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BRIEF COMMUNICATION

Transcriptomic signature of painful human neurofibromatosis type 2 schwannomas

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Abstract

Schwannomas are benign neoplasms that can cause gain- and loss-of-function neurological phenotypes, including severe, intractable pain. To investigate the molecular mechanisms underlying schwannoma-associated pain we compared the RNA sequencing profile of painful and non-painful schwannomas from NF2 patients. Distinct segregation of painful and non-painful tumors by gene expression patterns was observed. Differential expression analysis showed the upregulation of fibroblast growth factor 7 (FGF7) in painful schwannomas. Behavioral support for this finding was observed using a xenograft human NF2-schwannoma model in nude mice. In this model, over-expression of FGF7 in intra-sciatically implanted NF2 tumor cells generated pain behavior compared with controls.

Introduction

Why do a significant proportion of patients with schwannomas have pain[1] and others do not? Schwannomas are peripheral nerve sheath tumors derived wholly from Schwann-lineage cells.[2] While virtually always benign, the tumors and their treatment are associated with substantial morbidity and a shortened life expectancy for many affected individuals.[2] Schwannomas cause a variety of serious neurological defects including hearing loss, imbalance, tinnitus, paralysis, and persistent severe pain.[3] Interestingly, schwannoma-associated pain is often not relieved by tumor removal and does not appear to be well correlated with tumor size, thus suggesting that the pain can be due to mechanisms other than nerve compression.[4] Nonetheless, the standard of care for the treatment of schwannoma is operative resection. Pain treatments are frequently inadequate and efficacious drug therapy for tumor control is essentially non-existent.[5]

We investigated the molecular mechanisms of schwannoma-associated pain by conducting RNA sequencing of painful and non-painful schwannomas from NF2 patients. Two transcriptomic analyses independently demonstrated self-segregation of samples based on

pain phenotype. A potential role of increased FGF7 production in schwannoma-associated pain was supported using a xenograft human NF2-schwannoma mouse model.

Subjects and Methods

Patient tumor samples

Patient-derived tumors were formalin-fixed paraffin-embedded (FFPE) and these tumor blocks along with pain status were obtained from Pathology Core, MGH, USA. All FFPE schwannoma tissues utilized were from NF2 patients treated by one of the authors (SRP). Tumor samples were de-identified and used in accordance with MGH institutional policy.

Schwannoma model and pain assessment

Sciatic nerve schwannomas were generated by direct injection of HEI-193-FC human schwannoma cells, previously transfected with lipofectamine-FGF7 or GFP (1-10) construct (pcDNA3.1-GFP (1-10), into the left sciatic nerve of anesthetized mice (*nu/nu*, 5- to 7-week-old males, Charles River Lab)). Bioluminescence imaging was performed to monitor tumor-cell burden.[6,7] Hindpaw thermal sensitivity was quantified via foot withdrawal latency to radiant heat (Hargreaves test, Ugo Basile)[8] by investigators blind to group. All in vivo experiments were approved by and conducted under the oversight of the MGH Institutional Animal Care and Use Committee (Protocol Number: 2014N000211).

Results

Differential transcriptomic profile of painful and non-painful human NF2-schwannomas

Genomic studies of schwannoma are limited by the availability of human tumor samples reflecting both the rarity of the disease and absence of a common tumor tissue bank. We conducted two separate RNA sequencing experiments on small sets ("set 1" & "set 2") of FFPE samples from painful (set 1: $n = 3$; set 2: $n = 4$) and non-painful (set 1: $n = 8$; set 2: $n = 3$) peripheral human NF2-schwannomas. These sample sets originated from different tumors and were processed using two different protocols for the preparation of RNA-seq libraries. These protocol differences led us to perform separate differential gene expression analyses between painful and non-painful schwannomas for each of the two sets, followed by focused experiments on specific genes that showed consistent expression changes in both sets. Patient pain features, tumor histopathology, and location are described for each sample (Table S1). Hematoxylin & Eosin (H&E) staining with cresyl violet-guided macro-dissection was employed to locate tumor-rich areas and maximize tumor-derived total RNA utilized for library preparation (Fig. 1A). Consistent with prior reports, we did not identify any gross histological differences between painful and non-painful tumors.[4]

Set 1 of RNA-seq samples showed a distinct segregation of gene expression patterns between painful and non-painful schwannomas from NF2 patients (Fig. 1B), with approximately 100 genes and 417 transcripts differentially expressed with more than two-fold change in expression. Of these 417 transcripts, 303 were protein coding. Only a fraction of these genes (<15 genes) and transcripts (35 transcripts), however, passed the standard cutoff of statistical significance (false discovery rate (FDR) < 0.05), likely due to the small sample size and the variability between samples.

Set 2 of RNA-seq samples also showed a distinct segregation of gene expression patterns between painful and non-painful schwannomas from NF2 patients (Fig. 2A) and yielded a larger number (593) of differentially expressed transcript isoforms based on the cutoff of 2-fold change in gene expression and FDR<0.05 (~490 protein coding transcripts). Comparing the results of RNA-seq analyses in *sets 1 and 2*, revealed several secreted proteins that were upregulated in the painful schwannomas, including cytokines and growth factors, consistent with possible paracrine-like effects on primary nociceptive afferents and/or non-neuronal cells (e.g., immune pathways) that could result in nociceptor sensitization. We also found multiple microRNA, long non-coding and antisense RNA genes that were differentially expressed in painful compared to non-painful schwannomas (Tables S2–S5). To validate these RNA-seq results, we performed qRT-PCR on a panel of the most highly up- or downregulated protein-coding genes, based on both RNA-seq sets. qRT-PCR results for 8 out of 10 tested genes showed a good correlation with RNA-seq expression values (Figs. 1C and 2E). *Fibroblast Growth Factor 7 (FGF7)*, *periaxin* (*PRX*), *natriuretic peptide receptor 3* (*NPR3*), and *disks large-associated protein 1* (*DLGAP1*) were validated by qRT-PCR as upregulated in painful schwannomas.

Both RNA-seq experiments revealed several members of *FGF* gene family with large fold changes of gene expression between painful and non-painful schwannomas (Table S2 and S4; Fig. 2B and D). Members of the FGF family are generally secreted factors and have roles in wound healing, cell proliferation/differentiation, nervous system development, synaptogenesis, and regulation of voltage-gated channels.[9]

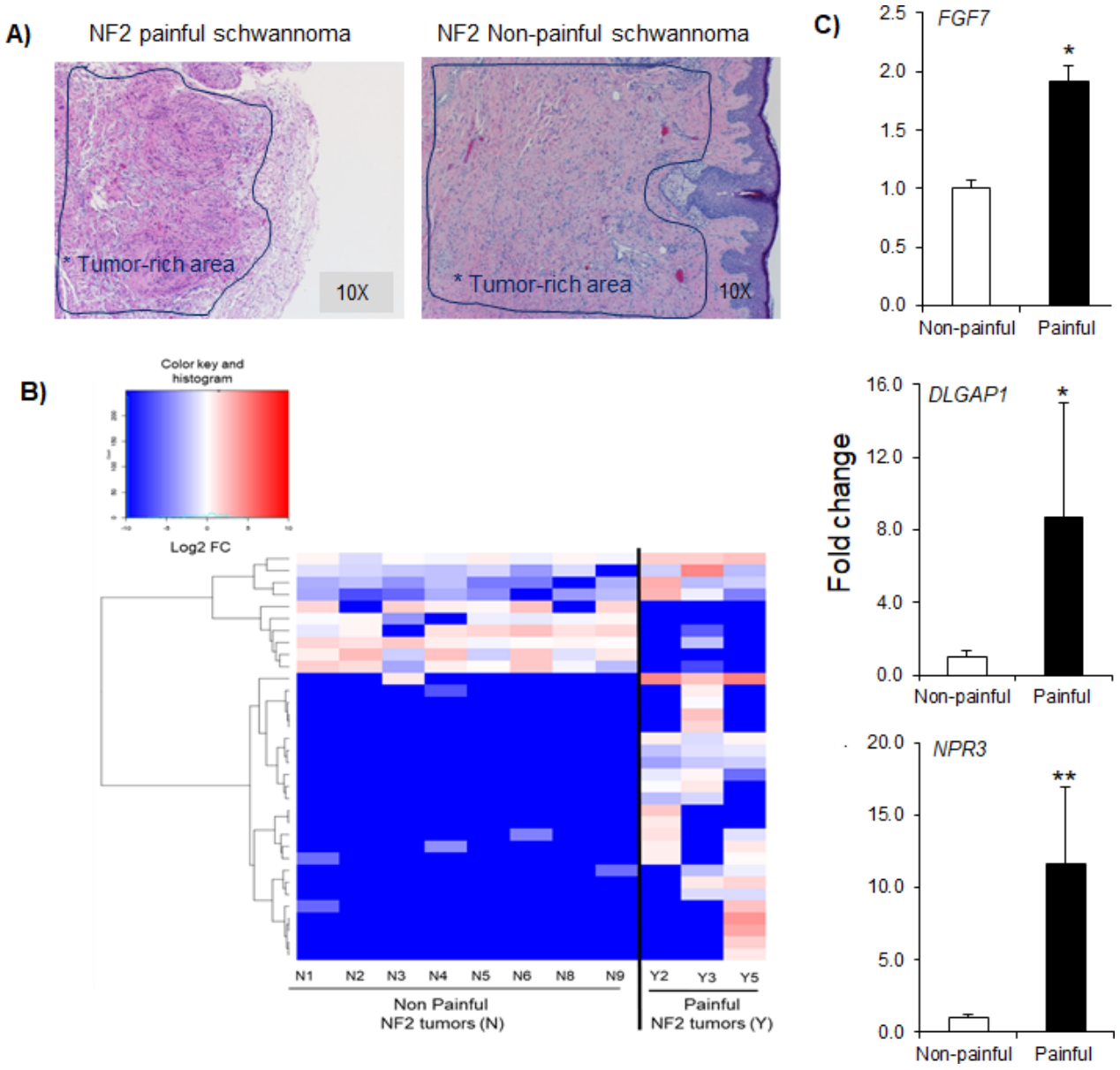

Figure 1. Patterns of differential gene expression between painful and non-painful schwannomas from NF2 patients based on set 1 of RNA-seq samples. (A) Representative images of Hematoxylin & Eosin staining of FFPE schwannomas. Marked regions indicate tumor-rich areas which were macrodissected used for RNA extraction. Macrodissection was performed blind to pain status and other patient information. (B) Heatmap of expression values of transcripts that were differentially expressed between non-painful (N) and painful (Y) schwannomas (fold change >2, FDR<0.05). (C) qRT-PCR validation of representative genes that RNA-seq set 1 indicated upregulation in painful schwannomas compared to control tumors. Data are presented as mean ± SEM $*p < 0.05$, $**p < 0.005$.

Tumor-cell FGF7 over-expression in a xenograft human NF2 model generates pain

To determine how FGF7 may contribute to pain, we investigated whether FGF7 signaling can sensitize peripheral sensory neurons. FGF7 is minimally expressed in HEI-193 NF-2 human-schwannoma-derived cells (data not shown); we utilized plasmid transfection to overexpress human *FGF7* (NM_002009.3) or control (*GFP1-10*). FGF7 was detected in cell lysates, supernatants, and extracellular vesicles (EVs) of the FGF7- but not GFP-transfected HEI-193 cells (Fig. 3A). We tested whether conditioned media from FGF7-transfected HEI-193 cells potentiates capsaicin-mediated nociceptor activation by

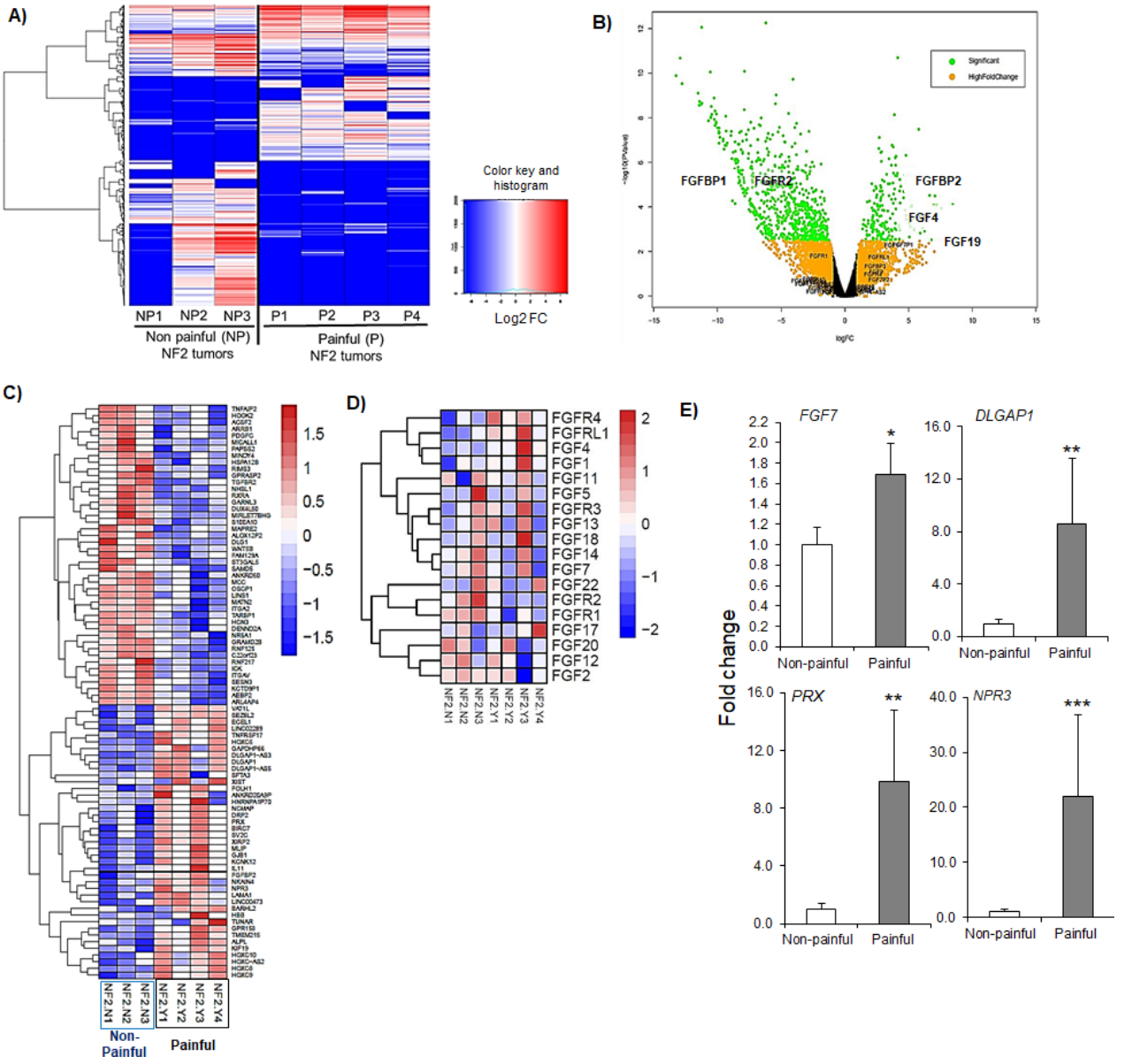

Figure 2. Patterns of differential gene expression between painful and non-painful schwannomas from NF2 patients based on set 2 of RNA-seq samples. (A) Heatmap of expression values of transcripts that were differentially expressed between non-painful (NP) and painful (P) schwannomas (fold change >2, FDR<0.05). (B) Volcano plot (log fold change against −10 log *P*-value) of differences in gene expression between non-painful (NP) and painful (P) schwannomas. Genes from fibroblast growth factor family are marked. (C) Heatmap of gene expression (represented as *Z*-scores across all expression values for a given gene) for top 100 differentially expressed genes in RNA-seq set 2. (D) Heatmap of gene expression (represented as *Z*-scores across all expression values for a given gene), for members of fibroblast growth factor family marked in volcano plot (B). (E) qRT-PCR validation of representative genes from all schwannoma samples indicated upregulation in painful schwannoma compared to controls. Data are presented as mean ± SEM, $*p < 0.05$, $**p < 0.05$, and $***p < 0.0005$.

conducting in vitro calcium imaging to asses both the percentage of responding cells and the amplitude of calcium responses. Conditioned media from FGF7-expressing HEI-193 cells resulted in a 20% and 42% increase in responding cells in independent biological replicates of sensory neurons cultured from separate mice (FGF7-conditioned media: 615/749 and 436/722 cells; GFP-conditioned media: 344/501 and 257/605 cells). Capsaicin-elicited calcium peaks, however, were of similar amplitudes following exposure to FGF7 and control-conditioned media (Fig. 3B). These data suggested that FGF7-mediated sensitization might occur via recruitment of normally silent nociceptors rather than increased amplitudes of responding cells.

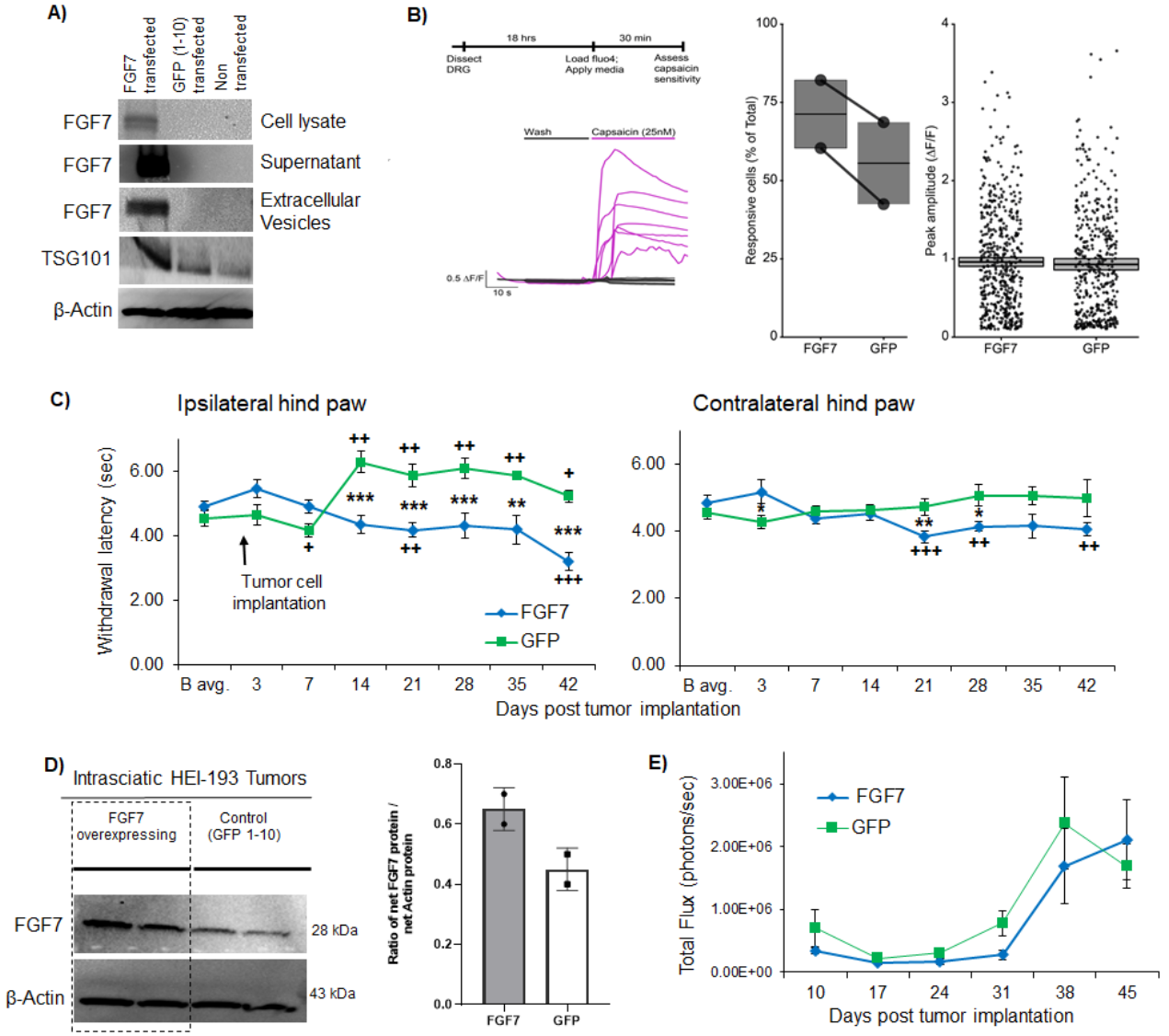

Figure 3. Tumor cell FGF7 over-expression in a xenograft human-NF2 model generates pain. (A) FGF7 was detected in cell lysates, supernatants, and extracellular vesicles of human HEI-193 schwannoma cell line following in vitro transfection with FGF7 plasmid, compared to GFP control plasmid (Western blot). (B) Incubation of cultured mouse dorsal root ganglion (DRGs) with conditioned media from FGF7 overexpressing cells increased frequency of capsaicin-sensitive sensory neurons. Calcium responses were identified by peak response amplitude above 0.1 DF/F and peak rise slope above 0.1 DF/F/s. Plots indicate quantification of percent responsive cells (each dot is independent experiment) and peak response amplitude (each dot is individual cell; line and shaded area for both are mean and SE by condition), ($N = 2$ independent experiments with 3 wells/each. (C) Hargreaves method indicating thermal sensitivity of the hindpaw ipsilateral (left) and contralateral (right) to HEI-193 schwannoma cell-line implantation. Animals implanted with FGF7-expressing HEI-193 cells developed pain-like behaviors (hyperalgesia) compared with control animals. Data are presented as mean ± SEM; $n = 8$ mice/group. Statistical significance was calculated using Student's *t*-test; plus-sign (+) indicates within group difference compared to baseline average; asterisk (*) indicates between group differences at the same timepoint. */+ $p < 0.05$, **/++ $p < 0.01$, and ***/+++ $p < 0.005$. (D) Intrasciatic tumors harvested from animals in panel "C" 7-week post-tumor implantation indicates overexpressing of FGF7 in the ipsilateral nerves ($n = 2$ animals) compared to the control group nerves ($n = 2$ animals). The graph indicates quantification of western blot image; data are shown as mean with SD for two data points. (E) In vivo bioluminescence imaging to monitor tumor growth for the same animals shown in panel "C" at weekly intervals starting 3 days post intrasciatic tumor implantation indicates no differences between the two groups. Data are presented as mean ± SEM.

To further investigate FGF7 as a putative mediator of schwannoma-associated pain, we utilized a xenograft human-NF2 schwannoma model.[7] FGF7- or GFP-transfected HEI-193 cells were implanted into the sciatic nerve of nude (*nu/nu*) mice ($n = 8$/group). Mice with FGF7-overexpressing schwannomas developed thermal hyperalgesia (Hargreaves method) in the hindpaws both ipsilateral and contralateral to tumor compared to controls ($p < 0.05$, Fig. 3C). Thermal sensitization was apparent 2-week post tumor implantation and persisted more than 40 days (Fig. 3D). Other than at day 31 post-implantation, there was no difference in bioluminescent signal between groups (Fig. 3E). Thus, tumor burden cannot explain the development of pain behavior associated with FGF7 over-expression.

Discussion

Here we report next-generation RNA-sequencing data from painful, and non-painful FFPE schwannoma samples from NF2 patients that suggest significant transcriptomic differences between painful and non-painful schwannomas, suggesting several pathways that have not been implicated in tumor-associated pain. Schwannoma tumor-associated pain is common, and there are multiple mechanisms through which tumors are thought to activate and/or sensitize primary sensory afferents including mechanical compression of nerves, direct cell-cell signaling, and release of secreted factors.[10,11] Tumor pain may be due to alterations in activity/function of non-neuronal cells including immune cells and glia.[12] Although a recent study of schwannomatosis demonstrated a correlation of tumor pain with genetic mutations in *LZTR1* and *SMARCB1*,[13] there are no published reports of mechanisms responsible for NF2-schwannoma pain.

Our RNA-seq data revealed increased expression of FGF7 in painful schwannomas. In vitro calcium imaging showed that conditioned media from FGF7 over-expressing cells augmented responses to capsaicin, consistent with nociceptor sensitization.[14] Using a xenograft human-NF2 model we demonstrated that FGF7 over-expression in schwannoma cells generated thermal sensitization. FGF7 was of interest as (1) FGF7 is a secreted factor, (2) FGF7 is upregulated in primary sensory neurons following pain-associated peripheral nerve injury,[15] and (3) primary sensory afferents possess FGF7 receptors.[16] Additionally, conditional disruption of FGF receptor signaling in Schwann cells causes neuropathy and lack of thermal sensitivity.[17] Interestingly, FGF7 overexpression in HEI-193 human-schwannoma cells augmented thermal sensitization not only in the hindpaw ipsilateral to the intrasciatic schwannoma, but also in the contralateral hindpaw as observed previously with peripheral nerve neuropathic pain models,[18] although the mechanism is not well understood.

While the specific role of FGF7 in schwannoma-inducted pain in humans requires further study, using transcriptome analysis we have uncovered novel molecular pathways of schwannoma-associated pain as a first step in generating novel candidate targets for therapeutic development. RNA-seq data GEO accession number: GSE138347.

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Authors' Contributions

G.J.B., P.K., G.F., and S.A. carried out the overall design of the project. G.J.B., P.K., and S.A. wrote the manuscript. P.K., M.C., and B.A. analyzed the sequencing data and performed the bioinformatics analysis. R.S. designed the bioinformatic analysis pipeline and consulted for the project, assisted with manuscript preparation. S.P. contributed clinical samples and information. A.S. provided and performed a pathological evaluation of FFPE samples. P.K., S.A., and A.A. performed in vitro experiments involving HEI-193 cells and conducted in vivo xenograft schwannoma modeling and behavioral experiments. B.W. and D.D. designed and performed calcium imaging experiments and related data analysis, assisted with manuscript preparation. P.K. and B.A. performed extracellular isolation and quantification. G.J.B. involved in concept initiation and overall supervision of project.

Conflict of Interest

The authors declare no competing financial interests or conflicts of interest. GJB has a financial interest in Mulberry Biotherapeutics, Inc, a company developing novel biologic therapies for schwannoma and related neoplasms. GJB's interests were reviewed and are managed by

Massachusetts General Hospital and Mass General Brigham in accordance with their conflict of interest policies.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. RNASeq Supplementary tables 1–6_An Cl Tr Neurol.

Data S2. RNASeq Supplementary materials and methods_An Cl Tr Neurol.

