopic study, at the end of the experimental procedure, antitumoral effect was measured by PET with the radiotracer 18F-fluorodeoxyglucose (18F-FDG). Overnight prior to PET, mice were fasted but allowed water. On the day of PET, mice were anesthetized with 2% isoflurane in 100% O₂ and 18F-FDG (17.7 MBq ± 2.6 in 80–100 μL) was injected into the tail vein. To avoid radiotracer uptake in the hindlimb muscle, 18F-FDG uptake was performed under continuous anesthesia for 50 minutes. PET imaging was performed in a dedicated small-animal Philips Mosaic tomograph with 2 mm resolution, 11.9 cm axial field of view (FOV), and 12.6 cm transaxial FOV. Anesthetized mice were placed horizontally on the PET scanner bed to perform a static acquisition (sinogram) of 15-minute duration. Images were reconstructed using the 3D Ramla algorithm (a true 3D reconstruction) with two iterations and a relaxation parameter of 0.024 into a 128 x 128 matrix with a 1 mm voxel size; corrections were applied for dead time, decay and random, and scattering corrections. For the assessment of 18F-FDG uptake, all studies were exported and analyzed using the PMOD software (PMOD Technologies Ltd.). Regions of interest (ROI) were drawn on coronal 1-mm thick small-animal PET images on consecutive slices including entire hindlimbs. Finally, maximum standardized uptake value (SUV_MAX) was calculated using the formula SUV = [tissue activity concentration (Bq/cm³)/injected dose (Bq)] x body weight (g).

Immunohistochemical analysis

Paraffin-embedded sections of mouse leg, lung, and liver were immunostained with specific antibodies for adenoviral mouse–hexon (Chemicon International, Inc.), E1A (Santa Cruz Biotechnology), biotinylated hyaluronan binding protein (AMS biotechnology), and vimentin clone V9 (US30, Dako Denmark A/S). Conventional procedures were followed in all cases (27). For immunohistochemical staining, Vectastain ABC kits (Vector Laboratories Inc.) were used according to the manufacturer’s instructions.

Statistical analysis

Data were expressed as mean ± SD. For the in vitro experiments, data were evaluated with the two-tailed Student t test. The same test was used to compare data for treatment and control groups in the in vivo experiments. The program GraphPad Prism 5 (Statistical Software for Sciences) was used for the statistical analysis.

Results

Characterization of the in vitro antosteosarcoma effect of VCN-01

As mentioned in the introduction, VCN-01 is an improved genetically modified adenovirus that presents in its genome several modifications that renders a more specific and potent virus; E2F-binding sites in the E1A promoter, a 24-bp deletion in the E1A gene, modified fiber, and finally, VCN-01 has an expression cassette inserted in its genome that allows the virus to express PH20 (hyaluronidase; Fig. 1A).

First, we wanted to evaluate the in vivo antosteosarcoma effect of VCN-01. To this end, we performed MTT analyses in 4 primary (531 MII, 588M, 595M, and 689R) and one established (143B) osteosarcoma cell lines. MTT assays showed that VCN-01 induced cell death in a dose-dependent manner in all cell lines. The IC₅₀ of VCN-01 ranged between 1 and 30 MOIs. 531MII was the most sensitive line and 588M, the most resistant (Fig. 1B and Supplementary Fig. S1A).

Assessment of viral protein levels in cells previously infected with VCN-01 revealed robust expression of E1A, an early viral protein, and fiber, a late protein. Levels of both proteins followed a dose-dependent pattern (Fig. 1C and Supplementary Fig. S1B). These data suggest that there was effective viral transcription, and that the virus was replicating.

To confirm the existence of an effective viral cycle in osteosarcoma cell lines, we used a replication assay based on anti-hexon staining. Viral counts ranged from 10⁴ to 10⁷ pfu/mL, indicating efficient replication in all cell lines (Fig. 1D and Supplementary Fig. S1C). In 531MII and 678R cell lines, the strength of cytotoxic effect of VCN-01 correlated with viral titers.

Finally, we assessed the expression levels of the PH20 produced by the virus and whether those levels were correlated with fiber expression. We performed mRNA expression analysis, and we observed that PH20 and fiber mRNA levels correlated with each other in both cell lines tested (R² = 0.99, P < 0.0001) indicating that VCN-01, when transcribed, can express the PH20 cassette inserted in the viral genome (Fig. 1E and Supplementary Fig. S1D).

All together these data indicate that, in pediatric osteosarcoma–derived cell lines, VCN-01 replicated in vitro, expressed the PH20 enzyme, and exerted a robust oncolytic effect.

Evaluation of viral toxicity in vivo

To evaluate the potential toxicity of local and systemic viral administration, weight was controlled every week after local (intratumoral) or systemic (tail vein) viral administration. Mice did not lose weight with VCN-01 administration; route of administration (intratumoral in the intratibial model versus tail vein in the lung-metastatic model) did not affect weight loss and neither did viral concentration (Fig. 2A and B).

Hematoxylin–eosin staining of liver sections did not reveal morphologic alterations associated with hepatotoxicity (Fig. 2B and C). We observed no Councilman bodies, macrosteatosis, or necrotic areas. IHC did not detect expression of E1A and hexon.
Thus, in mice, locally or systemically administrated VCN-01 was not significantly toxic and was well tolerated.

**Antitumor effect of VCN-01 in an orthotopic osteosarcoma model**

Next, we analyzed the therapeutic effect of VCN-01 in an orthotopic osteosarcoma model. For that purpose, we inoculated 531MII cells in the tibial tuberosity of nude mice followed by VCN-01 injection at days 7 and 21 administered at two different dosages (10^7 pfu/animal or 10^8 pfu/animal). PBS was administered to the control group (Supplementary Fig. S3A). Tumor development was monitored every week until the end of the experiment (day 90 after cell implantation), and mice were then sacrificed.

By the end of the experiment, there were clearly visible differences in tumors in the three groups of mice (Fig. 3A). PET imaging also showed that tumor volumes were significantly bigger in the control group relative to those in the 10^8 pfu group (Fig. 3B and C). All control mice (5 mice × 10 tibias) developed visible tibial tumors, whereas only 3 out of 10 tibias in the group treated with 10^7 pfu VCN-01 and none of the mice in the group treated with 10^8 pfu VCN-01 did (Fig. 3D and E). Tumor volumes of control mice ranged between 100 and 500 mm^3; volumes for mice in the 10^7 pfu group were between 50 and 400 mm^3.

Pathologically, at the end of the experiment, tumors had the characteristic morphology of osteosarcoma and a notable production of malignant osteoid (Fig. 3F). In some control mice, tumors had grown such that they had crossed the epiphysis of the tibiae, resulting in transarticular tumors. In the VCN-01 lower dose treated group, there was evidence of a certain tumoral burden in some tibiae. Mice in the higher dose treated group showed no evidence of tumor.

At the end of the experiment, approximately 70% of control mice presented lung metastases derived from the primary osteosarcoma tumor (Supplementary Fig. S4B). Of mice treated with VCN-01, only one in the 10^7 pfu–treated group developed lung metastasis (Fig. 3E and F and Supplementary Figs S4B and S4C).

Hexon staining verified viral presence (Supplementary Fig. S4A). In hexon-positive areas, IHC indicated decreased hyaluronic acid expression and a lower density of cancer cells. These
observations, beyond an indirect demonstration of the oncolytic effect of VCN-01, suggest that VCN-01 was able to express PH20, and that this PH20 was functional at digesting hyaluronic acid (Supplementary Fig. S4A).

RNA extracted from tibial sections was used to assess the expression of fiber and PH20. Expression of fiber and hyaluronidase mRNA was higher in mice treated with VCN-01–10^8 pfu group relative to those in the VCN-0 10^7 pfu–treated group (Fig. 3G). As in the in vitro experiments, there was a significant correlation between the levels of expression of hyaluronidase PH20 and fiber mRNA.

In conjunction, these results show that VCN-01 replicated in vivo inside tumors, where it produced functional hyaluronidase and had a potent antiosteosarcoma effect.

**Therapeutic effect of VCN-01 in a metastatic osteosarcoma model**

As the lungs are preferred metastasis organs for osteosarcoma, we evaluated the antitumor effect of VCN-01 in a lung metastatic osteosarcoma model using the cell line 531MII. This cell line recapitulates the pathology found in osteosarcoma lung metastasis in patients. We administrated 2 × 10^6 cells in the tail vein of athymic nude mice and, seven days later, mice were treated with four intravenous injections at doses of 10^7 pfu/animal of VCN-01 or 10^8 pfu/animal of VCN-01. Mice were sacrificed at day 60 (Supplementary Fig. S3B). The objective of this experiment was the assessment of tumor burden in mice lungs and the evaluation of the capacity of VCN-01 to reach the tumors and to replicate in tumor cells after systemic administration. This virus has a modification in the fiber that improves its half-life in blood, which might improve the antitumor effect compared with the other tested virus.

The pathologic analysis of lung metastases showed the characteristic appearance produced by 531MII. This phenotype was verified by V9-positive staining (Fig. 4A and C). Lung tumor burden was significantly reduced in both VCN-01–treated groups compared with the control group (PBS-treated mice): by the end of the experiment (day 60), a mean of 2.06% of lung surface area in untreated mice presented tumor, whereas this percentage in the 10^8 pfu and 10^7 pfu groups was 0.4 and 0.14%, respectively (Fig. 4B). In addition, mice treated with VCN-01 had less tumor nodules (Fig. 4C).

Viral fiber and PH20 expression in the lungs was analyzed by qRT-PCR, which demonstrated that the virus was able to
target and to replicate in tumor cells after systemic administration (Fig. 4D). The overall mRNA expression of viral fiber and PH20 in the lungs of mice treated with VCN-01 10^7 pfu/animal was higher than that in mice treated with VCN-01 10^8 pfu/animal. This result can be explained by hypothesizing that at 10^8 pfu/animal, VCN-01 was left with few tumoral cells in which to replicate.

In summary, VCN-01 displayed a significant and potent antisarcoma effect in the 531MII lung metastatic osteosarcoma model.

Discussion

Previously, others and ourselves have shown that oncolytic adenoviruses present a potential therapeutic approach for treatment of pediatric osteosarcoma (28–31). This study found that VCN-01, a new generation genetically modified oncolytic adenovirus, administered locally or systemically, had a potent antisarcoma effect in vitro and in vivo in mouse models of intratibial and lung metastatic osteosarcoma.

VCN-01 was engineered by inclusion of eight E2F response elements inside its genome and the deletion of 24 bp in the E1A gene, to replicate preferentially in cancer cells with an aberrant RB pathway. We observed VCN-01 to have its strongest antitumor effect in the 531MII cell line, which harbors an Rb mutation. Because Rb mutations are associated with malignance and poor prognosis in osteosarcoma patients (32), that VCN-01 worked well in this scenario is of particular relevance.

Several studies have found a relationship between levels of hyaluronic acid and tumoral resistance to chemotherapy, with associated poor prognosis for patients (17, 18). Therefore, in the treatment of osteosarcoma, VCN-01 can have two therapeutic effects: an intrinsic oncolytic effect on tumor cells and
the effect of hyaluronidase on the extracellular matrix (33). In respect of the latter effect, inhibitors of hyaluronic acid retention have been shown to be able to modify the pericellular matrix in an osteosarcoma, restraining tumor formation in vivo and also reducing the occurrence of metastasis (34). In addition, several works have found that hyaluronidase disturbed cell–cell interaction within the tumor mass, thereby enhancing the diffusion of antitumor agents (33, 35). Following this line of thinking, further studies are needed to assess the promising possibility that the complementary effects of VCN-01 and chemotherapy used in conjunction will result in enhanced diffusion of both virus and drugs and an improved antisarcoma efficacy.

An important limitation of therapy with systemically administered adenovirus is clearance of virus by the immune system (36). Virtually, the entire human population has IgG antibodies to adenovirus serotype 5, and 55% of these antibodies effectively neutralize adenoviruses (37). The insertion of an RGDK sequence into the fiber protein produced by VCN-01 considerably improved its biodistribution after systemic administration and enhanced the therapeutic effect of VCN-01 in vivo in two immunocompetent models (11, 38). Currently, there are two clinical trials underway to evaluate the therapeutic effect of systemically administered VCN-01; one of these concerns VCN-01 alone and the other VCN-01 in combination with gentamicin (NCT02045602 and NCT02045589).

In summary, VCN-01 exerted a potent anti-osteosarcoma effect in vitro and in vivo and was safe in terms of toxicity. Our results consolidate the case for pursuing VCN-01 as a realistic therapeutic option for kids with osteosarcoma.

Disclosure of Potential Conflicts of Interest

M. Cascallo has ownership interests (including patents) in and reports receiving commercial research support from VCN Biosciences. R. Alemany is a consultant/advisory board member for VCN Biosciences. No potential conflicts of interest were disclosed by the other authors.

Figure 4.

VCN-01 administered systemically reduced osteosarcoma lung metastasis in vivo. Lung metastases were induced by endovenous injection of $2 \times 10^5$ S31-MII cells in the tail of female nude mice. Animals were randomized as positive control (no treatment), VCN-01 group $10^7$ pfu ($10^7$ pfu/week × 4 weeks) and VCN-01 group $10^8$ pfu ($10^8$ pfu/week × 4 weeks). At day 60 animals were sacrificed. A, Quantification of lung tumor burden (S31-MII) in mice at the end of the experiment. Bar representation of tumor area relative to total lung area. The values represent mean percentage tumor area of animals from each group, as obtained from a specifically designed program that measured both tumor area and total lung area from which was calculated the percentage of tumor area. B, Representative H&E photomicrographs at 200× of lung lesions from mice of the three groups described. C, Quantification of number of lung tumor nodules. Bar representation of tumor number represent different quantifications of different observers. D, 200× vimentin (V9antigen) immunohistochemistry images showing metastatic implants in lung of mice from untreated and VCN-01 treated groups. E, Quantification of fiber and PH20 mRNA expression of cDNA from osteosarcoma tumors by qRT-PCR and its correlation graph. mRNA levels are presented as $2^{-\Delta\Delta C_t}$, standardized with their constitutive gene and compared to an uninfected control.
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