Nerve-Sparing Therapy with Oncolytic Herpes Virus for Cancers with Neural Invasion

Ziv Gil,1 Avigail Rein,1 Peter Brader,2 Sen Li,1 Jatin P. Shah,1 Yuman Fong,2 and Richard J. Wong1

Abstract

Purpose: The invasion of cancer cells along nerves is an ominous pathologic finding associated with poor outcomes for a variety of tumors, including pancreatic and head and neck carcinomas. Peripheral nerves may serve as a conduit for these cancers to track into the central nervous system. Cancer progression within nerves and surgical resection of infiltrated nerves result in a permanent loss of neural function, potentially causing cosmetic and functional morbidity. Herpes simplex viruses (HSV) have utility for gene transfer into nerves and as oncolytic agents. We studied the use of an attenuated HSV, NV1023, as a treatment for cancers with neural invasion.

Experimental Design and Results: NV1023 injection into the sciatic nerves of nude mice had no toxic effect on nerve function, whereas similar doses of wild-type HSV-1 (F strain) caused complete nerve paralysis within 4 days and 100% mortality at day 6. NV1023 showed effective cytotoxicity in vitro on three neurotrophic human carcinoma cell lines, including pancreatic (MiaPaCa2), squamous cell (QLL2), and adenoid cystic (ACC3) carcinomas. A model of neural invasion was established by implanting human carcinoma cells in the sciatic nerves of nude mice. All control group mice developed left hind limb paralysis 5 to 7 weeks after tumor injection, whereas animals treated with NV1023 maintained intact nerve function and showed significant tumor regression (P < 0.0001).

Conclusions: These results show that NV1023 oncolytic therapy may effectively treat cancers with neural invasion and preserve neural function. These findings hold significant clinical implications for patients with cancer neural invasion.

Certain tumors, including pancreatic and head and neck carcinomas, have the ability to track along nerves and significantly impair the clinical outcome of patients. Pancreatic cancer invasion of the celiac ganglion at an early clinical stage is a major contributor to cancer progression and mortality, and can induce severe pain, resulting in poor quality of life for patients (1). In the head and neck region, minor salivary gland adenoid cystic carcinomas and squamous cell carcinomas have a 70% and 30% incidence of perineural invasion, respectively (2). Significant correlations between perineural invasion and poor outcome have also been shown in patients with biliary, prostate, bladder, gastric, esophageal, colorectal, and uterine carcinomas (3, 4).

Perineural invasion by these cancers leads to progressive dysfunction of the infiltrated nerve, and may manifest as pain and numbness in sensory nerves or paralysis of motor nerves. In the head and neck region, cranial nerve invasion may result in significant impairment of cosmetic, swallowing, speech, and visual function. Furthermore, attempted surgical resection of tumors with perineural invasion necessitates complete nerve resection and induces a complete loss of neural function. The exploration of alternate cancer therapies that may potentially preserve neural function could have a significant effect on the quality of life of patients afflicted with these cancers.

Oncolytic herpes viruses are a promising form of cancer therapy. Genetically designed, replication-competent viruses with attenuated toxicity have been shown to retain effective lytic activity against a wide range of neoplasms (5). Oncolytic herpes viruses based on herpes simplex type 1 (HSV-1) can effectively treat pancreatic and head and neck carcinomas in flank tumor models (6, 7). Early clinical trials recently showed encouraging safety profiles by several oncolytic herpes viruses (8, 9). HSV is also a neurotropic virus, which can pass selectively across synapses and infect a broad range of neuronal cell types (10). Wild-type HSV-1 may infect neurons and be transported retrograde within the nervous system to enter a latent state within the neuronal soma. Herpes viruses have been shown to have utility as a means of gene transfer into the nervous system (11). Direct injection of herpes vectors can transduce neurons of the dorsal root ganglion to provide a therapeutic effect in models of polynuropathy and chronic regional pain (12). A prior study showed that the injection of an oncolytic HSV, G207, into a murine sciatic nerve model of neuroblastoma was able to result in a reduction of functional impairment (13). We sought to determine if cancer infiltration...
of peripheral nerves by pancreatic, squamous cell, and adenoid cystic carcinomas can be effectively targeted with NV1023, a replication-competent attenuated oncolytic HSV, to (a) significantly reduce cancer burden and (b) preserve nerve function.

**Materials and Methods**

**Cell lines.** The human pancreatic carcinoma cell line MiaPaCa2 was grown in DMEM, the head and neck squamous cell carcinoma QLL2 in MEM, and the salivary gland adenoid cystic carcinoma ACC3 in RPMI 1640. All media also contained 10% FCS, penicillin, and streptomycin. Cells were maintained in 5% CO2 in a 37°C humidified incubator.

**Viruses.** NV1023 is an attenuated, replication-competent oncolytic HSV whose construction was previously described (14). In brief, NV1023 was derived from R7020, an HSV-1 (F) strain vector originally designed as an HSV-1/2 vaccine candidate (15). This virus carries a 5.2-kb fragment of HSV-2 DNA (containing HSV-2 genes US2-2 through US2-5) inserted in the UL/S junction. Attenuation was achieved by a 15-kb deletion in the inverted repeat region that extends from the 3′ end of UL55 to the promoter for ICP4, deleting UL56 and one copy of the diploid genes ICP0, ICP4, and γ34.5, a neurovirulence gene. *Escherichia coli* β-galactosidase gene (*LacZ*) at the US10-12 locus was inserted as an infection marker. NV1023 was provided by Medigene, Inc. NV1066 was used for fluorescent imaging of viral infection of tumor cells (16). This vector is an attenuated herpes virus similar to NV1023, which carries a transgene for an enhanced green fluorescent protein (eGFP). HSV-1(F) is a wild-type HSV-1 strain (17). Viral titers were determined by plaque assays on Vero cells.

**Cytotoxicity assays.** Cancer cells were plated at 2 × 10⁵ per well in 12-well plates in 2 mL medium. After incubation for 6 h, NV1023 (100 μL) was added to each well at multiplicities of infection (MOI, or the ratio of infectious viral units per tumor cell) of 0, 0.1, 1, 5, or 10. Viral cytotoxicity was measured at 48-h intervals. On day 4, 1 mL of fresh medium was added to the wells to feed viable cells. Cells were washed with PBS and lysed with Triton X (1.5%) to release intracellular lactate dehydrogenase, which was quantified with a Cytotox 96 kit (Promega) by spectrophotometry (EL321e, Bio-Tek Instruments) at 450 nm. Results are expressed as the ratio of surviving cells determined by comparing the measured lactate dehydrogenase of each infected sample relative to control untreated cell samples, which were considered 100% viable. All samples were analyzed in triplicate.

**In vivo model of neural invasion.** Six-week-old athymic nude mice (National Cancer Institute, Bethesda, MD) were anesthetized with inhalational methoxyflurane for all procedures and the left sciatic nerve was exposed deep to the femorococcygeal and biceps femoris muscles. QLL2, MiaPaCa2, and ACC3 cells were microscopically injected into the perineurium of the sciatic nerve, distal to the bifurcation of the tibial and common peroneal nerves. Slow microinjection of 3 μL of cell suspension at a concentration of 1 × 10⁶/jL was done using a 10 μL Hamilton syringe over a 2-min period (17). Sciatic nerve–infiltrating tumors were established and then injected at day 7 with NV1023 at 5 × 10⁷ plaque-forming unit (pfu) or saline for control animals. For tissue histochemistry, mice were euthanized after 48 h and the sciatic nerve was excised, frozen in Tissue Tek solution, and cut into 8-μm-thick sections. Other groups were observed for up to 7 weeks for assessment of tumor response, nerve function, or other signs of morbidity.

**Measures of sciatic nerve function.** Sciatic nerve function was measured weekly as described previously (18). The sciatic nerve innervates the hind limb paw muscles. Functional measures for monitoring tumor neural invasion included (a) gross behavior—signs of motor weakness or repetitive biting of the hind limb were monitored for 10 min weekly; (b) limb function—function was graded according to hind limb paw response to manual extension of the body, from 0 (normal) to 1 (total paw paralysis); (c) sciatic nerve function index—calculated as the spread length (in mm) between the first and fifth toes of the mouse hind limbs.

**Ultrasound assessment of neural invasion.** A high-resolution small-animal ultrasound (55 MHz) was used to measure the (a) proximal sciatic nerve diameter and (b) primary sciatic nerve tumor diameters on a weekly schedule. The proximal sciatic nerve diameter is a measure of the nerve thickness and reflects the degree of neural infiltration by cancer cells. The proximal sciatic nerve was measured 4-mm proximal to the cancer cell injection site, just before the nerve enters the spinal column. Using this method, we are able to noninvasively image neural invasion with a spatial resolution of 50 to 100 μm.

**X-gal histochemistry.** Sciatic nerve sections were stained with H&E or X-gal for assessment of β-galactosidase expression. Cells were stained for 4 h with X-gal (1 mg/mL) in an iron solution of 5 mmol/L K3Fe(CN)6, 5 mmol/L K4Fe(CN)6, and 2 mmol/L MgCl2, as previously described (19). Counterstaining of background cells with nuclear fast red was done. Virally infected cells expressing β-galactosidase were identified histologically as blue-staining cells.

**Quantitative real-time PCR.** Sciatic nerves of athymic nude mice were injected with NV1023 at 5 × 10⁵ pfu or with saline for control animals. At 24 h or 14 days after injection, mice were euthanized by CO2 inhalation and brain, spinal cord, and sciatic nerve tissues were excised and homogenized separately in 1.2 mL TRIZol reagent (Ambion) according to the manufacturer's directions. cDNA was reverse transcribed from tissue RNA using random hexamer priming. For each sample, 250 to 500 ng of total RNA was added to 4 μL of 5x reverse transcriptase buffer (Invitrogen), 10 pmol random hexamers, 12.5 μmol/L each of dATP, dTTP, dGTP, and dCTP, 200 units of reverse transcriptase (Invitrogen); and 20 units of RNasin (Promega) to a final volume of 20 μL.

Reverse transcription-PCR was done on the total RNA extracts from mice treated with saline or NV1023. Real-time PCR was run in triplicate on an ABI Prism 7700 thermal cycler (Applied Biosystems Foster City) and contained cDNA or tail DNA, TaqMan universal PCR mix (Applied Biosystems), and target-specific TaqMan dye-labeled primer/probe (Assays by Design; Applied Biosystems). The primers used for RT-PCR were for expression of the early HSV-1 gene ICP6 and late gene LAT (20). Each sample was measured quantitatively by real-time RT-PCR and standardized to an 18s rRNA control. Probes used for ICP6 included ATAGCCCAATCCATGACCTGTAT (forward), GGTTGGAG-GCTGAGGAG (reverse), and CACGAGAAAGCCTGAGG (probe). Probes used for LAT included EXON included, GCCAGACTTCCCGGAGG (forward), AGACCGAAGATACAGGACG (reverse), and GCCACCGC- CCGTGTTTTTGT (probe). Standard curves were generated from serial dilutions. PCR was done under the following conditions: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3 (35 cycles), 95°C for 15 s and 60°C for 1 min; and stage 4, 25°C.

**Immunohistochemistry.** Samples were frozen in OCT embedding medium and sectioned by crytome for histologic examination. Slides were stained with H&E or with rabbit polyclonal HSV-1 antibody using a Histomouse-SP Bulk Staining Kit (Zymed Laboratories, Inc.). A biotinylated secondary antibody was added and visualized with streptavidin-labeled horseradish peroxidase and chromogen solutions (Super Sensitive Ready-to-Use Detection System, Biogenex). Counterstaining with Harris hematoxylin was done.

**In vivo imaging.** Tumors were established in the left sciatic nerve as described earlier. At day 7, the left and right nerves were injected with NV1066 (5 × 10⁵ pfu). Imaging was done 48 h later (n = 4 per group) using the Leica MZFL3 stereomicroscope (Leica Microsystems) in both bright-field and fluorescent modes. The Retiga EX digital charge coupled device camera (QImaging) was used for image capture. The GFP images were taken with minimal background illumination to illustrate the surrounding organs.

**Statistical analysis.** Student's t tests or ANOVA tests were used for statistical analysis as appropriate. Mantle-Haenszel and Fisher exact tests were used for evaluating differences in toxicity between groups.
Differences were considered significant at $P < 0.05$. All data are represented as mean ± SE, unless indicated otherwise. All experiments were repeated in triplicate. Data from representative experiments are shown.

Results

Sciatic nerve function with wild-type and NV1023 exposure. We first evaluated the safety of oncolytic therapy by direct viral injection into murine sciatic nerves. The left sciatic nerve of nude athymic mice was injected with NV1023 or wild-type F strain at $5 \times 10^7$ pfu. The right sciatic nerve was injected with saline and served as control. Measures of nerve function were assessed daily and included (a) signs of motor weakness or repetitive biting of the hind limb and (b) sciatic nerve score. Mice treated with NV1023 had normal sciatic nerve function and showed no change in behavior for up to 60 days after treatment (Fig. 1A; $n = 6$). However, all mice treated with F strain HSV showed complete sciatic nerve paralysis within 4 days posttreatment (Fig. 1B; $n = 6$). Two of the F strain–treated mice died at postinjection day 6, and the rest were moribund, requiring euthanasia at day 7. Four of the NV1023–treated mice were observed for 60 days, and none of these showed any sign of morbidity at the time the experiment was concluded.

RT-PCR was done on the RNA extracts from sciatic nerves treated with an injection of NV1023 at $5 \times 10^7$ pfu. The primers for the HSV-1 ICP6 and LAT genes were used to assess for presence of NV1023. At 1 and 14 days after NV1023 viral injection, ICP6 and LAT mRNA expression was detected in the sciatic nerve, but not in the brain, spinal cord, or saline-treated sciatic nerves (Fig. 1C). The ICP6 mRNA levels decreased to 34% 14 days after viral infection and the LAT mRNA levels decreased to 6.2% within the same period.

NV1023 cytotoxicity in vitro. Human carcinoma cell lines with a propensity to invade nerves were examined. These included QLL2 (head and neck squamous cell carcinoma), ACC3 (salivary adenoid cystic carcinoma), and MiaPaCa2 (pancreatic adenocarcinoma). The cytotoxic effects of NV1023 on these cell lines were assessed in vitro by lactate dehydrogenase assays (Fig. 1D-F). QLL2 showed similar sensitivities to NV1023 at a MOI of 1 and 5. MiaPaCa2 showed an intermediate cytotoxic response at MOI of 1, and greater response at MOI 5. ACC3 was sensitive to the virus only at a higher MOI of 10. At maximal doses tested, all cell lines showed complete cell death by day 7.

Fig. 1. Safety of sciatic nerve treatment with NV1023 in vivo and oncolytic effects in vitro. Representative pictures of mice injected with $5 \times 10^7$ pfu of NV1023 (A) or F strain HSV (B) to the left sciatic nerve. Four days after injection, six of six mice treated with F strain HSV developed left hind limb paralysis, whereas six of six mice treated with NV1023 had normal function. C, quantitative RT-PCR analysis of nerves treated with NV1023 was done 1 to 14 d after viral injection. ICP6 and LAT gene expression was detected in the sciatic nerve but not in the spinal cord and brain of treated mice. Sciatic nerve injected with saline served as control (D, day; Spine, spinal cord; Control, treatment with saline). D to F, lactose dehydrogenase cytotoxicity assays of three human carcinoma cell lines demonstrating sensitivity to NV1023. D, the head and neck squamous cell carcinoma cell line QLL2 showed high sensitivity to NV1023 at a MOI of 0.1 (●), 1.0 (●), and 5.0 (○). The pancreatic cell line MiaPaCa2 (E) and the adenoid cystic carcinoma cell line ACC3 (F) also showed sensitivity to the virus at higher MOIs (●, 0.1; ●, 10.0; ○, 10.0). All cell lines showed complete cell death by day 7 at MOI 10.
Treatment of sciatic nerve cancer invasion in vivo. A murine model of neural invasion was established by implanting tumor cells in the left sciatic nerve. We assessed the propensity of MiaPaCa2, QL12, and ACC3 tumors to induce sciatic nerve paralysis in nude mice. After establishing neurally invasive tumors (7 days after injecting cancer cells into a distal site of the sciatic nerve), animals were randomized into two groups: (a) intratumoral injection of $10^7$ viral pfu of NV1023 or (b) intratumoral injection of saline. Sciatic nerve function was measured weekly and included monitoring of gross behavior, limb function, and sciatic nerve index. The Escherichia coli $\beta$-galactosidase gene ($\beta$-gal) inserted at the US10-12 locus was used as an infection marker for animals treated with NV1023. Histochemical staining for X-gal confirmed the presence of significant expression of LacZ by NV1023 within tumors at 48 to 72 h posttreatment (Fig. 2A and B). No X-gal staining was evident in tumors that were treated with saline or in nerves without tumors treated with NV1023 ($n = 4$). Similar results were found with immunostaining with anti HSV-1 antibodies (Fig. 2C and D). In another set of experiments, a similar attenuated herpes virus, NV1066, was injected to nerve infiltrated by cancer cells and to nerves devoid of tumors ($5 \times 10^7$ pfu). NV1066 is a viral vector that carries a transgene for an eGFP instead of LacZ. This virus was previously shown to have similar oncolytic properties as NV1023 (21). Fluorescence imaging was done 48 h after injection with NV1066 and showed significant expression of eGFP (Fig. 2E), confirming viral infection of cancer cells. Injection of NV1066 to the right nerve that was devoid of cancer cells, as well as saline injections to nerves infiltrated by tumors, resulted in no fluorescence (Fig. 2F; $n = 4$-6 per group).

In the control group, mice began to develop left hind limb paralysis 2 to 4 weeks after tumor implantation (Fig. 3). Significant differences in the limb function and sciatic nerve index (hind paw width) were found between saline- and NV1023-treated mice (Fig. 3). High-resolution ultrasound images (55 MHz) done in vivo showed significantly lower primary sciatic nerve tumor and proximal sciatic nerve diameters in the NV1023-treated group ($n = 7$ per group, $P < 0.05$; see Fig. 4). All mice were euthanized when >70% of them developed nerve paresis, and the sciatic nerve was excised for histopathologic analysis. Tumor diameter was measured at the injection site, and proximal nerve diameter was measured 4 mm medially. Histopathologic analysis showed that the NV1023-treated mice had significantly decreased sciatic nerve diameters compared with controls both at the primary tumor site, and at 4 mm proximally along the sciatic nerve (Fig. 5). Most importantly, in six of seven mice with MiaPaCa2 tumors treated with NV1023, no tumor cells were detected. We observed no significant toxicity attributable to NV1023 administration such as change in body weight, animal behavior, skin condition, infections, other morbidity, or mortality.

Discussion

Neural invasion by carcinoma is an ominous finding that causes progressive loss of nerve function and heralds aggressive disease with poor clinical outcomes. The identification of novel therapies that have antitumoral activity combined with selectivity to preserve involved nerves would have clinical utility in the treatment of these patients.

In this study, we show that a single injection of an attenuated, replication-competent, oncolytic HSV can effectively treat nerves infiltrated with carcinomas by significantly reducing tumor burden along the invaded nerves while also preserving physiologic nerve function. All tumors showed significant reduction in tumor size. Our group and others have...
shown that multiple viral dosing, concomitant radiation, chemotherapy, and cytokine expression may all enhance viral therapeutic efficacy without adding observed toxicity in animal models (14, 22–24). Remarkably, six of seven MiaPaCa2 animals showed a complete response following a single viral administration without evidence of residual viable tumor at 7 weeks. These findings underscore the exquisite sensitivity of some tumors to oncolytic herpes viral therapy, and the ability of selected tumors to strongly support multiple cycles of herpes viral infection, replication, and cancer cell lysis.

G207, an oncolytic HSV attenuated by deletions in both copies of the ®134.5 gene, has previously been shown to be safe for intraneural injections and able to reduce both tumor volume and functional impairment of murine sciatic nerves infiltrated by human neuroblastoma (13). In this study, we show that the attenuation of NV1023 for decreased neurovirulence by deletion of one copy of the ®134.5 gene seems to be sufficient to preserve peripheral nerve function and to prevent central nervous system dissemination. In contrast, nerve infection with wild-type HSV-1 (F strain) is lethal to athymic nude mice at similar doses. RT-PCR results are consistent with other studies showing that attenuated HSV may establish latency in neurons, as suggested by the production of ICP6 and LAT (25). However, these mRNA levels dropped dramatically by 2 weeks, and histochemical staining failed to show visible lacZ expression in NV1023-treated sciatic nerves, and eGFP in NV1066-treated mice, suggesting minimal viral gene expression within nerves devoid of tumors. These findings suggest that the attenuation of NV1023 is sufficient to allow for the preservation of peripheral nerves from oncolytic effects.

Head and neck carcinomas invading cranial nerves may cause significant cosmetic and functional morbidity by altering facial motion, speech, swallowing, vision, and airway stability. Surgical therapy for cancers with neural invasion deemed to be resectable typically requires resection of the infiltrated nerves, committing the patient to a permanent loss of neural function. One implication of this study is the potential application of oncolytic HSV as an intraoperative adjuvant therapy. Such an approach might allow for tumor resection and intraoperative administration of oncolytic herpes virus to the infiltrated nerve rather than resection. Neural injection with replication-competent oncolytic viruses may also prove advantageous by being able to potentially treat anatomic areas inaccessible by surgery, such as the celiac ganglion and spinal cord in cases of pancreatic carcinomas, and the cranial nerve ganglia and skull base in head and neck carcinoma cases. Local recurrence at sites of perineural invasion is a common occurrence in this clinical scenario. For patients undergoing surgical resection of tumor and involved nerves, herpes oncolytic viruses administered intraoperatively to the amputated proximal nerve stump might treat residual tumor cells infiltrating these nerves and tracking proximally toward the brain or spinal cord. Whether nerves themselves can serve as
means for delivery of oncolytic therapy requires further studies.

Advanced tumors with neural infiltration cause a significant degree of pain in >60% of patients (26). Neuropathic pain is a common cause of morbidity in patients with pancreatic and head and neck cancers. Although treatment with surgery, chemotherapy, or radiation can be undertaken with palliative intent, these measures are often ineffective for management of neuropathic pain. For these patients, treatment with NV1023 or related viruses may be considered by computed tomography–or ultrasound-guided injection of tumor-infiltrated nerves or ganglia. Further studies are required to evaluate this approach as a potential strategy for palliative pain treatment.

None of the NV1023-treated animals suffered from clinically apparent side effects attributable to viral administration. Our RT-PCR data indicate that the NV1023 virus has a limited ability to spread to the central nervous system via the sciatic nerve. NV1023 carries the thymidine kinase (TK) gene, making it sensitive to acyclovir as a potential additional safety mechanism for eradicating the virus. The parent virus from which NV1023 was derived, R7020, has a highly favorable safety profile in Aotus monkeys, a primate exquisitely sensitive to herpes viral infections (13). At a 10,000-fold higher dose than wild-type HSV-1, NV1020 still remained less toxic than HSV-1 to Aotus monkeys. NV1020 was recently studied in a phase I trial for patients with hepatic colorectal metastases. Doses of up to $1 \times 10^8$ pfu were administered by hepatic infusion pump without dose-limiting toxicity or significant adverse events attributable to the virus (8). These findings for NV1020 have encouraged our investigation of its related vectors such as NV1023 for clinical application.

In conclusion, this study suggests that neural invasion by cancer cells can be safely and effectively treated with an attenuated, replication-competent, and oncolytic HSV. Oncolytic HSV therapy seems to be safe in animal models and early clinical trials. Neural invasion by cancer cells is an ominous finding that heralds aggressive disease with a propensity for local recurrence, and an impending loss of neural function through either tumor progression or surgical resection. These findings hold clinical implications for improving patient care by demonstrating (a) effective therapy of nerves infiltrated by cancers and (b) functional preservation of infiltrated nerves. Additional studies are warranted to further investigate the potential effect of oncolytic HSV therapy on the challenging clinical scenario of neural invasion.

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Fig. 5. Representative in situ and histopathologic photomicrographs of tumors treated with saline or NV1023. In situ image of the left sciatic nerve of a saline-treated mouse (A) and of NV1023-treated mouse (B). Arrowheads, area of tumor injection. Notice the infiltrated proximal end of the nerve in the control mouse. C to K. H&E staining. Saline-treated (C) and NV1023-treated (D and E) QLL2 tumors. Saline-treated (F) and NV1023-treated (G and H) MiaPaCa2 tumors. Saline-treated (I) and NV1023-treated (J and K) ACC3 tumors. Bar, 1 mm (except in E, H, and K —0.25 mm). L to O, diameters of tumors and proximal nerves in mice treated with NV1023 or saline. The tumor diameter was measured at the tumor implantation site, and the proximal sciatic nerve diameter was measured 4 mm proximally at its insertion to the spinal cord. L. QLL2 tumor diameter. M. QLL2 proximal nerve diameter. N. MiaPaCa2 tumor diameter. O. MiaPaCa2 proximal nerve diameter. P. ACC3 tumor diameter. Q. ACC3 proximal nerve diameter. Open circles, mean; bars, SE.

References
