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Research Article

Integrating Ataxia Evaluation into Tumor-Induced Hearing Loss Model to Comprehensively Study NF2-Related Schwannomatosis

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Abstract

NF2-related Schwannomatosis (NF2-SWN) is a disease that needs new solutions. The hallmark of NF2-SWN, a dominantly inherited neoplasia syndrome, is bilateral vestibular schwannomas (VS), which progressively enlarge, leading to sensorineural hearing loss, tinnitus, facial weakness, and pain that translates to social impairment and clinical depression. Standard treatments for growing VS include surgery and radiation therapy (RT); however, both carry the risk of further nerve damage that can result in deafness, facial palsy, blindness, and hemiparesis. The resultant suffering and debility, in combination with the paucity of therapeutic options, make the effective treatment of NF2-SWN a major unmet medical need. A better understanding of these mechanisms is essential to developing novel therapeutic targets to control tumor growth and improve patients' quality of life. Previously, we developed an orthotopic cerebellopontine angle (CPA) mouse model of NF2-SWN-related VS, which faithfully mimics tumor-induced hearing loss. In this model, we observed that mice exhibit symptoms of ataxia and vestibular dysfunction. Here, we described our methodology using a panel of 5 tests to evaluate tumor-induced ataxia and how anti-angiogenic and anti-fibrotic treatments improve coordination and gait. These methods paired with hearing tests allow us to comprehensively evaluate tumor-induced neurological deficit and evaluate the efficacy of novel therapeutics to improve neurological function.

INTRODUCTION

NF2-related Schwannomatosis (NF2-SWN) is a dominantly inherited neoplasia syndrome, resulting from a germline mutation of the *NF2* tumor suppressor gene [1]. NF2-SWN has an incidence of 1 in 25,000 persons and a penetrance of nearly 100% [1]. The hallmark of NF2-SWN is bilateral vestibular schwannomas (VS), which progressively enlarge, leading to sensorineural hearing loss, as well as other neurological symptoms, including tinnitus, facial weakness, impaired balance, and pain [2]. These neurological symptoms can translate to social impairment and clinical depression [2]. VS can also cause brainstem compression resulting in severe morbidity and mortality [3]. Standard treatments for growing VS include surgery and radiation therapy; however, both carry the risk of further damaging the nerve [4-6]. The resultant suffering and debility, in combination with the paucity of therapeutic options, make the effective treatment of NF2-SWN a major unmet medical need.

The goal of our research is to find novel treatments that can simultaneously control tumor growth and alleviate neurological symptoms to improve patient quality of life. To better understand the biology of VS tumor progression and tumor-induced hearing loss, we established an

orthotopic VS mouse model by directly implanting tumor cells into the cerebellopontine angle (CPA) in mice. This model faithfully reproduced tumor-induced hearing loss [7]. Using this model, we identified cMET blockade as a potential treatment for VS that can sensitize VS to radiation therapy without any adverse effects on hearing [8]. We also found that losartan, an FDA-approved anti-hypertensive drug, via blocking the inflammatory angiotensin signaling, reduces neuroinflammation and prevents tumor-induced hearing loss [9]. In the CPA model, in addition to hearing loss, we also observed that mice exhibit symptoms of ataxia and incoordination, which recapitulate the symptoms observed in patients with NF2- SWN. Patients with bilateral VS may experience damage to the vestibular nerve [10] and the vestibule, leading to debilitating vestibular deficits [11]. A recent paper characterized vestibular dysfunction in NF2 patients using a panel of tests. They demonstrated that vestibular tests evaluating dizziness, gait, vestibular-ocular reflex and balance could reflect disease progression and treatment efficacy [12].

Although the tumors in our CPA model may lead to ataxia and incoordination through multiple mechanisms including direct vestibular dysfunction and cerebellar or brainstem compression, there is nonetheless a need for systematic measurement of gait and coordination. In two VS mouse models, we used a panel of tests to evaluate these variables both before and after treatment. We used the CPA hearing loss model that mimics the intracranial microenvironment of VS [7,9]; and the sciatic nerve model that reproduces the microenvironment of peripheral schwannomas [13]. With our panel of five tests, we characterize how tumor growth and treatments affect gait and coordination. Our study suggests that by incorporating these tests with tumor growth measurement and hearing test, our VS mouse models can comprehensively reproduce the tumor-induced neurological disability observed in patients with VS, and provide the NF research and clinical community with a robust tool to explore new therapeutic targets to tackle this devastating disease.

MATERIALS AND METHODS

Cell lines and reagents

Mouse *Nf2*$^{-/-}$ Schwann cells were maintained in 10% Schwann cell medium containing Schwann cell growth supplement (SCGS, ScienCell, Carlsbad, CA) [14]. Losartan Potassium was obtained from TCI America (Boston, MA). Anti-mouse and human VEGF antibody (B20-4.1.1) was provided by Genentech under a Material Transfer Agreement [13].

Animal models

Male and female syngeneic immune-competent FVB/C57BL/6 mice (10-12 weeks of age) were used. We breed and maintain our mice at the Gnotobiotic Cox 7 mouse colony at the Massachusetts General Hospital (MGH). All animal procedures were performed following the guidelines of the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the MGH. We used two xenograft models in our study. In both models, $1x10^5$ mouse *Nf2*$^{-/-}$ cells were implanted per mouse.

i) Cerebellopontine angle (CPA) model: to recapitulate the intracranial microenvironment of VS we implanted tumor cells injected into the CPA region of the right hemisphere [7]. The syringe was fixed on the stereotactic device at an angle of 15 degrees perpendicular to the work surface. The needle was moved 2.2 mm right to the confluence of sinuses, then moved backward to the transverse sinus, and lastly moved 0.5 mm to the dorsal side. The syringe was then lowered until it touched the surface, moved 3.7 mm downward, retracted 1mm, and then 1 microliter of tumor cell suspension was injected slowly over 45-60 seconds.

ii) Sciatic nerve model: to reproduce the microenvironment of peripheral schwannomas, we implanted tumor cells into the mouse sciatic nerve [8,13]. Three microliters of tumor cell suspension were injected slowly (over 45- 60 seconds) under the sciatic nerve sheath using a Hamilton syringe to prevent leakage.

Treatment protocols

In the CPA model, when the plasma Gluc level reaches $2x10^6$ RLU; and in the sciatic nerve model, when the tumor reaches 4 mm in diameter, mice were randomized into groups to receive the following treatment:

i) Losartan treatment. Losartan (40 mg/kg) was administered by oral gavage daily; treatment continued until mice in the control group became moribund [15,16].

ii) B20 treatment. B20 (5 mg/kg) was administered *i.p.* once a week; treatment continued until tumors reached 1 cm in diameter [13].

Measurement of tumor growth

Nf2$^{-/-}$ cells were infected with lentivirus encoding secretive Gaussia luciferase reporter gene (Gluc). The measurement of plasma Gluc was performed as previously described [7,8]. Briefly, 10µl of whole blood was collected from a slight nick on tail veils and mixed with 5 µl 50mM

EDTA immediately to avoid clotting. Blood samples were transferred to a 96-well plate and Gluc activity was measured using a plate luminometer (GloMax® 96 Microplate Luminometer, Promega, Madison, WI). The luminometer was set to automatically inject 100 µl of 100 mM coelenterazine (CTZ, Nanolight, Pinetop, AZ) in PBS and photon counts were acquired for 10 sec.

Gait and coordination tests

We tested the severity of gait and coordination symptoms using a group of tests. These tests are derived from previously published phenotype assessments used in mouse models of Huntington's disease, spinocerebellar ataxias, and spinobulbar muscular atrophy [17].

i) Ledge test was carried out by lifting the mouse and placing it on the cage's ledge and observing it as it walked along the cage ledge. Score 3: Normal mice will typically walk along the ledge and attempt to descend back into the cage using their paws. Based on the time that the mouse stayed on the ledge, a ledge test score was given: Score 2: mouse stayed on ledge > 10 seconds but lost its footing and fell off the ledge; Score 1: mouse stayed on ledge < 10 seconds and lost its footing and fell off the ledge; and Score 0: mouse could not stay on the ledge for over 3 seconds, or shook and refused to move at all.

ii) Hind-limb clasping test was performed to assess whether the mouse clasped its forelimbs and hindlimbs into its body or splayed its limbs when suspended by tail. The mouse was lifted clear of all surrounding objects by grasping its tail near its base. The hindlimb position was observed for 10 seconds and recorded. Score 3: Normal mice will typically splay out their hindlimbs. Score 2: if one hindlimb is retracted to the belly. Score 1: if both hindlimbs are partially retracted. Score 0: if both hindlimbs are entirely retracted and touching the belly.

iii) Gait is a measure of coordination and muscle function. To obtain footprints, hind- and forepaws were dipped in black ink. Then, animals were allowed to walk, and footprint patterns made on white paper lining the floor were obtained. Score 3: In normal mice, there is no limp and the belly does not touch the ground. Score 2: if the mouse walked slowly with slight limping. Score 1: if the mouse showed severe limping and lost coordination. Score 0: if the mouse had difficulty moving forward, dragged its body along the ground and could not walk a straight line.

iv) Kyphosis refers to an abnormally curved spine. For this test, the mouse was removed from its cage and placed on a flat surface. We assessed the characteristic dorsal curvature of the spine. Score 3: Normal mice will straighten their spine as they walk. Score 2: if a mouse could straighten its spine and exhibited mild hunchback. Score 1: if a mouse could not straighten its spine and exhibited a mild hunchback. Score 0: if a mouse exhibited a pronounced hunchback.

Rotarod test

Rotarod performance was evaluated using an automated Rotarod (Rotamex 4/8 4-Lane Treadmill Shock Grid; Columbus Instruments, Livoniz, MI). Rotarod test was performed in animals bearing size-matched tumors. Tumor size was measured every 3 days by caliper; when tumor size reached ~3 mm in diameter, mice were trained on the rotarod every day for 3 days. Three days later, we performed the rotarod test. Three tests were performed for each animal; each test began with a 30 second acclimation period at 4 rpm followed by an acceleration of 4 rpm every 60 seconds to a maximum of 10 min and 40 rpm. The amount of time that elapsed before the mouse fell off was recorded as rotarod endurance. To avoid heterogeneity between animals, the average time to fall from the rotating cylinder was normalized to the value from each mouse on the first day and presented as normalized rotarod endurance [13].

Statistical analysis

Efforts were made to minimize animal suffering and to reduce the number of animals used according to the 3R' principles. The primary endpoints of our study are to assess the ataxia score and rotarod performance and compare the gait and coordination function between different treatments. We determined whether growth curves significantly differed from each other by log-transforming the data, fitting a linear regression to each growth curve, and comparing the slopes of the regression lines (using an equivalent of ANOVA). Significant differences between the two groups were analyzed using the Student's *t-test* (two-tailed) or Mann-Whitney *U* test (two-tailed). All calculations were done using the GraphPad Prism Software 6.0 and Microsoft Excel Software 2010.

RESULTS

A panel of functional tests was established to evaluate the severity of gait and coordination symptoms in the VS mouse model

To evaluate for ataxia and incoordination, we performed a series of tests, including: i) ledge test to measure coordination, which is the symptom most directly related to signs of ataxia in patients (Video 1a), (Video 1b), (Video 1c), (Video 1d); ii) hind limb clasping test, which has been

used to evaluate disease progression in several mouse models of neurodegeneration (Video 2a), (Video 2b), (Video 2c), (Video 2d); iii) gait test, which measures coordination and muscle function (Video 3a), (Video 3b), (Video 3c), (Video 3d); and iv) kyphosis test, which assesses for a characteristic dorsal curvature of the spine, a common manifestation caused by a loss of muscle tone in the spinal muscles secondary to neurodegeneration (Video 4a), (Video 4b), (Video 4c), (Video 4d). Each test is scored from 3 to 0, with 3 being the highest score in normal animals and 0 being the most severe manifestation of symptoms in tumor-bearing mice. The scores from all 4 tests were combined to create a composite Ataxia score.

In the CPA model, the surgery and tumor implantation procedures do not result in ataxia symptoms (Figure 1a). Mice develop symptoms of ataxia two weeks after tumor implantation. The severity of ataxia increases as tumor growth progresses; and the ataxia score inversely correlates with tumor size (Figure 1b, Figure 2a).

(b) Compared to non-tumor-bearing mice (n=12), mice bearing *Nf2*$^{-/-}$ tumor in the CPA model (n=8) started to show decreased ataxia score 2 weeks after tumor implantation, which deteriorated over time. Institutional regulatory board permission was obtained for all procedures performed within this protocol from MGH IACUC.

Evaluate treatment effects on ataxia symptoms in mouse models of NF2-related VS

Next, we characterize the treatment effects on motor function. Treatment with bevacizumab, a humanized monoclonal antibody that specifically neutralizes VEGF-A, has been associated with a reduction in the volume of most growing VS. More importantly, bevacizumab treatment improved hearing function in 57% of patients [18]. A recent study showed that bevacizumab improves vestibular precision and clinical disability in patients with NF2–SWN [12]. Therefore, we tested the effects of anti-VEGF treatment on relieving neurological symptoms. In the sciatic nerve model, we have previously demonstrated that anti-VEGF treatment improves neurological function via reducing neuro- edema [13,19,20]. To characterize the anti-VEGF effects on gait and coordination, in the *Nf2*$^{-/-}$ CPA model, groups of mice were treated with: i) control, ii) anti-VEGF therapy using an antibody (B20, Genentech) that neutralizes mouse VEGF, and iii) anti-fibrotic treatment using losartan, an FDA-approved anti-hypertensive agent that blocks angiotensin II receptor 1 and reduces extracellular matrix content. Tumor growth was evaluated by measuring blood Gausia luciferase (G-Luc) levels, and the severity of motor symptoms was evaluated every 3 days. In the control group, we observed that as tumor size increased, ataxia score decreased, indicating tumor growth induced more severe ataxia (Figure 2a). In mice treated with anti-VEGF antibody, *Nf2*$^{-/-}$ tumor growth was inhibited and the ataxia score remained unchanged over time (Figure 2b). Previously we demonstrated that losartan, via reducing the angiotensin inflammatory signaling, prevented tumor-induced hearing loss in the VS model [9]. In the group of mice treated with losartan, tumor growth was not affected and the ataxia score decreased over time (Figure 2c).

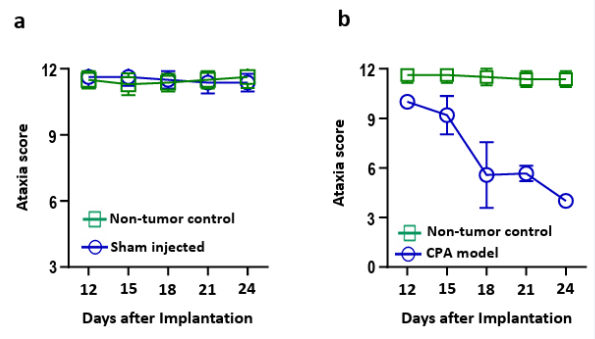

Figure 1 A panel of functional tests was established to evaluate the severity of ataxia symptoms in the VS mouse model. (a) Non-tumor-bearing normal mice without surgery or injection (Control, n=5) and mice that underwent unilateral sham surgery and saline injection (Sham, n=3) showed no differences in ataxia scores.

Rotarod performance inversely correlates with tumor size in mouse models of NF2-related VS

In the same experimental setting, we used a rotarod performance test to evaluate changes in the motor function of mice in the sciatic nerve model (Video 5a), (Video 5b). We found that rotarod performance decreased as tumor size increased in the control (Figure 3a); in anti-VEGF treated group, where the treatments inhibited tumor growth, rotarod performance significantly increased (Figure 3b); and in losartan treatment groups, tumor size is not reduced, rotarod performance decreased as tumor grow (Figure 3c).

DISCUSSION

Patients with brain tumors face serious challenges in maintaining quality of life. Primary brain tumors originate either in the brain parenchyma (e.g., gliomas, which include astrocytomas, oligodendrogliomas, ependymomas, and medulloblastomas) or in the extraneural structures (e.g., meningiomas and schwannomas). Secondary brain tumors develop when cancer cells metastasize to the brain (e.g., lung and breast cancer brain metastasis). These patients experience general symptoms resulting from increased

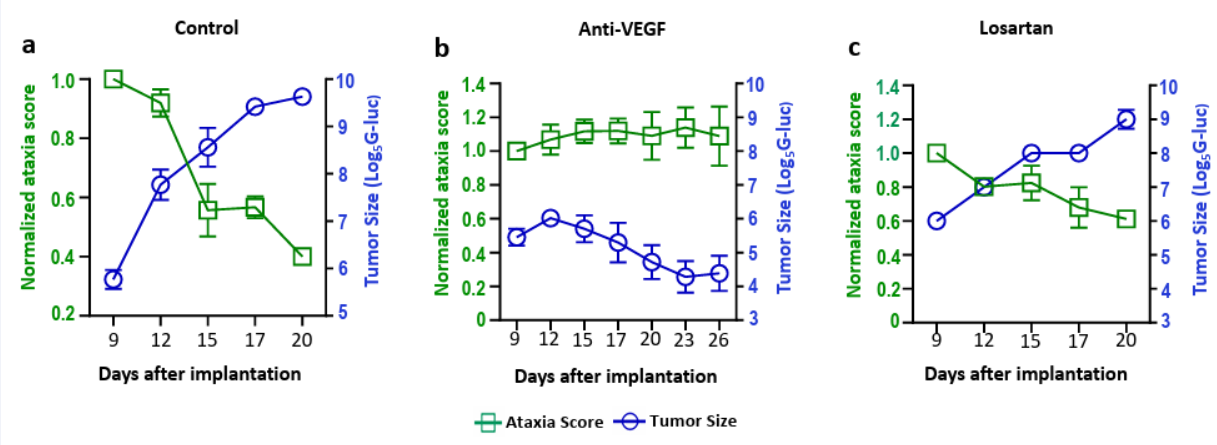

Figure 2 Evaluate treatment effects on vestibular symptoms in mouse models of NF2-related VS. $Nf2^{-/-}$ tumor cells ($1x10^5$ cells/mouse) were injected into the CPA region of the right hemisphere of mice. When blood G-Luc level reached $2x10^6$ RLU, mice were randomized to receive (a) isotype control IgG (n=12), (b) Anti- VEGF antibody (B20, n=6) and (c) Losartan (n=6) treatments. Ataxia tests and tumor measurement by blood G-Luc assay were performed every 3 days until the end of the experiment. Institutional regulatory board permission was obtained for all procedures performed within this protocol from MGH IACUC.

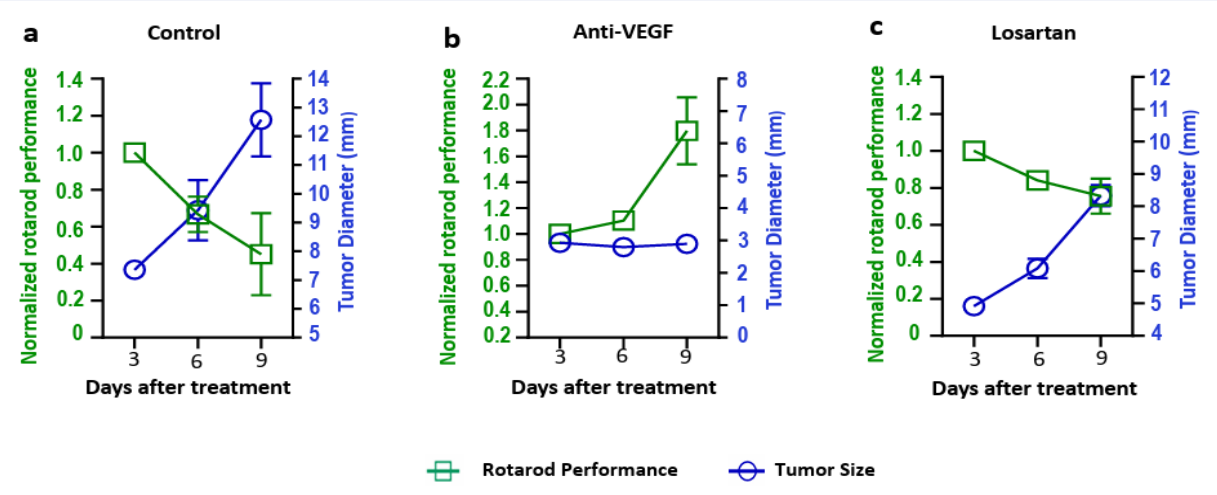

Figure 3 Rotarod performance correlates with tumor size in mouse model of NF2-related VS. $Nf2^{-/-}$ tumor cells ($1x10^5$ cells/mouse) were injected into the sciatic nerve of mice. When tumors reached 4 mm in diameter, mice were randomized to receive (a) Control (n=7), (b) Anti-VEGF antibody (B20, n=8) and (c) Losartan (n=6) treatment. Rotarod performance test and tumor measurement by caliper were performed at 3 time points. Institutional regulatory board permission was obtained for all procedures performed within this protocol from MGH IACUC.

intracranial pressure, such as headache, anorexia, nausea, vomiting, seizures, blurred or double vision, and insomnia [16,17]. There are few well-tested interventions to improve the neurological function and quality of life among patients with brain tumors. A major limitation in studying tumor-induced neurological deficits and developing new therapeutics is the lack of orthotopic mouse models that allow assessment of function.

To faithfully recapitulate human disease, orthotopic animal models have been developed for glioblastoma [18], medulloblastoma [19], and NF2-related vestibular schwannomas [9,20]. However, most of these preclinical animal studies have focused on tumor growth; few studies investigated the biology and mechanisms of tumor-induced neurological symptoms. In two mouse models for NF2-related vestibular schwannomas, we observed that as the tumors progress, tumor-bearing mice develop ataxia.

The severity of the ataxia and incoordination worsens as tumors grow, demonstrating lack of coordination, gait abnormalities, and difficulty walking, which mimic the clinical disability induced by progressively enlarging VSs in patients with NF2-SWN . Recently, Madhani et. al., evaluated vestibular-mediated behaviors (eye movements, motion perception, and balance) and clinical vestibular disability (dizziness and ataxia) in 8 untreated patients with NF2-SWN, and 2 bevacizumab-treated patients. They demonstrated that, coupled with imaging and hearing results, these tests showed VS tumor degraded vestibular precision and caused clinical disability, and bevacizumab improved vestibular precision and clinical disability in both patients [12].

In our VS mouse models, we compiled a panel of functional tests to comprehensively evaluate the vestibular function, ataxia, and motor function in mice. The first goal of our study is to identify tests that allow rapid and sensitive quantification of gait and motor dysfunction in the mouse models. Previously published protocols have reported assessments of cerebella ataxia in several neurological disease models, including spinocerebellar ataxias, Huntington's disease, and spinobulbar muscular atrophy [15]. Based on these published protocols, we combined five tests to evaluate tumor-induced neurological dysfunction and treatment effects. We reported that:

i) the surgery and tumor implantation procedures used to create our animal models do not result in ataxia or incoordination, and ii) the severity of motor symptoms correlates with tumor size. Furthermore, we demonstrated that anti-VEGF treatment inhibited tumor growth and prevented deterioration of vestibular and motor function. This reproduces the improvement in vestibular function observed in bevacizumab-treated VS patients [10].

Our second goal is to determine the best tests to evaluate neurological function in NF2-SWN models. In our NF2-SWN CPA model, we found that the ledge test - a direct measure of coordination - is the most sensitive test, and changes in ledge test scores can easily be observed in the early tumor stage. Gait test that measures coordination and Kyphosis test that evaluates the characteristic dorsal curvature of the spine show differences with late to end-stage tumors. We used the rotarod performance test to evaluate motor function. The rotarod test is a relatively straightforward procedure, and can accurately reflect motor function in both CPA and sciatic nerve models.

SUMMARY

In summary, we report a group of tests that will allow us to fully utilize orthotopic animal models to characterize brain tumor biology and neurological function. By studying brain tumors from multiple perspectives, we can unravel the basic tumor pathobiological underpinnings of these tumors and develop novel therapeutic approaches.

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