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Assessment of Fine Needle Aspiration Feasibility and Specimen Adequacy for Molecular Diagnostics of Benign Vocal Fold Lesions

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Abstract

Objectives/Hypothesis—The use of molecular testing is becoming more significant for the diagnosis and classification of disease. The application of fine-needle aspiration (FNA) biopsy as the means of sampling lesions in union with molecular testing could be a powerful combination in laryngology. The objectives of this study were to investigate 1) if FNA was feasible to sample benign vocal fold lesions; 2) if FNA samples provided sufficient RNA quality for molecular analysis; and 3) if gene expression of FNA samples matched paired surgical excised specimens.

Study Design—Prospective cross-sectional.

Methods—Fifteen vocal fold specimens were obtained from adult patients undergoing routine surgical removal for benign vocal fold lesions using FNA and surgical excision. Comparisons were made between FNA and excision biopsies for RNA quality. Correlative analysis was completed for RNA expression of nine genes, including decorin (DCN), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), collagen type VI alpha 3 (COL6A3), superoxide dismutase 1 (SOD1), glutathione S-transferase (GST2), collagen type I alpha 2 (COL1A2), ATP binding cassette (ABC), and procollagen I alpha 1 (COL1A1).

Results—FNA and excision samples demonstrated similar RNA quality ($P > 0.05$). Per gene expression, four out of nine genes were moderately correlated between the paired samples ($P < 0.05$).

Conclusions—FNA of the vocal fold lamina propria is technically feasible to perform. Further improvement in the FNA technology is desirable to optimize RNA quality for reliable gene expression analysis.

Keywords

Fine needle aspiration; vocal folds; benign lesions; gene expression

INTRODUCTION

Differential diagnosis of benign vocal fold lesions, such as nodules, polyps and cysts, remains a challenge in laryngology. The phenotype of benign lesions are similar and their nomenclature is yet to be standardized.¹ At the same time, various treatment responses are indicated among the lesions. Nodules are generally more responsive to voice therapy, whereas some (e.g., polyps or cysts) require surgical intervention. An accurate diagnosis of benign vocal fold lesions is needed for a more effective clinical management and to spare patients from unnecessary and costly treatments. Although the diagnostic criteria for benign vocal fold lesions have advanced since the publication of Classification Manual for Voice Disorders-I (CMVD-I),² the majority of the classification criteria is based on subjective evaluation of the macroscopic appearance of benign lesions. While clinical pathology has made significant advancements in the diagnosis of many diseases using molecular diagnostics without the need of surgical biopsy, such precedent has not progressed in laryngology. Existing techniques of obtaining diagnostic specimens require an excisional biopsy of the vocal folds for clinical pathology. However, such biopsy is generally performed to discriminate epithelial disease from stromal or lamina propria changes; that is, malignant versus benign pathology at best. Current pathological techniques do not comment on the few cells and the alteration of extracellular matrix composition present in the histologic evaluation of the lamina propria, as featured in benign lesions. Further, any intentional excision of vocal fold tissue could cause tissue scarring to the vocal fold mucosa. Vocal fold scarring causes a deformity of the vocal fold edge, a disruption of the viscoelastic properties of the vocal fold tissue, increased stiffness, and glottal incompetence resulting in dysphonia.³ Decreasing the invasiveness of vocal fold biopsy would be an obvious improvement in minimizing the risk of scarring. We here propose that the use of fine needle aspiration (FNA) could meet this need.⁴

The advent of cytologic examination from over a decade ago that used FNA has revolutionized diagnostic protocols for major diseases such as thyroid disease,⁵⁻⁷ head and neck cancer,^{8,9} and parotid disease.¹⁰ One major application is to differentiate if palpable soft tissue lesions (e.g., thyroid nodules) are benign or malignant.^{5,6} In these diseases, diagnosis is made based on cellular examination of a small number of cells, as the diseased cells are either morphologically different (i.e. a different size or shape from normal) or could be identified by cell markers. FNA employs a thin, hollow needle that is placed into the target tissue, and cells are drawn up into the needle core. These cells are then spread onto a slide and a diagnosis is made. Generally hundreds of cells from the target tissue are obtained through FNA. Because cells in benign vocal fold diseases are not unlike normal cells, cytologic examination is not very helpful for differential diagnosis of benign lesions. Cells extracted via FNA, on the other hand, could be used to obtain molecular profiles of benign diseases. Molecular profiles have been utilized to establish profiles among diseases with similar phenotypes, namely multiple sclerosis,^{11,12} diffuse large B-cell lymphoma,¹³⁻¹⁶ acute leukemia,¹⁷ melanoma,¹⁸ and breast cancer.¹⁹ We proposed that such establishment of patterns of gene transcript expression can be accomplished for benign vocal fold lesions as well.^{20,21} Utilization of FNA, coupled with gene expression profiling, would allow for differential diagnosis without excisional biopsy, providing a new tool for diagnosis,

treatment, and prognosis of benign vocal fold lesions. The objectives of this study were 1) to determine the technical feasibility of using FNA for vocal fold specimens from benign lesions; 2) to measure RNA quantity and quality of FNA samples; and 3) to compare transcript profiles of vocal fold cells obtained through FNA, versus those obtained during surgical biopsy.

MATERIALS AND METHODS

Subjects and Sample Procurement

Twelve patients, aged 24 to 45, were recruited from the clinical population at the University of Wisconsin–Madison Voice and Swallow Clinic and Division of Otolaryngology–Head and Neck Surgery. The study was approved by the Institutional Review Board at the University of Wisconsin–Madison. Participants were identified as candidates if they were scheduled for microlaryngoscopic excision of a benign vocal fold lesion, namely nodule, polyp, cyst, or Reinke edema. Lesions that were hemorrhagic were excluded because blood in the lesion might confound the cell mix for the microarray. During the procedure to excise the vocal fold lesion, two FNAs were performed of the benign lesion under direct microlaryngoscopic view through a laryngo-scope, by means of a 26-gauge bayonet aspiration needle (Medtronic-Xomed MicroFrance, Jacksonville, FL) attached to a 3-ml syringe. Care was taken to perform needle placement in the line of the proposed microflap incision to limit trauma. Bilateral lesions were indicated in three of the patients, and thus a total of 15 paired FNA and excised tissue samples were obtained for this study. A portion of the excised lesion was sent for standard histopathology, as per current standard of care and a portion for molecular analysis. Excised lesions and FNA samples were separately stored immediately in RNeasy Lysis Buffer (Qiagen, Valencia, CA) and then transferred to minus 80°C until processing. Feasibility of the FNA procedure was noted at the time of surgery.

RNA Extraction, Quantity and Quality Tests

Total cellular RNA was extracted from both the FNA and excision samples using an RNeasy Micro Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. RNA quantity (ng/nl) and quality (A260/280 ratio) were determined for every RNA sample by spectrophotometric analysis using a Nanodrop 1000 (Thermo Scientific, Barrington, IL). In addition, RNA quality of these samples were further evaluated using Agilent 2100 Bioanalyzer. Onboard software of Agilent 2100 computes an RNA Integrity Number (RIN) based on the full electrophoretic trace generated during analysis of the RNA species, as opposed to focusing solely on the 18s and 28s rRNA bands. RIN ranges from 1 (totally degraded RNA) to 10 (completely intact RNA) as an estimate of the extent of degradation of the RNA sample.

Gene Expression Analysis

Real-time quantitative PCR (qRT-PCR) was performed using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics Corporation, Indianapolis, IN) to quantify mRNA expression of the genes of interest, with amplification of GAPDH as control. Copy DNA (cDNA) was synthesized from 2 lg of total RNA by using a QuantiTect Reverse Transcription Kit (Qiagen, CA). The mRNA from the cDNA sample was amplified with

specific primer pairs for decorin (DCN), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), collagen type VI alpha 3 (COL6A3), superoxide dismutase 1 (SOD1), glutathione S-transferase (GST2), collagen type I alpha 2 (COL1A2), ATP binding cassette (ABC), procollagen I alpha 1 (COL1A1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These genes were chosen as they have been previously shown to be differentially expressed between benign vocal fold lesions.²⁰ Primer sequences, gene bank access number, and expected PCR product sizes are in Table I. Real time quantitative PCR was performed in 20 µL reactions containing 50 ng of cDNA as template, using a LightCycler 1.5 (Roche Diagnostics Corporation, Indianapolis, IN), as previously described.²⁰ Negative controls with water instead of template and initial mRNA were included in each run to rule out DNA and genomic DNA contamination. The exact amplification efficiencies of target and reference genes were assessed by LightCycler software (version 4.0) before any calculation of the normalized gene expression was completed. Results were shown by the fold of control (polystyrene or Matrigel) of target gene mRNA concentration (ng/l) normalized by housekeeping gene GAPDH mRNA (ng/l). Each individual sample was tested in duplicate.

Statistical Analysis

Nonparametric Wilcoxon signed ranked tests were performed to compare the RNA quality between FNA and excision samples. Spearman's rank correlation was used to evaluate the correspondence of gene levels between FNA and excision samples. The overall α -level for these tests was set at 0.05.

RESULTS

Feasibility

The FNA procedure was noted to be straightforward to perform and no additional trauma to the surgical site was noted that prevented successful completion of the operation.

RNA Quantity and Quality

Table II lists patient information, as well as RNA quantity and quality of individual FNA and excision samples. All 15 FNA samples, except for eight (53.5%), matched excision samples and had sufficient RNA for quantity and quality evaluation and transcript analysis. Thus, the eight paired FNA and excision samples were selected for further analysis. For these paired samples, mean ($\pm$ SE) RNA quantity was 6.64 ($\pm$ 1.88) ng/nl and 75.3 ($\pm$ 37.53) ng/nl for FNA and excision samples respectively. RNA quality (A260/280 ratio) was satisfactory (mean [$\pm$ SE]: FNA: 2.60 [$\pm$ 1.01]; excision: 2.00 [$\pm$ 0.03]), indicating minimal protein contamination in RNA samples. No significant difference was found between the paired FNA and excision samples in RNA quality ($P = 0.31$). RNA quantity was significantly different due to the differences of sample size between FNA and excision samples ($P = 0.04$). Regarding RNA quality of FNA samples using Bioanalyzer (Table III), two FNA samples had above zero RIN (RNA integrity number), indicating most RNA extracted were degraded. Despite that, transcript expression of FNA samples were detected from FNA samples.

Transcript Expression in Paired FNA and Excision Samples

Transcript analysis was performed on eight pairs of FNA and excision samples (Fig. 1). Four out of nine genes showed significant moderate to strong association ($\rho = 0.64\text{--}0.84$) between FNA and excision samples (Table IV). Five genes, CTGF, GST2, COL1A2, ABC, and COL1A1, showed weak yet nonsignificant association. Out of these five genes, no particular pathology was indicated as outliers leading to weak correlation. From the FNA samples, differential patterns of gene expression were observed (Fig. 2). Only qualitative analysis was carried out herein due to small and unequal samples size across pathologies (nodules $n = 3$; polyps $n = 7$; cyst $n = 4$; Reinke edema $= 1$). In general, nodules were characterized by high expression of COL1A1. Polyps were characterized by high expressions of both COL1A2 and COL1A1. Cysts were characterized by high expression of COL1A2, COL1A1, and SOD1. Reinke edema was characterized by high expression of multiple genes, including COL1A2, COL1A1, SOD1, DCN, and GST2.

DISCUSSION

FNA is a simple and safe procedure that has been performed in the office setting for differential diagnosis of breast disease,²² thyroid, and parathyroid diseases.⁵ The proposed FNA biopsy method offers the potential to be much less invasive, with minimal damage for multiple sampling of vocal fold tissue. Pathologists currently categorize lesions based on the morphology of the cells or the tissue under a microscope. However, the histopathologic appearance of the specimen only allows distinguishing benign from malignant vocal folds and does not inform gene and protein activity underlying a disease. Molecular diagnostics, on the other hand, measures levels of genes and proteins or specific mutations, which increases the accuracy of diagnosis and prognosis with high sensitivity and specificity. Molecular markers, as obtained via FNA shown in this study, would open our existing diagnostic ability of vocal fold diseases from merely distinguishing benign versus malignant to subtyping phenotypically similar benign vocal fold lesions. The FNA data will also be useful for researchers to identify the genetic sources of population variations in the susceptibility to diseases, severity of illness, as well as response to treatments. For example, by comparing genetic profiles of patients who are responsive to voice therapy versus those are not, we will be able to identify if specific gene expression patterns are associated with individual differences in treatment response through genotype-phenotype mapping.

In this study, we demonstrated that acquisition of in vivo vocal fold samples is feasible by endoscopic-assisted FNA in the operating room. We also examined if the gene expression of FNA samples corresponded to those of the excised tissue, with potential for both diagnostic and investigative use. Both FNA and excision samples yielded similar quality of RNA. Analysis of gene expression data indicated that four of nine genes (DCN, VEGF, COL6A3, SOD1) were moderately correlated.

Due to the unequal and small sample size for the pathology obtained, it is not feasible to evaluate whether the strength of the correlation was related to the type of pathology. At the same time, qualitative analysis of FNA gene expression showed distinctive patterns of gene expression across the pathologies. In concordance with our previous findings,²⁰ SOD1 and GST2 were over-expressed in Reinke edema. These two genes were expressed in cells to

protect them from oxidative stress such as smoking or reflux. In this study, cysts were also characterized by SOD1, suggesting that patients with cysts might share certain pathogenesis with Reinke edema. Polyps were found to have high expressions of both COL1A2 and COL1A1, whereas nodules were mainly characterized by COL1A1 only. COL1A2 and COL1A1 is collagen type I alpha 2 and its precursor pro-collagen I alpha 1 respectively. Both of these collagen proteins are secreted by fibroblasts. Results suggested that an ongoing fibroblast-mediated repair process underlies the formation of vocal fold nodules and polyps with excessive collagen accumulation. Our results also provided insights in a long-standing argument in the field of laryngology—whether nodules and polyps are in a continuum of chronicity of the lesions. Histopathologic results suggested that nodules are younger lesions than polyps.^{23,24} We propose that collagen might be a differentiating marker for polyps and nodules, two clinically similar pathologies. A full microarray analysis will confirm the aforesaid postulations, and such investigation is now ongoing in our laboratory.

Of interest, we demonstrated that more FNA samples than excision samples had sufficient RNA for subsequent quality and transcript analysis. The finding is counterintuitive because excision samples are usually expected to be larger in size, and thus more RNA to be extracted. One possibility is that part of the tissue was aspirated during FNA, preceding the excision procedure in this research design. As a result, less tissue become available for the excision sample than the FNA sample. Subsequent analyses indicated that RNA had degraded in most FNA samples. Degradation of RNA from FNA samples have been currently reported from FNA of other soft tissue such as pancreas,²⁵ skin,²⁶ breast,²⁷ and lung.²⁸ Although degraded RNA may affect the accuracy and sensitivity of transcript measurements, studies also indicated that moderately degraded RNA samples could still give reliable qRT-PCR results with the selection of short target amplicons (<100bp) and proper normalization to a housekeeping gene.^{25,29,30} Although the amplicons used were between 189bp and 311 bp, we were able to measure transcript expression from FNA samples successfully using qRT-PCR, and data were normalized to the housekeeping gene GAPDH. Long amplicons were opted for this study because of attempts to protect the specificity of the genes to be amplified and measured at this initial stage of work. Future work will optimize the length of amplicons for the specificity and sensitivity of gene expression from FNA samples.

Further, RNA degradation in FNA samples was shown to be dependent on needle length and bore diameter.²⁵ A short, large bore (18-gauge) needle were shown to have higher RNA quantity and quality in FNA aspirates than with a standard fine needle (22-gauge). Long needles, however, are necessary to pass through the laryngoscope to reach the larynx. The bore diameter used was 26-gauge in this study. Our previous work showed that a 26-gauge needle was feasible for obtaining vocal fold specimens without causing structural injury and biomechanical deterioration in a rabbit model.³¹ Increasing the needle diameter will likely increase RNA quantity and quality; however, the likelihood of inducing an inflammatory response might also increase as well. Further investigation will be identifying the optimal needle diameter, allowing satisfactory RNA quantity and quality with minimal risk of complications.

CONCLUSION

In this study, we demonstrated the feasibility of attaining in vivo vocal fold specimens by FNA in an operative setting. FNA samples provided sufficient RNA material to generate transcript data that were comparable to those from excision tissue samples. Ongoing work is presently being completed to advance FNA technology to make it feasible for office-based diagnostic evaluations and to minimize RNA degradation for high-quality FNA procurement.

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Fig. 1.

Gene expression levels (mRNA, log scale) of nine genes for fine needle aspiration (FNA) and excised tissue samples. Each gene was normalized by housekeeping gene, GAPDH mRNA. The bars and the error bars represent the mean and the standard errors of the data representatively.

Fig. 2.

Gene expression levels (mRNA, log-log scale) of nine genes for each pathology from fine needle aspiration (FNA) samples. Each gene was normalized by housekeeping gene, GAPDH mRNA. The bars and the error bars represent the mean and the standard errors of the data representatively. RE = Reinke edema.

TABLE 1

Primer Sequences and Products of RT-PCR.

Gene	Forward Primer	Reverse Primer	Size of PCR Product (bp)
DCN	5'-gatgcagctagcctgaaagg-3'	5'-tcacaccgaataagaagcc-3'	274
CTGF	5'-ggcttaccgactggaagaca-3'	5'-tgagattttgggagtagcg-3'	251
VEGF	5'-ctacctccaccatgccaagt-3'	5'-tctctctatgtgctggcct-3'	311
COL6A3	5'-attcgagagaactgcctgga-3'	5'-gaagaaggagtcagccatcg-3'	189
SOD1	5'-tgcccgatgtgtctattgaa-3'	5'-gggcctcagactacatcaa-3'	217
GST2	5'-atcgctactccctgccttt-3'	5'-cactgtgggcaggttactga-3'	194
COL1A2	5'-acattggcccagctgttttc-3'	5'-agcgggggaaggagtaaat-3'	189
ABC	5'-cctttcactccctccatca-3'	5'-acaggtcttgagctggaga-3'	185
COL1A1	5'-gttggtgctaagggtgaagc-3'	5'-accagtgtctccttgctgc-3'	294
GAPDH	5'-accagaagactgtggatgg-3'	5'-tgagcttgacaaagtgtcg-3'	382

TABLE II

RNA Quantity and Quality of FNA and Tissue Biopsies Using Nanodrop.

Sample ID	Age	Gender	Pathology	RNA Quantity (ng/nl)		RNA Quality (260/280)	
				FNA	Excision	FNA	Excision
1	31	F	Cyst	22.20	-	1.60	-
2	31	F	Nodule	1.75	4.99	8.63	2.07
3	35	F	Polyp	23.10	-	1.39	-
4	45	M	Polyp	2.56	-	2.06	-
5	31	M	Polyp	8.80	17.16	1.47	1.88
6	37	M	Polyp	1.80	184.13	1.01	2.08
7	40	F	Cyst	9.40	-	1.46	-
8	47	M	Polyp	8.90	-	1.68	-
9	21	F	Cyst	5.00	19.42	1.58	1.91
10	38	M	Polyp	11.80	7.80	1.69	1.98
11	38	M	Polyp	5.80	-	1.79	-
12	42	F	Reinke's edema	3.20	251.19	2.31	2.09
13	24	F	Cyst	16.30	-	1.40	-
14	24	F	Nodule	14.10	42.42	1.42	2.02
15	24	F	Nodule	18.60	-	1.55	-
Mean (standard error) for paired samples				6.64 (1.88)	75.3 (37.53)	2.60 (1.01)	2.00 (0.03)

FNA = fined needle aspiration.

TABLE III

RNA Quality of FNA Biopsies using Bioanalyzer.

Sample ID	Gender	Pathology	RNA Quality (RIN)
1	F	Cyst	0.9
3	F	Polyp	0
8	M	Polyp	2.7
10	M	Polyp	0
11	M	Polyp	0
13	F	Cyst	0
14	F	Nodule	0
15	F	Nodule	0
Mean (standard error)			0.45 (0.34)

TABLE IV

Spearman's Rank Correlation of Gene Expression Between Paired FNA and Excision Samples.

Gene	Spearman's Rho
DCN	0.66*
CTGF	0.46
VEGF	0.84*
COL6A3	0.64*
SOD1	0.82*
GST2	0.50
COL1A2	-0.20
ABC	0.44
COL1A1	-0.04

*
P < 0.05.