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Improved Facial Nerve Identification During Parotidectomy With Fluorescently Labeled Peptide

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Abstract

Objectives/Hypothesis—Additional intraoperative guidance could reduce the risk of iatrogenic injury during parotid gland cancer surgery. We evaluated the intraoperative use of fluorescently labeled nerve binding peptide NP41 to aid facial nerve identification and preservation during parotidectomy in an orthotopic model of murine parotid gland cancer. We also quantified the accuracy of intraoperative nerve detection for surface and buried nerves in the head and neck with NP41 versus white light (WL) alone.

Study Design—Twenty-eight mice underwent parotid gland cancer surgeries with additional fluorescence (FL) guidance versus WL reflectance (WLR) alone. Eight mice were used for additional nerve-imaging experiments.

Methods—Twenty-eight parotid tumor-bearing mice underwent parotidectomy. Eight mice underwent imaging of both sides of the face after skin removal. Postoperative assessment of facial nerve function measured by automated whisker tracking were compared between FL guidance (n = 13) versus WL alone (n = 15). In eight mice, nerve to surrounding tissue contrast was measured under FL versus WLR for all nerve branches detectable in the field of view.

Results—Postoperative facial nerve function after parotid gland cancer surgery tended to be better with additional FL guidance. Fluorescent labeling significantly improved nerve to surrounding tissue contrast for both large and smaller buried nerve branches compared to WLR visualization and improved detection sensitivity and specificity.

Conclusions—NP41 FL imaging significantly aids the intraoperative identification of nerve branches otherwise nearly invisible to the naked eye. Its application in a murine model of parotid gland cancer surgery tended to improve functional preservation of the facial nerve.

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Drs. Timon Hussain and Quyen T. Nguyen had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Keywords

Fluorescence-guided surgery; nerve labeling; facial nerve; parotid gland

INTRODUCTION

Electromyographic (EMG) monitoring techniques, first established more than 4 decades ago,¹ are routinely used by a majority of head and neck surgeons during parotid gland surgery today.² Although EMG monitoring can provide immediate feedback on the proximity of the facial nerve, its limitations include low specificity and its dependence on intact axons that might have already been affected by the disease process.^{3,4} Reported rates of iatrogenic injury to the facial nerve incurred during parotid gland surgeries still range up to 40% for temporary and about 5% for permanent facial dysfunction.^{4–11} Other smaller nerves in the anatomical region, such as the great auricular nerve, are routinely sacrificed to reduce operating time and enable better exposure of the gland despite reported long-term sensory deficiencies.¹² Improved intraoperative visual identification of nerve branches could decrease the incidence of iatrogenic injury and associated morbidity, while also reducing operating time and associated cost. We have previously described the fluorescently labeled peptide NP41, which binds to nerves after systemic administration and aided facial nerve identification during nerve repair surgeries in mice.^{13–15} Compared to retrograde or anterograde tracers, which travel slowly and label a single nerve fiber at a time after local injection,^{16,17} NP41 labels all peripheral sensory nerves and motor pathways within hours after systemic injection. This could be particularly valuable for the identification of nerve branches during surgery, especially if anatomical landmarks are distorted (e.g., in the presence of a large tumor). Therefore, in this study, we first evaluated the intraoperative use of NP41 imaging in a model of murine parotid gland cancer. We compared postoperative facial nerve function following parotidectomy with NP41 fluorescence (FL) guidance compared to white light reflectance (WLR) alone using whisker movement amplitude with a fully automated tracking system.

Second, we aimed to determine the potential benefit of fluorescent labeling for intraoperative identification of smaller nerves in head and neck surgery. Therefore, we also measured sensitivity and specificity, identifying nerves that are on the surface of the face as well as nerves that are buried with other overlying tissue such as muscle using NP41 FL imaging compared to WLR imaging.

MATERIALS AND METHODS

Animals

Twenty-eight female Swiss-Webster mice (Jackson Laboratories, Bar Harbor, ME) served as experimental animals for parotid gland cancer surgeries. Eight female SKH-1 nude albino wild-type mice were used for nerve imaging experiments. Nude SKH-1 mice were used because no removal of potentially autofluorescent hair was necessary prior to imaging, whereas Swiss-Webster mice were used for the surgical experiments because malignant salivary gland cancer cells used in this study originated from this strain. All animal studies

were approved by the University of California San Diego Institutional Animal Care and Use Committee (protocol number S05536).

Fluorescently Labeled Nerve Probe

FAM-NP41 was synthesized as previously described.¹³ The fluorescent dye carboxyfluorescein was conjugated to a C-terminal lysinamide, resulting in Ac-SHSNTQTLAKAPEHTGK (5,6FAM)-amide. Before the surgical procedures, all mice were injected with FAM-NP41 (15 nmol/g body weight) via the tail vein. Imaging and surgical procedures were performed 2.5 hours after injection to allow sufficient wash-out of the probe.

Salivary Gland Cancer Model and Surgery With Intraoperative FL Nerve Imaging

Murine salivary gland cancer cells (SCA-9 clone 15, ATCC CRL-1734; American Type Culture Collection, Manassas, VA) were transfected with firefly luciferase enzyme (Luc2) as described in detail elsewhere.¹⁸ Luc2 is a codon-optimized luciferase gene with an increased expression in mammalian cells; cancer cell detection has been reported to be possible at a single cell level.¹⁹ There were 6×10^6 murine Luc2-transfected salivary gland cancer cells injected into the right parotid gland of every mouse. For the injection, mice were anesthetized, and a small skin incision of about 0.5 cm was made over the parotid gland region after facial hair removal with depilatory cream. Luc2-positive SCA-9 cells were then injected into the gland, and the incision was closed in a single layer.

To confirm the presence of parotid gland tumors, preoperative images were taken on the day of surgery using an in vivo bioluminescence imaging system (IVIS Spectrum; Caliper Life Sciences, Waltham, MA). After a brief inhalational anesthesia with isoflurane, mice were intraperitoneally injected with 150 mg/kg of D-luciferin in phosphate-buffered saline and imaged 10 minutes after injection. The same imaging procedure was performed on the day following tumor removal surgery to assess the completeness of tumor resection.

After a 7-day growth period, tumors were surgically removed. Mice were randomly allocated to one of two groups: FAM-NP41-aided tumor removal surgery (n = 13) or surgery performed under WLR alone (n = 15). For the surgeries, animals were anesthetized. Following facial hair removal with depilatory cream, an infra-auricular skin incision of about 2.0 cm was made and the facial skin was retracted. Surgical removal of the large, invasively growing tumors was performed with FL guidance for facial nerve preservation or under WLR alone, depending on the allocation to the respective experimental groups. For the FAM-NP41-guided surgeries, continuous fluorescent intraoperative imaging was performed. A strong effort was made to preserve the facial nerve and its branches in both groups, while at the same time achieving complete tumor resection; heat cauterization was used when necessary. The skin was closed in a single layer. All procedures were performed by a single surgeon with experience in microscopic surgery.

Postoperative Assessment of Facial Nerve Function by Whisker Movement Analysis

The difference in whisker movement amplitude (defined as the difference between maximal retraction and protraction in degrees) between the operated and healthy side served as an

indicator of the degree of postoperative functional facial nerve injury. Vibrissal pilorector muscles are innervated by the facial nerve,²⁰ and following facial nerve injury, whisker movement is impaired.²¹ To measure whisking, vibrissa motion was recorded in awake nonsedated head-fixed mice, a method which has been previously described.^{15,22–25}

Measurements of whisker movement were performed 2 days after tumor removal surgery to assess nerve function. For measurements of whisker movement, mice were gradually acclimatized to head restraint and immobilization in a plastic tube (Tailveiner restrainer; Braintree Scientific, Braintree, MA). During the recording session, mice were head-fixed using a custom-made head restraining mount. All but one corresponding whisker on the right and left mystacial pad were trimmed down to the skin. Vibrissa motion was monitored with a Basler 602f camera (Basler Inc., Exton, PA). Pixel planar images (360×250) were acquired at 250 Hz with a WL-emitting backlight for trials of 10 seconds each. After video acquisition, vibrissa angle was automatically tracked using custom-written MATLAB software (MathWorks, Natick, MA) by fitting a line to the spatially contiguous pixels comprising the initial 5-mm segment of the vibrissa base. For evaluation of postoperative function, mice were allocated to one of three groups: 0% to 33% versus 34% to 66% versus 67% to 100% movement amplitude on the operated side compared to the injured side.

Nerve Imaging and Intensity Quantification

To assess sensitivity and specificity of NP41 for nerve labeling, eight non-tumor-bearing SKH-1 mice were injected with a nerve probe, and both sides of the face were surgically exposed after anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg). A 2.5-cm infra-auricular skin incision was made, and the facial skin was retracted. Using a customized Olympus FL dissecting microscope (Olympus, Tokyo, Japan), nerve branches in the field of view were imaged with WLR and with FL. Peptide FL was imaged with a light-emitting diode source (450–490 nm excitation and 500–550 nm emission). Videos were acquired for subsequent signal to background ratio analysis.

Using images acquired as described above, for four SKH-1 mice, regions of interest (ROI) on the three main facial nerve branches on corresponding white light (WL) and FL images were selected using the arbitrary shape tool in ImageJ (National Institutes of Health, Bethesda, MD). The mean pixel intensities within the selected areas were compared for individual nerve branches against adjacent muscle tissue. All values were normalized to background signal to take noise into account. Exactly the same ROIs were evaluated on corresponding FL and WL images. Results of signal-to-adjacent tissue ratios for reflectance and FL were compared.

To measure the intensity of small nerve branches within the field of view, for four SKH-1 mice, all visually identifiable nerve branches except the three main facial nerve branches were marked on corresponding WL and FL images by a surgeon blinded to the experimental conditions. For image acquisition, the parotid gland was retracted to unveil small nerve branches located deep and inferior to the gland. The number of visible branches was compared to evaluate whether FL guidance aided the detection of small nerve branches. For every branch detected, signal-to-adjacent tissue ratios for reflectance and FL were quantified

by selecting three corresponding ROIs on FL and WL images per individual nerve branch. All values were normalized to background.

To histologically confirm visually identified structures as nerve tissue, hematoxylin and eosin staining was performed on representative samples. A receiver operating characteristic (ROC) curve analysis was performed to determine sensitivity and specificity of the NP41 FL labeling compared to WL.

Data Analysis

Statistical data analysis was performed using SigmaPlot software (Systat Software Inc., San Jose, CA). An unpaired *t* test was used to compare nerve to surrounding tissue intensities and number of nerves visible under WL versus FL. Postoperative whisker mobility data were compared using a χ^2 test. An ROC analysis was conducted to analyze sensitivity and specificity of NP41.

RESULTS

Parotid Gland Cancer Surgeries

Preoperative in vivo imaging system (IVIS) imaging confirmed the presence of parotid gland tumors in all experimental mice (see Supporting Fig. 1 in the online version of this article). Postoperative imaging with an IVIS for presence of residual tumor showed no remaining bioluminescence signal in 15/15 mice from the WL group and 13/14 mice from the FL group. One mouse in the FL group showed a slight remaining bioluminescence signal in the parotid region postoperatively, potentially indicating minimal remaining tumor.

Intraoperative FL guidance increased nerve to surrounding tissue contrast and helped to accurately identify the facial nerve during parotid gland cancer removal surgery. Particularly where the facial nerve was encapsulated by tumor tissue, fluorescent labeling aided nerve identification through overlying tissue (Fig. 1). The use of both FL and WLR imaging increased the duration of the procedure by 27% (26.3 ± 3.2 minutes for FL versus 20.7 ± 4.0 minutes for WLR, $P = .007$).

Postoperative analysis of whisker movement amplitude showed that facial nerve function was slightly improved after FL-guided parotid gland cancer surgery compared to surgery performed under WLR alone (Table I), although the functional difference was not statistically significant (χ^2 test, $P = .31$), most likely due to limited sample size.

Postoperatively, no adverse effects of NP41 were observed, in line with prior experiments.^{13–15}

Improved Facial Nerve Contrast With FL Versus WL Nerve Imaging

Intraoperative fluorescent labeling significantly increased nerve to surrounding tissue contrast for nerve branches in the head and neck. Contrast was enhanced by FL compared to WL imaging for nerves located on the surface, and particularly under overlying tissue where small nerve branches were mostly invisible under WLR.

For the three main branches of the facial nerve, nerve branch to surrounding tissue contrast was increased 2.86-fold (4.13 ± 9.08 for FL vs. 1.44 ± 4.11 for WL, $P = .005$) (Table II) in this experiment, which is in line with prior experiments with NP41. The average nerve diameter for those branches was 0.2 mm in our model. Although the main facial nerve branches uncovered by tissue were visible under WLR alone, their visual identification was improved by FL guidance.

The surgically exposed side of the face was explored for further nerve branches. With FL guidance, 7.50 ± 1.60 small nerve branches were visually identified in the field of view in addition to the three main facial nerve branches by blinded study personnel versus 3.25 ± 0.89 under WL ($P < .0001$, $n = 8$) (Fig. 2). The average nerve diameter for those branches was 0.05 mm. Nerve branches that were visually identifiable under WLR were located on the surface, whereas FL imaging enabled the identification of additional branches through overlying tissue, such as muscle, fat, or connective tissue. On average, for small nerve branches, FL guidance improved nerve to surrounding tissue contrast 2.05-fold (2.08 ± 1.29 for FL vs. 1.07 ± 0.52 for WLR, $P = .002$). Nerve to surrounding tissue contrast of 1.07 under WLR (i.e., a contrast enhancement of merely 7%) illustrates that small nerve branches were hardly distinguishable from surrounding tissue. For those branches, particularly if buried under tissue, FL guidance substantially improved visual identification.

We confirmed by histological analysis that fluorescently labeled structures were nerves, particularly in samples where nerves were identified under FL guidance but were not visible under WLR.

Improved Identification Accuracy With FL

ROC analysis was performed to evaluate whether improved nerve contrast with FL guidance also led to improved identification accuracy.

For the main facial nerve branches located on the surface, the area under the curve (AUC) was increased with FL (0.90 vs. 0.80 for WLR, $P < .001$) emphasizing the value of increased contrast with intraoperative FL guidance even for branches visible under WLR. Added FL guidance significantly increased detection sensitivity and specificity (Fig. 3A).

For the smaller nerve branches identified in the field of view, the AUC was 0.79 for nerve identification with FL versus 0.48 with WL ($P < .001$) (Fig. 3B). ROC analysis showed that visual identification of small and buried nerve branches with WLR did not provide sufficient sensitivity and specificity to ensure reliable nerve identification intraoperatively. FL guidance significantly increased identification accuracy of small and buried nerve branches.

DISCUSSION

Although 15% to 30% of parotid gland cancer patients already exhibit facial nerve palsy at the time of presentation,^{26,27} indicating tumor involvement of the nerve and warranting sacrifice of the involved segment,^{28,29} in the majority of cases the nerve is not yet infiltrated by malignant cells. Here it is essential to preserve as much nerve as possible during surgery. Currently, EMG monitoring is the only tool available to aid intraoperative identification.

With regard to future clinical translation of the previously described nerve labeling peptide NP41, in this study, we determined whether intraoperative nerve visualization with FL would affect postoperative nerve function following parotid gland cancer surgery. We showed that added intraoperative FL guidance tended to improve postoperative facial nerve function without achieving statistical significance in a mouse model, as assessed by postoperative ipsilateral whisker mobility. Because the surgeon could not be blinded as to whether FL guidance was used or not, all mice underwent pre- and postoperative imaging to ensure completeness of tumor resection in both groups. Imaging results showed a complete resection in nearly all mice from both groups, indicating a lack of experimenter bias.

The observed functional improvement in the FL versus WL group is encouraging, although not statistically significant, most likely due to limited sample size. A caveat that limits this allograft model is that although we intentionally placed the parotid carcinoma around the proximity of the facial nerve, which impedes visualization of the nerve, once the parotidectomy begins, the tumor typically can be removed off the nerve with a clean dissection plane, resulting in functional preservation. This is in contrast to malignant parotid neoplasms in patients in which a high percentage of the tumor invades the nerve, complicating nerve dissection. Ideally, future studies will focus on comparing functional results in mice with tumor involvement of the lower branch, which is mainly responsible for whisker mobility. Particularly when assessing long-term recovery, recent findings showing evidence of non-facial nerve-mediated whisker movement after chronic denervation by reinnervation from axons within the infraorbital branch of the trigeminal nerve need to be taken into consideration.³⁰ Finally, in rodents, the anatomical course of the facial nerve is less complex than in humans, where it separates the superficial and the deep lobe of the parotid gland. Therefore, identification can be more challenging in humans than in our animal model, particularly in the presence of a large tumor, suggesting that additional intraoperative guidance may be more beneficial. Also, although permanent facial nerve paralysis after parotid gland cancer surgery is not common, its consequences are highly debilitating to patients, warranting all efforts to improve nerve preservation during surgery. Intraoperative EMG monitoring is routinely used during parotid gland cancer surgery today despite a lack of randomized prospective studies demonstrating its benefit.^{5,31} Additional FL guidance could potentially further reduce the rate of iatrogenic injury by complementing today's technology. Although EMG monitoring has the potential to indicate relative proximity of nerve branches with high sensitivity, FL imaging improves visualization when dissecting in immediate proximity to the nerve, where EMG monitoring does not provide any added benefit. Also, as we have previously shown, transected nerve branches are also reliably labeled by NP41 many months after injury, due to uptake in the perineurium/epineurium, whereas EMG monitoring depends on intact axons.¹⁵ Axons may be compromised, particularly in the presence of a large proximally situated tumor.

In the second part of this study, we found that smaller nerves (less than 0.05 mm) and buried nerve branches in the operative field were more visible with FL guidance due to improved contrast to surrounding tissue compared to WL. Notably, nerve to surrounding tissue contrast for those branches was only 1.07 under WL, making them practically indistinguishable from the surrounding tissue with the naked eye. Small nerve intensity

compared to surrounding tissue was increased by more than two-fold with FL guidance, and ROC analysis showed that high levels of sensitivity and specificity were attainable.

It is important to clarify that the small nerve branches imaged in this study were at least an order of magnitude smaller than surgically relevant human facial nerve branches, and their functional relevance in the mouse model remains unclear. Although facial nerve diameters in humans increase with age, even in young children they are substantially larger than in our model.³² Nevertheless, intraoperative visual identification can be tedious. Surgeons have to rely primarily on their knowledge of the anatomy, which may be substantially altered in the presence of a tumor, and excessive searching may damage other tissues. Therefore, iatrogenic injury to smaller nerve branches, such as the great auricular nerve, is common during head and neck surgery. Although functional consequences may not be as debilitating as those caused by damage to larger nerves in the head and neck, postoperative quality of life can be improved by preservation of small nerves.^{12,33,34} Importantly, we were able to show significantly improved small nerve visualization with FL through thin layers of overlying tissue. Covered small nerve branches were mostly not visible at all under WL; therefore, this particular finding may have substantial value in the more complex human anatomical setting, even for larger nerve branches, particularly where the anatomy has been distorted.

Regarding further future applications of NP41, the feasibility of improved intraoperative visualization of very small nerves is also highly intriguing for labeling of nerves outside the field of head and neck surgery (e.g., during radical prostatectomy). Cavernosal nerves cannot be visualized with the naked eye and are therefore oftentimes resected during surgery, with highly debilitating consequences for affected patients.³⁵ The broad potential field of application of NP41 should help to promote clinical translation of NP41.

CONCLUSION

We show that fluorescent facial nerve labeling with NP41 aids intraoperative facial nerve identification during parotid gland cancer surgery. This may improve postoperative functional outcome, but larger studies are needed to confirm this finding and assess the potential clinical benefit. The identification of small and buried nerve branches, which are barely visible under WL, is significantly improved by NP41 with high sensitivity and specificity, potentially facilitating their intraoperative identification and preservation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Intraoperative nerve visualization during parotid gland cancer surgery. Prior to tumor removal (stippled line indicating tumor extent), the main branch of the facial nerve is covered by tissue and barely distinguishable under white light (A, arrows pointing to nerve). Fluorescent labeling improves nerve visualization (B). After tumor removal with fluorescence guidance, the intact nerve is shown under white light (C) versus with fluorescence (D). Scale bars: 1 mm.

Fig. 2.

Improved visualization of small nerve branches with fluorescence labeling. Intraoperative view of the left side of the face. Under white light (A), three small nerve branches can be visually identified aside from the three main branches of the facial nerve (yellow arrows pointing to visible nerve branches). With fluorescent labeling (B), four additional small and buried nerve branches (red arrows) can be identified, potentially enabling their preservation during surgery. Scale bars for A and B: 1 mm. Histological analysis was performed to confirm the presence of nerve tissue. Tissue was cut along the stippled line (C), and presence of nerve was confirmed histologically (black arrows pointing to nerves in C, scale bar: 200 μm). Higher magnification image (D, scale bar: 50 μm) of stippled outline in C, shows the presence of a small nerve branch detectable with fluorescence but not under white light reflectance.

Fig. 3.

Improved identification accuracy with fluorescence nerve labeling. Receiver operating characteristic analysis showing significantly improved identification accuracy for the main facial nerve branches (A) and small and buried nerve branches (B) with fluorescence (FL, black line) versus white light (WL, red line). $P < .001$ for both curves.

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TABLE I

Postoperative Whisker Movement Analysis.

Postoperative Whisker Movement Amplitude (% of Contralateral Side)	Degree of Functional Deficit	NP41 (n = 13)	%	WL (n = 15)	%
0%–33%	Complete to severe	1	7.7	3	20.0
34%–66%	Moderate	3	23.1	4	26.7
>66%	Mild to none	9	69.2	8	53.3

Postoperative whisker movement tended to be improved after surgery with NP41 guidance versus surgery under WL alone.

WL = white light

TABLE II

Fluorescence Labeling Enhances Nerve Contrast.

	Main Facial Nerve Branches (Nerve Intensity/Adjacent Tissue Intensity $\pm$ SD)	Small and Buried Nerve Branches (Nerve Intensity/Adjacent Tissue Intensity $\pm$ SD)
Fluorescence	4.13 $\pm$ 9.08	2.08 $\pm$ 1.29
White light	1.44 $\pm$ 4.11	1.07 $\pm$ 0.52
<i>P</i> value	.005	.002

Relative nerve intensity was significantly increased for large nerve branches (i.e., facial nerve branches), as well as smaller nerve branches in the field of view with fluorescence compared to white light imaging.

SD = standard deviation.