Cutaneous targets for topical pain medications in neuropathic pain patients: individual differential expression of biomarkers supports the need for personalized medicine

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SUPPLEMENTAL MATERIAL

Details of the immunolabeling methodology and antibodies utilized for this study

running title: Cutaneous Targets for Topical Pain Medications - SUP

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Supplemental METHODS

The INTiDYN ChemoMorphometric Analysis (CMA) methodology platform was designed to provide comprehensive immunolabeling analysis in complex tissues. The use of the standard innervation biomarker PGP9.5 for skin biopsy and quantification of IENF provides the only objective biomarker accepted by the FDA (and covered by insurance for testing) that has been shown to document chronic pain-related pathologies, diagnosed as small fiber neuropathy (SFN).

Tissue Collection. For this study, 3mm human skin punch biopsies were collected from 1) the ipsilateral painful rash area, and 2) the contralateral non-pain mirror dermatome area from each PHN patient. Each biopsie was immediately fixed by immersion in ice-cold 4% paraformaldehyde (PFA) in PBS buffer for a minimum of 4 hours and maximum of overnight (<24hrs) at 4°C. Biopsy specimen were then rinsed in 3 changes of fresh PBS, and transferred to fresh PBS for storage/shipment to INTiDYN at 4°C. Biopsies were shipped on cold packs (unfrozen) by overnight courier to INTiDYN for tissue processing.

Upon arrival at INTiDYN, biopsies were cryoprotected overnight in 30% sucrose/PBS, mounted in optimal cutting temperature media, frozen, and sectioned by cryostat at a 14µm thickness. Consecutive sections were quickly thaw-mounted and rotated sequentially across a series of at least 20 slides, such that each slide contained sections from equally spaced intervals throughout the entire biopsy. Importantly for this study, the two separate biopsy specimen designated as left or right from the same patient were mounted on the same microscope slide to assure identical immunolabeling procedures across each patient biopsy pair.

Immunofluorescence. Following the CMA platform for multi-label immunofluorescence, all specimen were processed for integrated immunolabeling assessments using several primary antibodies on alternating sections. The primary antibodies utilized here were chosen to target the active signaling systems of commonly utilized topical active compounds. Previous work from this tissue had documented the Protein Gene Product 9.5 (PGP) antibody (directed against ubiquitin-C-terminal hydrolase (UCHL1) and labels all cutaneous innervation) innervation results, and that data was utilized to enrich this study cohort for patients with similar innervation patterns. All primary antibodies for this study were incubated at 4°C overnight. Following primary antibody incubations, slides were rinsed in excess PBS and then incubated with the appropriate species secondary antibody conjugated with Cy3 (red fluorescence; Jackson ImmunoResearch, ab2307443 or ab2315778; 1:500) at room temperature for 2 hrs. The DNA binding protein DAPI (4',6-Diamidino-2-Phenylindole) was also included in the secondary antibody mix to stain cell nuclei (blue fluorescence). After secondary antibody labeling, slides were rinsed in excess PBS and mounted under coverslips with 90% glycerin in PBS with .05% sodium azide, and stored at -20°C until analysis.

Analysis. Following established ITD-CMA procedures, the indirect immunofluorescence method was used for microscopic evaluation of immunolabeled tissue. Immunofluorescence-labeling intensity evaluations were conducted on digital images captured at identical camera settings across the entire slide series utilizing a high-intensity camera (Hammamatsu ER) mounted to a standard microscope (Olympus BX51) equipped with conventional fluorescence filter cubes for specific excitation and emission spectra required for red/green/blue fluorophore channel separation, a linear focus encoder, and a 3-axis stage, controlled with Neurolucida software (MBF Bioscience, Essex, VT). This system

produced seamless high resolution whole section montages for image mapping/measuring of selected elements. Captured images were analyzed using Photoshop (Adobe Systems, San Jose, CA) routines consisting of a standard size pixel marquee to measure the average pixel intensity (API, 0-256) across the vital epidermal keratinocyte strata (corresponding to stratum basalis, stratum spinosum, stratum granulosum). Immunolabeling controls have previously been conducted on human biopsy tissue slides omitting the primary antibodies and using the rabbit secondary antibodies conjugated to Cy3. These controls routinely demonstrate no fluorescent signal (data not shown). For all immunolabeling, DAPI staining (blue fluorescence) was utilized to identify epidermal keratinocyte cell nuclei. For the use of primary antibody biomarkers, all were purchased from commercial sources and the specificity was expected to be as declared. The reagents were used in good faith and trusted as such. For those biomarkers not utilized routinely in the lab, specificity was determined empirically based from experience with immunolabeling skin, for which our group has seen numerous examples of non-specific labeling or labeling in locations that are not supported in the literature, in addition to simple high-background cellular labeling that would be indicative of specificity issues. None of the antibody biomarkers utilized in the present study demonstrated any signal anomalies that would be indicative of non-specific labeling.

Measures of epidermal keratinocyte API were taken at 5 evenly spaced locations on each of three different sections for each biopsy analyzed (n=15 measures/biopsy). Human epidermis contains a majority of keratinocyte cells, along with several additional cell types, including Langerhan's Cells, melanocytes, and Merkel cells that also reside in the epidermis. For this study, data was collected such that each region of interest (ROI) was selected to contain only a majority of observed keratinocyte cells. Immunolabeling by additional non-keratinocyte cell types, identified morphologically within the epidermis, was not readily observed. Ipsilateral (pain) and contralateral (nonpain) biopsy API averages were calculated from each slide, analyzed for differences by paired Student's T-test, and within patient API ratios [Ipsilateral/Contralateral] were created for cohort analysis. A ratio value of 1 indicated no differences between sides, whereas ratio values less than 1 indicated an increased contralateral expression, and ratio values greater 1 indicated increased ipsilateral expression. The API % difference of ipsilateral (pain) to contralateral (nonpain) biopsy expressions were also calculated as [(ipsilateral - contralateral)/contralateral x 100]. A paired Student's T-test statistic was used to determine significant differences ($p \leq 0.05$) between contralateral and ipsilateral biopsies from each slide (within patient), and to determine ratio differences among the cohort (between patient).

Model: As a proof-of-principle, this study conducted a multi-molecular immunofluorescence survey of existing unprocessed, archived tissue slides of previously characterized PHN patients. The archived biopsy tissue slides were collected (for an unrelated study) from approximately 300 patients afflicted with PHN whose pain intensities were assessed by 11-point Visual Analog Scale (VAS) and daily diary Numerical Rating Score (NRS) measures (0-10, with 0 – no pain to 10 – unbearable pain). The PHN model was chosen for this initial investigation because each patient provided two punch biopsies prior to any treatment: one from the painful dermatome (disease site; ipsilateral), and one from the opposite non-painful dermatome (mirror site; contralateral), providing a perfect within-patient reference. For this study, a cohort (n=20 PHN patients) of unprocessed slides was selected based from the previously determined cutaneous innervation densities and the clinical pain intensity scores,

such that this tissue study cohort had similar innervation patterns and pain scores. All of the PHN patients had been diagnosed with clinically significant pain defined as a VAS >3/10. Importantly, for this PHN study, tissue was mounted onto slides utilizing a novel technique to enhance the detection of difference and validity of any immunolabeling results, whereby each slide contained a single row of sections collected from both biopsies (ipsi and contra) from the same patient. Therefore, although only 20 slides (1/patient) were utilized for each biomarker, this study generated results from the analysis of 40 human biopsies. In designing the slides this way, each slide was evaluated as individual patient ipsi/contra measurements, then expressed as a ratio for patient-to-patient comparisons. This type of enriched-subject experimental design, coupled with innovative techniques, builds on the importance of personalized medicine and assures a more consistent result from each immunolabel measure, thereby increasing the validity of the CMA platform technology.

The cutaneous innervation profiles were previously determined in these specimen using the biomarker PGP, which is uniquely expressed at high levels in all types of peripheral innervation and indiscriminately labels all types of sympathetic and sensory innervation, including large caliber, heavily myelinated Aβ fibers implicated as low threshold mechanoreceptors, and small caliber, lightly myelinated Aδ fibers and unmyelinated C fibers, which are implicated in a variety of non-noxious and noxious thermo-, chemo-, mechano-, and polymodal-receptor functions [1]. Previous research from this cohort of tissue has quantified the epidermal and subepidermal innervation for the ipsilateral and contralateral biopsies using NeuroLucida software (MBF Bioscience, Essex, VT) according with ITD-CMA procedures.

REFERENCES

1. Albrecht PJ, Rice FL. Role of small-fiber afferents in pain mechanisms with implications on diagnosis and treatment. *Current pain and headache reports*. 2010;14(3):179-88.

ANTIBODIES UTILIZED:

For five of the nine tissue biomarker antibody targets, INTiDYN tested 3 specific antibodies on separate human tissue to determine the best primary antibody for labeling the entire cohort of study slides. These test slides were evaluated for label intensity and quality among the epidermal keratinocytes and the antibody that provided the best results was subsequently used for the study. The other four biomarkers are utilized routinely in the lab. The following nine primary antibody biomarkers were used to investigate a cohort of innervation-characterized PHN patient skin biopsies:

Antibody	***Species***	***Supplier/Cat no.***	***Dilution***
NMDAR2b	Rabbit anti-rat	Abcam/ab65783	1:500
CACNA1S	Rabbit anti-human	Abcam/ab96413	1:100
Alpha2AR	Rabbit anti-rat	Neuromics/RA14110	1:2,000
$GABA_BR2$	Rabbit anti-human	Abcam/ab52248	1:300
CGRP	Sheep anti-rat	Abcam/ab22560	1:500
CB2	Rabbit anti-human	Abcam/ab150569	1:100
TrpV1	Rabbit anti-human	Abcam/ab3487	1:500
ETA	Rabbit anti-rat	Alomone/AER-001	1:200
Nav1.6	Rabbit anti-rat	Alomone/ASC-009	1:200