

ECM1 and Tmprss4 Are Diagnostic Markers of Malignant Thyroid Neoplasms and Improve the Accuracy of Fine Needle Aspiration Biopsy

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Objective: The objective of this study was to determine whether genes that regulate cellular invasion and metastasis are differentially expressed and could serve as diagnostic markers of malignant thyroid nodules.

Summary and Background Data: Patients whose thyroid nodules have indeterminate or suspicious cytologic features on fine needle aspiration (FNA) biopsy require thyroidectomy because of a 20% to 30% risk of thyroid cancer. Cell invasion and metastasis is a hallmark of malignant phenotype; therefore, genes that regulate these processes might be differentially expressed and could serve as diagnostic markers of malignancy.

Methods: Differentially expressed genes (2-fold higher or lower) in malignant versus benign thyroid neoplasms were identified by extracellular matrix and adhesion molecule cDNA array analysis and confirmed by real-time quantitative polymerase chain reaction (PCR). The area under the receiver operating characteristic (AUC) curve was calculated to determine diagnostic accuracy of gene expression level cutoffs established by logistic regression analysis.

Results: By cDNA array analysis, ADAMTS8, ECM1, MMP8, PLAU, SELP, and Tmprss4 were upregulated, and by quantitative PCR, ECM1, SELP, and Tmprss4 mRNA expression was higher in malignant (n = 57) than in benign (n = 38) thyroid neoplasms ($P < 0.002$). ECM1 and Tmprss4 mRNA expression levels were independent predictors of a malignant thyroid neoplasm ($P < 0.003$). The AUC was 0.956 for ECM1 and 0.926 for Tmprss4. Combining both markers improved their diagnostic use (AUC 0.985; sensitivity, 91.7%; specificity, 89.8%; positive predictive value, 85.7%; negative predictive value, 82.8%). ECM1 and Tmprss4 expression analysis improved the diagnostic accuracy of FNA biopsy in 35 of 38 indeterminate or suspicious results. The level of ECM1 mRNA expression was higher in TNM stage I differentiated thyroid cancers than in stage II and III tumors ($P \leq 0.031$).

Conclusions: ECM1 and Tmprss4 are excellent diagnostic markers of malignant thyroid nodules and may be used to improve the diagnostic accuracy of FNA biopsy. ECM1 is also a marker of the extent of disease in differentiated thyroid cancers.

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The incidence of thyroid cancer has increased over the last 3 decades and approximately 24,000 new cases of thyroid cancer occurred in 2004.¹ The incidence of thyroid nodules is even higher as a result of greater use of more sensitive imaging studies.^{2,3} Approximately 10% of the U.S. population will develop a significant thyroid nodule during their lifetime.⁴ Fine needle aspiration (FNA) biopsy has reduced the need for performing diagnostic thyroidectomies to rule out malignant thyroid neoplasms,^{5–10} but may show indeterminate or suspicious cytologic features in 15% to 20% of all thyroid nodules.^{5–7} This limitation is commonly the result of the overlapping cytologic features between benign (hyperplastic nodules, follicular adenoma, Hürthle cell adenoma) and malignant (follicular cancer, Hürthle cell cancer, the follicular variants of papillary cancer) follicular thyroid nodules.^{5,6} Because the risk of malignancy is approximately 20% for follicular and Hürthle tumors and approximately 50% in nodules suspicious for papillary thyroid cancer, thyroidectomy is generally the recommended treatment. Unfortunately, none of the preoperative clinical (age, sex, solitary vs multiple nodules), imaging (tumor size, nodule ultrasound characteristics), and cytologic (atypia, mitotic index) factors studied so far are accurate enough to determine which patients with suspicious or indeterminate FNA cytologic findings should undergo thyroidectomy.^{11–14}

Several genetic alterations (RET/PTC rearrangements, PAX8-PPAR γ , BRAF) and molecular markers (galectin-3, telomerase, lactoferrin, CD44, CK19, MET, HMBE-1, p21, p27, DAP4, HMG1, TTF-1, TPO) have been studied to help distinguish benign from malignant thyroid neoplasms.^{15–39}

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RET/PTC rearrangements, BRAF mutations, and MET overexpression are present mostly in papillary thyroid cancer and cannot discriminate benign from malignant follicular thyroid tumors.^{30,31,40–44} Although the initial report that identified the PAX8-PPAR γ fusion gene suggested that this chromosomal translocation primarily occurred in follicular thyroid cancer, subsequent studies have shown it to be present in up to 55% of follicular adenomas.^{35,45,46} Similarly, TPO, TTF1, p53 mutations, p21, and telomerase expression analysis have not been found to discriminate reliably between benign and malignant follicular tumors.^{47–52} Positive immunostaining for CK19, S-100 protein, HMBE-1, galectin-3, and p27 is helpful for identifying papillary thyroid cancer and its follicular variant.^{53–59} However, false-positive and false-negative results for these markers occur because there is considerable overlap in gene expression levels, especially in thyroid glands with lymphocytic thyroiditis.^{60–65}

cDNA array expression analysis, which has been used to analyze several human cancers, allows the correlation of gene expression profile with clinical variables, the classification or definition of different tumor types, and the identification of genes or networks of genes involved in carcinogenesis.^{66–69} cDNA array studies of thyroid cancer have identified 47 to 627 genes that are differentially expressed and distinguish benign from malignant thyroid neoplasms with a sensitivity of 87.5% to 93.0% and a specificity of 87.1% to 100%.^{23,24,26,70} However, these genes are variable and only a few studies of a limited number of genes confirmed the gene expression levels by RT-PCR.^{16,70} This is important given that some investigators have suggested that cDNA array analysis may incorrectly identify 30% of genes and gene expression levels.⁷¹ Furthermore, the clinical use of using cDNA array analysis on FNA samples to discriminate benign from malignant thyroid neoplasms is unclear because more total RNA is required than would be available from FNA samples. The conflicting results of the differentially expressed genes among the different cDNA array studies and platforms used is also a problem, and there appears to be no uniform method of data analysis that has yet been standardized.^{72,73}

Genes that regulate cell–cell and cell–matrix adhesion and degradation of extracellular matrix are critical in tumor cell invasion and metastasis, the hallmarks of malignant phenotype.⁷⁴ Extracellular matrix and adhesion molecules, therefore, represent excellent candidate genes to study as markers of malignant thyroid nodules. Accordingly, we used an extracellular matrix and adhesion molecule cDNA array to identify differentially expressed genes in thyroid tissue that would represent tumors that are indeterminate or suspicious on FNA cytology. The differentially expressed genes on cDNA array analysis were further evaluated by real-time quantitative polymerase chain reaction (PCR) to determine their diagnostic accuracy.

METHODS

Thyroid Tissue and Fine Needle Biopsy Samples

Thyroid tissue samples and clinical and histopathology data were obtained for 131 patients with informed consent. The Committee on Human Research at the University of California, San Francisco approved the study.

Tissue from patients with hyperplastic nodule (n = 28), follicular adenoma (n = 28), follicular thyroid cancer (n = 25), follicular variant of papillary thyroid cancer (n = 19), papillary thyroid cancer (n = 26), and anaplastic thyroid cancer (n = 5) was studied. All thyroid tissue diagnoses were confirmed by permanent histology. The tissues used in our experiments were snap-frozen in liquid nitrogen at the time of thyroidectomy and stored at -80°C until total RNA was extracted. In 12 patients undergoing thyroidectomy for thyroid nodules, a biopsy of the thyroid nodule was performed with a 25-gauge needle at the time of thyroidectomy. The biopsy samples were immediately stored on ice until total RNA extraction. These samples were evaluated as samples with “unknown” histology.

cDNA Array

A cDNA array containing 96 human extracellular matrix and adhesion molecule genes, 3 endogenous control genes, and 2 negative control genes was used according to the manufacturer’s instructions (HS-010 GEarray Superarray kit; Bioscience Co., Fredrick, MD). Equal amounts of total RNA from 5 randomly selected patient samples for each thyroid histology group (hyperplastic nodule, follicular adenoma, follicular cancer, follicular variant of papillary thyroid cancer, and papillary thyroid cancer) were pooled together.^{75,76} Total RNA was prepared by TRIZOL (Invitrogen, Carlsbad, CA) extraction and quantitated by spectrophotometry. Total RNA (1 μg total of 5 pooled samples for each histology) was prepared, labeled, and the cDNA array blots were hybridized according to the manufacturer’s instructions (GEarray; Bioscience Co.). The Alpha image analyzer was used for spot densitometry measurements of the quadruplicate signals per gene. Gene expression levels (ie, density measurements) were normalized to β -actin to control for total RNA and hybridization efficiency differences among the different blots.

Real-Time Quantitative Polymerase Chain Reaction

Genes that were up- or downregulated by greater than 2-fold in malignant thyroid tissue on cDNA array blots were further analyzed by quantitative PCR. Total RNA was prepared by TRIZOL extraction (Invitrogen). Total RNA (125 $\text{ng}/\mu\text{L}$) was reverse-transcribed using the RT script cDNA synthesis kit (USB Corp., Cleveland, OH). Real-time quantitative PCR was used to measure mRNA expression levels relative to β -gluc-

ronidase (GUS) mRNA expression. Normalized gene expression level = $2^{-(C_t \text{ of gene of interest} - C_t \text{ of GUS})} \times 100\%$, where C_t is the PCR cycle threshold.⁷⁷ The PCR primers and probes for the genes were purchased from Applied Biosystems (Assay-on-Demand kit, Foster City, CA).

All PCR reactions were performed in a final volume of 20 μL on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) with 1 μL cDNA template. The PCR condition was 95°C for 12 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All quantitative PCR reactions were done in triplicate and repeated at least twice.

Statistical Analysis

Nonparametric Kruskal-Wallis and Mann-Whitney rank-sum tests were used to determine differences in normalized mRNA expression levels. A P value <0.05 was considered statistically significant. Data are presented as mean \pm standard deviation unless otherwise stated.

We used logistic regression analysis to develop a scoring model and determine if each gene was an independent marker for malignant thyroid neoplasms and to define a cutoff point. To evaluate the performance of the logistic regression scoring model for gene expression levels as diagnostic markers of malignant thyroid neoplasms, we determined the area under the receiver operating characteristic (ROC) curve (AUC). ROC curves were plotted for individual variables and for scores derived from logistic regression fits. We identified points corresponding to equal penalties for misclassification and points with estimated sensitivity above 95%. Because logistic regression scores were constructed, we identified a cutoff point corresponding to 99% sensitivity. We divided the sample at random into 10 parts and used the same approach (fitting a logistic regression model and identifying the score corresponding to 99% sensitivity) on nine tenths of the data (training data) using the score to identify positive/negative on the remaining tenth of the data (test data), each time producing a table that cross-classified histology with test status. This procedure was repeated 10 times, each time testing on a different tenth. The 10 tables were summed and proportions derived from them were used to get unbiased estimates of sensitivity, specificity, positive predictive value, and negative predictive value.

RESULTS

Extracellular Matrix and Adhesion Molecule cDNA Array Analysis

In all of the cDNA array blots, the endogenous control genes were positive and the negative control genes had no hybridization signals. Comparison of cDNA array blots of hyperplastic nodules and follicular adenoma to follicular cancer, follicular variant of papillary thyroid cancer, and

papillary thyroid cancer samples identified 6 differentially expressed genes: ADAMTS8, ECM1, MMP8, PLAU, SELP, and TMPRSS4 (Table 1). The intraassay variability of the densitometry measurements was $\leq 5.0\%$.

Real-Time Quantitative Polymerase Chain Reaction

Quantitative PCR measurements of the 6 differentially expressed genes showed that ECM1, SELP, and TMPRSS4 mRNA expression were higher in malignant (follicular cancer, follicular variant of papillary thyroid cancer, and papillary thyroid cancer) than in benign (hyperplastic nodules and follicular adenoma) thyroid neoplasms ($P \leq 0.005$) (Table 2). When we excluded cases of papillary thyroid cancer, which usually are accurately diagnosed by FNA biopsy, the levels of ECM1 and TMPRSS4 mRNA expression were higher in follicular thyroid cancers and the follicular variant of papillary thyroid cancers than in hyperplastic nodules and follicular adenomas ($P < 0.003$). The overlap in gene expression levels between benign and malignant thyroid neoplasms was 12.6% for ECM1 (5 follicular cancer, 2 follicular variant of papillary cancer, 2 papillary cancer), 25.2% for TMPRSS4 (17 follicular cancer, 6 follicular variant of papillary cancer, 2 papillary cancer), and 34.7% for SELP (17 follicular cancer, 8 follicular variant of papillary cancer, 8 papillary cancer) (Fig. 1). The level of ECM1 mRNA expression was higher in TNM stage I differentiated thyroid cancers than in stage II and III tumors, and higher in low-risk AMES than in high-risk AMES differentiated thyroid cancers (Table 3).

TABLE 1. Differentially Expressed Extracellular Matrix and Adhesion Molecule Genes in Benign Versus Malignant Thyroid Tissue by cDNA Array*

Gene Symbol	Gene Name	Benign	Malignant
ADAMTS8	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 8	0	0.7694
ECM1	Extracellular matrix protein, 1	0	1.0294
MMP8	Matrix metalloproteinase, 8 (neutrophil collagenase)	0.2272	0.6398
PLAU	Plasminogen activator, urokinase	0	0.7685
SELP	Selectin P	0	0.6658
TMPRSS4	Transmembrane protease, serine 4	0	0.7178

*Benign thyroid tissues were hyperplastic nodules and follicular adenomas; malignant thyroid tissues were follicular thyroid cancer, follicular variant of papillary thyroid cancer, and papillary thyroid cancer. Gene expression levels represent the densitometry signal for each gene normalized to β actin densitometry signal.

TABLE 2. Extracellular Matrix and Adhesion Molecule Normalized mRNA Expression Level in Benign and Malignant Thyroid Tissue Measured by Quantitative Polymerase Chain Reaction*

Gene Symbol	Thyroid		Histology Type		
	Hyperplastic Nodule (n = 19)	Follicular Adenoma (n = 19)	Follicular Thyroid Cancer (n = 19)	Follicular Variant of Papillary Thyroid Cancer? (n = 19)	Papillary Thyroid Cancer (n = 19)
ADAMTS8 [†]	0.082 ± 0.195	0.016 ± 0.034	0.040 ± 0.077	0.082 ± 0.177	0.270 ± 0.571
ECM1 [‡]	0.469 ± 1.000	0.383 ± 0.272	60.000 ± 117.864	43.624 ± 77.417	57.435 ± 115.294
MMP8	0.201 ± 0.798	0.008 ± 0.034	0.002 ± 0.011	0.298 ± 0.792	0.095 ± 0.131
PLAU [§]	0.004 ± 0.013	0.000 ± 0.000	0.127 ± 0.328	0.018 ± 0.076	0.018 ± 0.043
SELP [‡]	8.965 ± 11.971	9.999 ± 10.522	5.889 ± 5.765	29.022 ± 30.531	26.518 ± 18.539
TMPRSS4 [‡]	0.054 ± 0.076	0.260 ± 0.530	0.816 ± 0.782	21.565 ± 27.872	29.930 ± 30.194

*Gene expression values in table were normalized to GUS expression level, mean ± standard deviation (see Materials and Methods).

[†]Significantly higher in papillary thyroid cancer than in follicular adenoma, hyperplastic nodule, follicular variant of papillary thyroid cancer, and follicular thyroid cancer ($P \leq 0.04$).

[‡]Significantly higher in malignant than in benign thyroid neoplasms ($P \leq 0.005$).

[§]Significantly higher in follicular thyroid cancer than in follicular adenoma, hyperplastic nodule, and follicular variant of papillary thyroid cancer ($P \leq 0.03$).

There was no difference in the level of ECM1, SELP, and TMPRSS4 mRNA expression by tumor size or by primary tumor versus lymph node metastasis (Table 3).

Diagnostic Accuracy of ECM1, SELP, and TMPRSS4 mRNA Expression Level as a Marker for Malignant Thyroid Neoplasms

Logistic regression analysis showed that ECM1 and TMPRSS4 mRNA expression levels were independent markers of malignant thyroid neoplasms but SELP mRNA expres-

sion level was not ($P < 0.003$). The scoring model generated was: total score = $1.78 + 4.234 \log_{10}(\text{ECM1} + 0.01) + 2.148 \log_{10}(\text{TMPRSS4} + 0.01)$ with a cutoff point of -1.344 , so that when the score is ≥ -1.344 , the case would be considered malignant. The AUC was 0.956 for ECM1 and 0.926 for TMPRSS4 (Fig. 2). Combining both markers improved their diagnostic use, yielding an AUC of 0.985 with a sensitivity of 91.7%, specificity of 89.8%, positive predictive value of 85.7%, and negative predictive value of 82.8%. Because the diagnostic accuracy of quantitative PCR depends on the reproducibility of the gene expression levels, we also compared the intraassay variability of normalized ECM1 and TMPRSS4 mRNA expression levels. The correlation of normalized ECM1 and TMPRSS4 mRNA levels between experiments was excellent ($R^2 = 0.938$, $P < 0.0001$).

Comparison of the ECM1 and TMPRSS4 scoring model results to the preoperative FNA biopsy results showed that gene expression analysis was more accurate than FNA cytology (Table 4). In 35 of 38 indeterminate or suspicious FNA biopsy results, ECM1 and TMPRSS4 correctly distinguished between benign and malignant thyroid neoplasms (Table 4). Furthermore, 3 of 6 nodules considered benign by FNA biopsy were correctly identified as malignant, and 11 of 32 nodules considered malignant by FNA were correctly identified as benign.

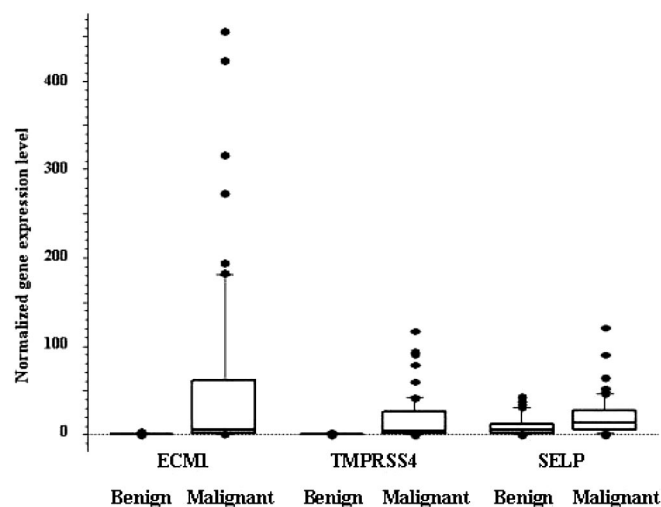


FIGURE 1. Box plot of ECM1, TMPRSS4, and SELP mRNA expression in benign and malignant thyroid neoplasms. The values are normalized ECM1, TMPRSS4, and SELP mRNA expression to GUS mRNA expression. The box represents 50% of data (above 25% percentile and below 75% percentile) and the line within the box is the median value.

Feasibility of ECM1 and TMPRSS4 mRNA Measurement in Fine Needle Aspiration Biopsy Samples

The accuracy of ECM1 and TMPRSS4 mRNA expression for distinguishing malignant from benign thyroid neoplasms was determined using frozen thyroid tissue. To ensure that FNA biopsy samples would yield enough total RNA for

TABLE 3. Level of ECM1, SELP, and TMPRSS4 mRNA Expression and Extent of Differentiated Thyroid Cancer*

Clinical Variables	ECM1	SELP	TMPRSS4
TNM stage [†]			
I (n = 27)	112.0 ± 136.1	21.1 ± 22.5	17.1 ± 30.6
II (n = 16)	5.4 ± 5.3	8.9 ± 8.4	13.0 ± 19.8
III (n = 14)	20.7 ± 53.3	25.6 ± 34.2	16.2 ± 14.9
AMES risk group [‡]			
Low (n = 38)	12.2 ± 38.9	18.9 ± 27.4	10.9 ± 13.6
High (n = 19)	74.4 ± 119.0	21.2 ± 20.8	20.7 ± 30.5
Tumor size [‡]			
T1 (n = 15)	93.9 ± 106.6	14.4 ± 12.4	11.4 ± 9.4
T2 (n = 27)	26.1 ± 62.3	24.4 ± 28.5	27.1 ± 32.2
T3 (n = 8)	43.9 ± 59.6	1.0 ± 0.9	0.4 ± 0.6
T4 (n = 3)	123.8 ± 197.2	20.6 ± 16.8	4.1 ± 8.1
Primary tumor vs lymph node metastasis [§]			
Primary (n = 15)	48.1 ± 119.5	24.9 ± 20.1	34.7 ± 32.1
Lymph node metastasis (n = 4)	92.6 ± 104.8	32.5 ± 10.8	12.2 ± 11.6

*Differentiated thyroid cancer samples analyzed include follicular thyroid cancer, follicular variant of papillary thyroid cancer, and papillary thyroid cancer.

[†]ECM1 mRNA expression was higher in stage I tumor than in stage II and III tumors, and in AMES low-risk group than in AMES high-risk group ($P \leq 0.031$). AMES is a prognostic scoring system used in patients with differentiated thyroid cancer. AMES, Age, distant Metastasis, Extrathyroidal invasion, and tumor Size.

[‡]The total number is 53 because 4 of the papillary thyroid cancers studied were lymph node.

[§]All the samples were papillary thyroid cancer.

real-time quantitative PCR gene expression analysis, we evaluated 12 intraoperatively obtained FNA samples of thyroid nodules and were able to obtain 406 ng/ μ L to 914 ng/ μ L of total RNA using a 25-gauge needle. This finding suggests that enough thyroid tissue would be available from FNA biopsy to test the accuracy of ECM1 and TMPRSS4 mRNA expression in distinguishing malignant from benign thyroid neoplasms. Most importantly, the scoring model for ECM1 and TMPRSS4 mRNA expression correctly identified 10 of 11 benign (4 hyperplastic nodules, 2 Hürthle cell adenoma, one follicular adenoma [false-positive], and one of one malignant [one papillary thyroid cancer] thyroid nodules).

Validation of Diagnostic Logistic Regression Scoring Model for ECM1 and TMPRSS4 mRNA Expression Levels

The accuracy of the logistic regression scoring model, which used normalized ECM1 and TMPRSS4 mRNA expres-

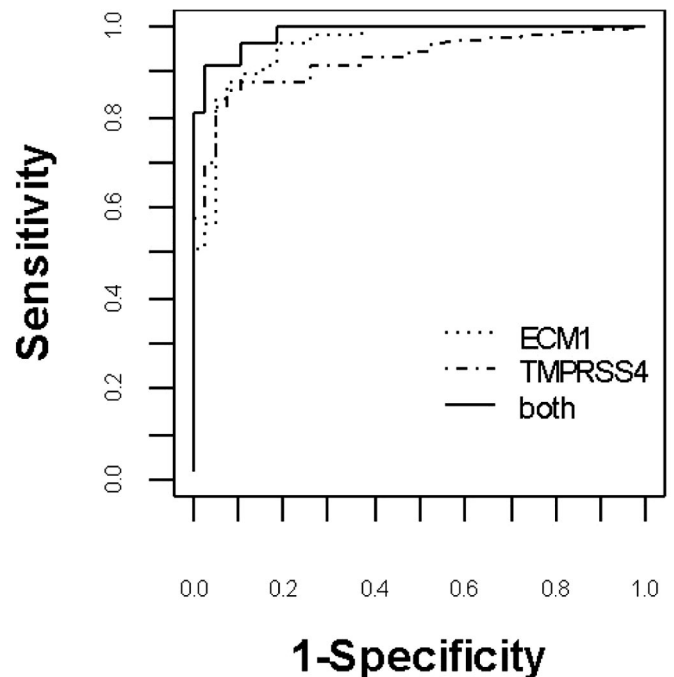


FIGURE 2. Receiver operating characteristic curve of normalized ECM1 and TMPRSS4 mRNA expression for distinguishing benign from malignant thyroid neoplasms. Combined use of both markers was more accurate than one marker alone. Area under the curve of 1.0 represents a “perfect” diagnostic test without any false-negative or false-positive results.

sion cutoff levels to distinguish malignant from benign thyroid neoplasms, was determined in 95 patients with known benign and malignant thyroid histologic diagnoses. A bias may have been introduced in measuring the accuracy because of the lack of blinding to the thyroid tissue histology. We therefore evaluated an additional 36 thyroid samples (9 hyperplastic nodules, 9 follicular adenomas, 6 follicular cancers, 7 papillary cancers, and 5 anaplastic thyroid cancer) as “unknown” to validate the regression scoring model and confirm the diagnostic accuracy of normalized ECM1 and TMPRSS4 mRNA expression levels. In these samples, the model had a sensitivity of 90.0%, a specificity of 92.6%, a positive predictive value of 81.8%, and a negative predictive value of 96.1%.

DISCUSSION

Our study focused on thyroid nodules that generally cannot be accurately diagnosed as benign or malignant neoplasms by preoperative FNA cytology.^{5,6,7,8} Of 96 extracellular matrix and adhesion molecule genes, 6 were differentially expressed by more than 2-fold in malignant thyroid neoplasms. However, only ECM1, SELP, and TMPRSS4 mRNA expression were significantly higher in malignant than in benign thyroid neoplasms by quantitative PCR mea-

TABLE 4. Comparison of ECM1 and TMRSS4 gene Expression Scoring Model Results With Preoperative Fine Needle Aspiration (FNA) Biopsy Result and the Permanent Histologic Diagnosis

FNA Biopsy		Scoring Model Result	Final Histology
6 benign	3 benign 3 malignant		3 benign 3 malignant (1 papillary, 2 follicular variant of papillary)
38 indeterminate/ suspicious	15 benign 23 malignant (3 false-positives were in hyperplastic nodules)		18 benign 9 follicular adenomas 9 hyperplastic nodules 20 malignant 7 follicular 6 follicular variants of papillary 7 papillary
33 malignant	11 benign (1 false-negative in follicular cancer) 22 malignant (3 false-positives in follicular adenomas)		13 benign 7 follicular adenomas 6 hyperplastic nodules 20 malignant 6 follicular 5 follicular variants of papillary 9 papillary thyroid cancers

surement. Of these 3 genes, ECM1 and TMRSS4 were independent markers of malignant thyroid neoplasms. The diagnostic accuracy of these 2 markers was best when used in combination.

The function of the ECM1, TMRSS4, and SELP genes in thyroid cancer is unknown. The ECM1 gene encodes an extracellular matrix protein and is located on chromosome 1q21.⁷⁹ ECM1 has several physiological functions that include bone development and maintenance of the extracellular matrix.^{80–83} It also regulates cell proliferation and angiogenesis in breast cancer cells. Upregulated ECM1 protein expression has been observed in a variety of human epithelial tumors.⁸⁰ In a study of serial analysis of gene expression (SAGE) to identify transcripts that may distinguish benign from malignant thyroid neoplasms, elevated ECM1 expression has been observed in thyroid tumors.⁸⁴ The level of normalized ECM1 mRNA expression was higher in low-risk tumors than in high-risk tumors. This finding is not surprising because more aggressive or transformed cancer cells are capable of growth without interactions between the cell surface and extracellular matrix for survival and cell cycle progression.⁸⁵ This would suggest then that the decreased ECM1 mRNA expression in more aggressive differentiated thyroid cancer tumors may reflect cell anchorage-independent tumor growth. The TMRSS4 gene, initially referred to as TMRSS3, is located on chromosome 11.q23.3 and encodes a member of the serine protease family.⁸⁶ This gene, to our knowledge, has not been previously reported to be differentially expressed in thyroid neoplasms, but is overexpressed in pancreatic carcinoma.⁸⁷ SELP is a member of the selectin

family adhesion molecules that mediate calcium-dependent cell–cell interactions in endothelial cells and activated platelets⁸⁸ and is thought to be necessary for tumor cell growth and metastasis.⁸⁹ Increased expression of SELP has been observed in endothelial cells of colorectal cancers, and elevated serum levels have been reported in patients with breast cancer, lung cancer, lymphoma, and melanoma.^{90–92} We found that ECM1, TMRSS4, and SELP were overexpressed in malignant thyroid neoplasms of follicular cell origin. These findings suggest that ECM1, TMRSS4, and SELP may play a role in thyroid carcinogenesis.

Although numerous molecular markers of thyroid cancer have been studied, they have had little clinical use in follicular tumors, and the clinical feasibility of performing cDNA microarray analysis on FNA samples remains unclear. In this context, the high sensitivity and specificity of ECM1 and TMRSS4 expression that we observed have important clinical ramifications. The need for “diagnostic” thyroidectomy can be reduced or eliminated if the risk of malignancy of a thyroid nodule can be reliably predicted by measuring ECM1 and TMRSS4 mRNA expression levels. As a result, some or all of the diagnostic thyroidectomies being performed for benign thyroid nodules could be eliminated, as could the morbidity associated with thyroidectomy. Reliable prediction would also reduce the cost of managing thyroid nodules because a more definitive operation can be performed in the first place without the need for reoperations in patients who are subsequently found to have a malignant thyroid neoplasm on permanent histology.

We have demonstrated in a limited number of FNA samples that quantitative PCR measurement for ECM1 and TMPRSS4 mRNA expression is feasible and that the logistic scoring model is accurate. These results suggest that ECM1 and TMPRSS4 would be useful diagnostic markers as an adjunct to FNA biopsy with a higher accuracy than that of FNA cytology. However, the diagnostic accuracy of these markers needs to be evaluated in Hürthle cell neoplasms, which are indeterminate on FNA cytology. Furthermore, our findings need to be confirmed by others. This should be readily accomplished because quantitative PCR is an easy and accurate technique, the primer and probes used in this study are commercially available, and our results were highly reproducible from experiment to experiment. In addition, quantitative PCR requires only a small amount of RNA, which would be readily available from FNA samples, as we and others have demonstrated.^{93,94}

In summary, we found that ECM1, SELP, and TM-PRSS4 are overexpressed in malignant thyroid neoplasms of follicular cell origin. ECM1 and TMPRSS4 appear to be helpful diagnostic markers for distinguishing malignant from benign thyroid follicular neoplasms. ECM1 is a marker of extent of disease for differentiated thyroid cancer.

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Discussions

DR. RICHARD A. PRINZ (CHICAGO, ILLINOIS): I would like to congratulate and compliment Dr. Kebebew and the San Francisco group for tackling an important clinical problem in surgical endocrinology. Fifteen-twenty% of patients having a diagnostic fine needle aspiration biopsy of a thyroid nodule will have an indeterminate result and require a diagnostic thyroidectomy to differentiate hyperplastic nodules and follicular and Hurthle cell adenomas from follicular and Hurthle cell carcinoma. A long and growing list of methods have been tested to see if this can be decreased. These have included imaging studies such as MRI and PET scanning, immunohistochemistry of various molecular markers, and, more recently, cDNA microarrays.

Dr. Kebebew used a directed cDNA microarray analysis to identify genes differentially expressed in benign and malignant thyroid nodules. This is followed by realtime quantitative PCR of two genes, ECM1 and TMPRSS4. He suggests that this technology can be used to improve the diagnostic accuracy of fine needle aspiration biopsy.

We know that the results of cytology depend heavily on the skill and experience of the cytologist. Do the results obtained in your study depend on the skill and experience of your lab? In other words, just how transferable are the results that you have shown us today?

Also, in comparing your technique to cytology, did a single experienced cytologist read the biopsies or were multiple cytologists involved?

I believe you are suggesting that this technique is a complement to conventional cytology. Do you have any experience showing that both can be done together in the preoperative evaluation, not just in the operating room? Since the biopsy material has to be handled differently for both tests, does it pose any problems either in the collection of the specimens or the performance of each test?

With regard to your methodology, does overlap in gene expression caused either by sampling issues or concomitant conditions in the thyroid such as thyroiditis diminish its accuracy?

Since the usual clinical issue is to distinguish whether a follicular neoplasm is actually a benign follicular adenoma or a malignant follicular carcinoma, what are your results if you exclude follicular variant of papillary cancer in addition to papillary cancer? Are they just as good?

The ECM1 levels seem to be higher in low risk early-stage thyroid cancers. Does this pose any problem with the identification or diagnosis of late-stage tumors with your methodology?

Finally, have you determined whether these genes have any functional activity either in the pathogenesis or in the progression of follicular thyroid cancer?

DR. ELECTRON KEBEBEW (SAN FRANCISCO, CALIFORNIA): I think your first question was whether these results would be reproducible in other laboratories. I believe that it is feasible. One approach would be to use a reference sample to standardize the results amongst different laboratories. We would be happy and interested to have our colleagues that practice endocrine surgery join us in doing a clinical trial to validate and confirm these results.

The second question was whether an experienced cytopathologist looked at all of these slides. And I think that is an important point, because we know when experienced cytopathologists look at the FNA biopsy, the diagnosis or the management approach might change. We have seen this to be the case at our institution.

To answer your question: no, the comparison was made based on what the FNA diagnosis was preoperatively without secondary review by an experienced cytopathologist. Some of these samples were not reviewed by our cytopathologists at our institution and were reviewed by other cytopathologists.

The other issue was, is it technically possible to do concurrently gene expression analysis on FNA biopsy samples? In the intraoperative FNA samples, we only performed four passes, and in general most cytopathologists are doing up to ten passes. Therefore I think it is technically feasible.

Yes, the segregation of AMES low-risk and high-risk differentiated thyroid cancer based on the level of ECM1

gene expression is interesting. We don't have a good explanation for why it is higher in low-risk tumors. But it might be that the more aggressive tumors undergo anchorage independent growth. And there are examples of this in other types of cancer. But we don't really know the functional consequence of the difference in expression levels, and that is certainly something to explore in the future.

I think the other question you asked was, what could account for the false positives? And we were interested in looking at the presence of chronic lymphocytic thyroiditis as a possible explanation for this because it has been reported in other molecular marker studies to account for false positives. Interestingly, we had 22 samples that did have thyroiditis present, but that didn't make a difference on whether the results were false positive, true positive, true negative or false negative.

DR. ASHOK R. SHAHA (NEW YORK, NEW YORK): We looked at the MUC1, and there was survival data, that showed some differences. The question I have is: I was curious, you included some anaplastic thyroid cancers in your study. That should be a different entity, and fine needle biopsy should at least give you some suspicion.

The second question I have is: Did you find any difference between minimally invasive follicular cancer and widely invasive follicular cancer? Because that is where probably the difference would be in the—follicular lesion which is diagnosed on needle biopsy.

Did the size or the age of the patient matter? If the lesion is more than 3 centimeters we are generally going to take it out most of the time no matter what the fine needle aspiration shows, especially if it is a solid thyroid nodule.

The last question: In the initial slide you mentioned this is probably the most rising type of cancer in the human body in the United States. More and more incidentalomas we are going to see now and would this technique help us in evaluating and managing these incidentalomas?

DR. ELECTRON KEBEBEW (SAN FRANCISCO, CALIFORNIA): Since we were blinded to the histologic diagnosis, these samples were evaluated as unknowns to validate the scoring model and thus account for the anaplastic thyroid cancer cases. Obviously this was an unintended consequence of being totally blinded to evaluating the unknown samples. As far as the question of minimally invasive versus widely invasive follicular thyroid cancer, I do agree with you that is an important issue. Unfortunately, the samples of follicular thyroid cancer that we studied were widely invasive, so that would be something that we would need to study further to see if it would be useful in distinguishing between minimally and widely invasive follicular thyroid cancer.

We did evaluate the level of expression as related to the age and the tumor size and didn't find a difference, except for ECM1. And surprisingly, we found it was higher in those that

were younger than those that were older. And that might account for the segregation or the differences we saw in the AMES high-risk and low-risk tumors.

The last question, I believe, was: could this be useful in the management of incidentalomas? I am sure you are familiar with the work from the Japanese group where they elected to observe some patients with occult papillary thyroid cancers that were less than 1 centimeter in size and found that 70% of these patients didn't have progressive cancers. I think these markers may be a way of segregating out those occult tumors that might be aggressive or not. A study like that probably would not be possible to do here in the U.S. with the standards of our IRB.

DR. CHRISTOPHER R. MCHENRY (CLEVELAND, OHIO): I wonder if the sensitivity for ECM1 or TMPRSS4 expression varies for specific subsets of patients with an indeterminate fine needle aspiration biopsy, specifically in the patient that has a fine needle aspiration biopsy that is suspicious but not definitive for papillary thyroid cancer. Would you predict similar or better results for distinguishing benign versus malignant disease?

My second question is: Can you improve the sensitivity or usefulness of these markers if you compared neoplastic versus nonneoplastic disease rather than benign versus malignant disease?

Finally, do you foresee there to be any role for evaluating ECM1 and TMPRSS4 expression in patients with a thyroid nodule and a benign fine needle aspiration biopsy to help reduce the incidence of false negative fine needle aspiration biopsy?

DR. ELECTRON KEBEBEW (SAN FRANCISCO, CALIFORNIA): I think it is true that the sensitivity and specificity depend upon the histologies that we have evaluated. And if we did exclude those suspicious that would be follicular variant of papillary thyroid cancer, I don't know if it would be as accurate. But the initial analysis did exclude typical papillary thyroid cancer samples. And that is how we arrived at those two genes out of the three that were significantly different between benign and malignant tumors.

It is an interesting question that you asked as far as the difference between neoplastic and nonneoplastic thyroid tissue. Clinically, however, we are dealing with patients that have a nodule either benign or malignant, so we never really are trying to exclude a malignancy in normal thyroid or thyroiditis glands without a tumor. To answer your last question, I think that would potentially be very useful as demonstrated in those 6 patients that had an initial benign FNA result where we found 3 of them were malignant and proven histologically to be malignant but they had other clinical indications that led them to have an operation. So I think that would be useful in that sense and we need to do

these studies to see if the accuracy is really superior to FNA biopsy and cytologic examination.

DR. HERBERT CHEN (MADISON, WISCONSIN): I see 1 potential limitation with your technology. Because you are looking for differences in RNA levels, you stick the nodule once for the FNA and a second time for the sample for your analysis, and theoretically you could be giving 2 separate distinct samples. So I was wondering if you have looked at expression levels of the proteins. That way you could possibly get around this by doing immunohistochemistry. You

could FNA once, look at it under the microscope, and then stain that same slide to make sure you are actually doing your test on the same sample that you are looking at by FNA.

DR. ELECTRON KEBEBEW (SAN FRANCISCO, CALIFORNIA): It is actually something we have thought about, that is a sampling error, whether the cytologic sample is the same as the sample for gene expression analysis. But there are investigators that have demonstrated it is possible to perform PCR on emptied FNA needle samples. This is because only a small amount of RNA is required for real-time quantitative PCR.