Genetic Classification of Benign and Malignant Thyroid Follicular Neoplasia Based on a Three-Gene Combination

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Thyroid carcinoma is a common endocrine cancer with a favorable prognosis if subjected to timely treatment. However, the clinical identification of follicular thyroid carcinoma (FTC) among patients with benign thyroid nodules is still a challenge. Preoperative fine needle aspiration-based cytology cannot always differentiate follicular carcinomas from benign follicular neoplasias. Because current methods fail to improve preoperative diagnosis of thyroid nodules, new molecular-based diagnoses should be explored. We conducted a microarray-based study to reveal the genetic profiles unique to FTC and follicular adenomas (FAs), to identify the most parsimonious number of genes that could accurately differentiate between benign and malignant follicular thyroid neoplasia. We confirmed our data by quantitative RT-PCR and immunohistochemistry in two independent validation sets with a total of 114 samples. We were able to identify three genes, cyclin D2 (CCND2), protein convertase 2 (PCSK2), and prostate differentiation factor (PLAB), that allow the accurate molecular classification of FTC and FA. Two independent validation sets revealed that the combination of these three genes could differentiate FTC from FA with a sensitivity of 100%, specificity of 84.7%, and accuracy of 96.7%. In addition, our model allowed the identification of follicular variants of papillary thyroid carcinoma with an accuracy of 85.7%. Three-gene profiling of thyroid nodules can accurately predict the diagnosis of FTC and FA with high sensitivity and specificity, thus identifying promising targets for further investigation to ultimately improve preoperative diagnosis.

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Abbreviations: CCND2, Cyclin D2; Ct, threshold cycle; FA, follicular adenoma; FNA, fine needle aspiration; FTC, follicular thyroid carcinoma; FV, follicular variant; HCC, Hurthle cell carcinoma(s); IHC, immunohistochemistry; MBEI, model-based expression index; PCSK2, protein convertase 2; PLAB, prostate differentiation factor; PTC, papillary thyroid carcinoma; ROC, receiver-operated characteristics.

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FNA cytology in the clinical setting (4, 11–13). A possible underlying cause for this clinical problem is the continued limited understanding of the biological relationship of the different benign thyroid neoplasias to each other and to thyroid carcinoma, despite much research in this field (11, 14–17). Therefore, to directly address the clinically relevant issue, we sought to elucidate further the molecular differences between benign follicular neoplasia and FTC. We took a global expression array approach to dissect out the minimal number of genes that can play a fundamental role in the early steps of FTC carcinogenesis, thus not only giving new biological insight but also allowing us to differentiate FTC, even at the minimally invasive stage, from benign follicular neoplasia based on a limited set of genes. We believe that this may form a basis for further investigation, in the hope that objective molecular markers will serve as an adjunct in the preoperative diagnosis of follicular thyroid cancer.

Materials and Methods

Tissue specimens

In total, 55 samples (24 FTC and 31 benign thyroid samples) were independently acquired for gene expression analysis in our training and preoperative diagnosis of follicular thyroid cancer. These samples were obtained as anonymized materials without linked identifiers, with the approval of The Ohio State University’s Institutional Review Board for Human Subjects’ Protection. Without linked identifiers, with the approval of The Ohio State University’s Institutional Review Board for Human Subjects’ Protection.

TABLE 1. Histopathological classification of 12 FTC samples used for microarray analysis

| Sample ID | Sex/age | Pathologic diagnosis | TNM |
|-----------|---------|----------------------|-----|
| 02E187    | n/a     | FTC—Hurthle cell type; capsular invasion | pT2 |
| 03E139    | F/61    | FTC—Hurthle cell type; widely invasive | pT2 |
| 03E077    | F/48    | FTC—Hurthle cell type; minimal invasive | pT2 |
| 03E193    | F/82    | FTC—Hurthle cell type; minimal invasive | pT3 |
| 03E041    | F/72    | FTC—Hurthle cell type; hepatic metastases | pT2 |
| 408       | F/71    | FTC—Hurthle cell type; recurrence | 95 |
| 409       | F/69    | FTC—recurrence | 95 |
| 22        | F/67    | FTC | pT4 |
| 177       | F/78    | FTC; widely invasive | pT3 |
| 52        | M/40    | FTC; recurrence | pT3 |
| 03E191    | F/62    | FTC; minimal invasive | pT2 |
| 03E192    | F/25    | FTC; minimal, angioinvasive | pT2 |

n/a, Not available; M, male; F, female.
Primers for quantitative RT-PCR were designed to span an exon-exon boundary or an intronic sequence, to avoid amplification of any genomic DNA. All quantitative RT-PCR products were initially visualized on a 2% agarose gel to ensure the presence of only a single amplicon product. The average sd between replicates was 0.15 and the average interassay sd for control genes was 0.32.

**IHC**

IHC was performed as described previously (22). Antibodies against CCND2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution 1:150 and against PCSK2A (US Biological, Swampscott, MA) were used at a dilution of 1:100. A total of 83 sections were analyzed, consisting of 57 FTCs and 26 benign nodules (17 FA and nine follicular hyperplasia). Additional sections from five normal thyroid glands and adjacent normal thyroid tissue were used for comparison. All slides were scored in a blinded fashion, and a second individual randomly validated the results. We regarded cells as immunoreactive when an obvious nuclear (CCND2) or cytoplasmic (PCSK2) expression was seen. We scored immunoreactivity as follows: retained (+) when more than 50% of nuclei/cytoplasm were strongly immunoreactive, reduced (+) when 10–50% of the nuclei/cytoplasm were immunoreactive, and absent (−) when less than 10% of the nuclei/cytoplasm were immunoreactive or all cells’ nuclei showed no immunoreactivity at all [supplemental Figs. 2 and 3 (published as supplemental data on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org)]. The absence of a commercially available antibody that could reliably allow staining of thyroid tissue led to refine the IHC analysis to CCND2 and PCSK2.

**Statistical methods**

Two-tailed Student’s t test for independent samples, assuming equal variance, was used to determine difference between mean gene expression determined by RT-PCR of the three selected genes with 22 degrees of freedom (Table 2).

The hierarchical cluster analysis we used to present our data are based on 96 probe sets that we filtered from the 22283 probe sets present on the HG-U133A chip by setting the thresholds to 2-fold expression changes at the lower 90% confidence bound in either direction, a P value less than 0.05 for the difference in expression and no less than 50% present call for each gene in all 24 arrays. For our cluster analysis we choose the commonly used average linkage method. The distance measure in the clustering analysis is 1 minus the correlation coefficient (23).

When the expression of a single gene is used for diagnosis, it becomes necessary to find a desirable threshold value that is used to distinguish the two groups. We obtained for each possible threshold value the sensitivity and specificity of diagnoses, which are percentages of FTC (“test positive”) and FA (“test negative”, i.e. not FTC) samples correctly identified, respectively. The best threshold value is the one that maximizes an appropriate combination of the two. To use multiple genes in combination for the purpose of diagnosis, we applied linear discriminant analysis, which is based on the assumption of multivariate normal distributions of the joint expressions, and finds the best linear combination of the expression values that discriminates the two groups. In a first round, we applied the technique of cross-validation to the training set to assess the performances of the diagnostic tests, in which each sample is in turn left out of the data, a test developed based on the remaining samples and then applied to the sample being left out. The diagnoses can be compared with the true classes of the samples to indicate the performance of the method leading to the diagnostic test. In a second round, we applied the same technique of linear discriminant analysis, but this time using our validation set, to independently confirm our findings from the first round.

**Results**

To dissect out the most parsimonious gene expression differences that accurately classify FTC from benign follicular neoplasias, in particular FAs, we used a global expression array approach on 12 FTCs and 12 FAs (“training set”). So that we could also differentiate the earliest signs of malignancy from benign neoplasia, we included two minimally invasive FTCs and two minimally invasive HCC within our set of FTCs (Table 1). Using the dChip compare sample function, we used, as a first step, a straightforward but conservative approach to identify those genes that could reliably differentiate between FTC and FA. Using these criteria defined in the Materials and Methods section, we identified 96 probe sets, which represent 80 genes. To statistically validate these findings, we performed a random permutation analysis, in which we randomly permuted the labels of FTCs and FAs a large number of times, repeated the gene selection procedure using the same criteria, and recorded the number of genes identified (24). It demonstrated that these 80 genes were uncovered due to biological relevance and not by random coincidence (i.e. chance). Hierarchical cluster analysis showed that based on this set of 80 genes, FTCs and FAs could be accurately classified according to their histological group (Fig. 1 and supplemental Fig. 1). Notably, three of four minimally invasive carcinomas and all HCC clustered within the FTC group. Only sample 03E192, a minimally invasive FTC, clustered with the FA group. From this set of 80 genes, we set out to find the smallest number of genes that could reliably classify FTC from FA in an independent validation set. After ranking the probe sets based on their fold change and significance (P value and t statistics), we identified those genes that also showed the highest difference in expression levels between minimally invasive FTCs and FA and we excluded expressed sequence tags and hypothetical proteins. Based on these criteria, we identified a list of 11 genes, and we focused, in the first instance, on the two highest ranking genes CCND2 (fold change −11.72; P value 0.0025), and PLAB (fold change 7.86; P value 0.0039; this gene has been annotated under different names, such as GDF-15, MIC-1, or com1) (25). Besides CCND2, we also found CD44, a gene targeted by the Wnt signaling pathway, markedly under-expressed in FTCs compared with FAs. We therefore focused on the first 11 genes to reliably classify FTCs and FAs.

**TABLE 2. Summary of quantitative RT-PCR data obtained for three genes, CCND2, PCSK2, and PLAB**

| Gene  | Affymetrix ID | NCBI public ID | ΔΔCt FTCa | ΔΔCt FAc | Fold change FTC vs. FA | Pb     |
|-------|--------------|----------------|-----------|--------|-----------------------|--------|
| CCND2 | 200952_s_at  | AW026491       | −4.03     | −0.68  | Down                  | 10.2-fold | 0.00001 |
| PCSK2 | 204870_s_at  | AL031664       | −7.46     | −0.58  | Down                  | 263-fold | <0.0001 |
| PLAB  | 221577_x_at  | AF003934       | 4.12      | 1.67   | Up                    | 5.5-fold | 0.0037  |

The approved gene symbol for PLAB by the Human Genome Organization Nomenclature Committee is GDF-15 (growth differentiation factor 15).

a Given are ΔΔCt as mean of each group and exact 95% confidence intervals in parentheses.
b P values are calculated with two-tailed Student’s t test for independent samples with 22 degrees of freedom.
FIG. 1. Supervised hierarchical cluster analysis based on a set of 80 genes differentiates FTC from FA. Expression values of each gene across all samples were linearly scaled (standardized) to have a mean of 0 and SD of 1. These standardized values were used to calculate the correlation between genes, based on the distance metric (1-correlation). The average linkage model was used for merging nodes. Red represents overexpression and green represents underexpression.
pressed in FTC (fold change $-4.5$; $P$ value 0.0016). In addition, Frizzled-1, the membranous receptor for Wnt ligands, is also dysregulated (fold change $-4.39$; $P$ value 0.0081). Neither CCND2 nor PLAB have been previously associated with thyroid carcinogenesis.

As a second step, we analyzed our gene expression data for probe sets with very high absent calls in only one group, either FTC or FA but not both, expecting that this approach will identify strongly under-expressed or silenced genes, which would in theory reliably differentiate these two histologies. Such high absent calls can lead to high $P$ values, and consequently, the gene will not be detected by standard selection process. This approach revealed the gene encoding PCSK2 [present call 7% (MBEI 12.05) in FTC vs. 75% (MBEI 1743.51) in FA; fold change 144.7, $P$ value 0.011] on further analysis. Expressional differences of each of the three genes between FTC vs. FA in the training set was confirmed using quantitative RT-PCR (summarized in Table 2).

**Genetic classification of FTC and FA**

Based on our microarray data from the training set of 12 FTCs and 12 FAs, we then employed different statistical methods to predict the performance of our selected three genes in the accurate and reliable classification of FTC and FA. We employed receiver-operated characteristics (ROC) curve analysis to evaluate the performance of our genetic classification using the expression of each of the three genes (CCND2, PCSK2, and PLAB) individually. The ROC curve shows the sensitivity (proportion of FTC samples correctly classified) and one minus the specificity (where specificity is defined as proportion of FA samples correctly classified, i.e. not carcinoma) from using all possible threshold values of expression in the classification (graph not shown). Because a very low false-negative rate is desired, and we noted that to perfectly identify all FTC samples (12 of 12), the minimum proportions of misclassified FA samples based on our data are 33% (four of 12), 16.7% (two of 12), and 75% (nine of 12) when the expression values of CCND2, PCSK2, and PLAB are used separately. Of significance, when expression values of CCND2 and PCSK2 were used jointly in the classification by applying the method of linear discriminant analysis, the two groups of samples, FTC and FA, can be distinguished perfectly (24 of 24) (Fig. 2A). PCSK2 and PLAB have the same joint effect (Fig. 2B). To validate this microarray-based classification, we blindly analyzed the expression levels of CCND2, PCSK2, and PLAB in an independent validation set of 12 FTCs, 12 nonfunctioning thyroid nodules (five FAs and seven follicular hyperplasia), two normal thyroids and five autonomous adenomas (hot nodules). Linear discriminant analysis of this independent validation series confirmed that dual combinations of CCND2 and PCSK2 or PCSK2 and PLAB were able to distinguish between FTCs and FAs with an accuracy of 87.1% (exact 95% confidence interval 70.2–96.4%) (27 of 31 samples) and 93.5% (exact 95% confidence interval 78.6–99.2%) (29 of 31 samples), respectively (Fig. 2 and Table 3). Furthermore, because both hot as well as cold nodules could be accurately identified, we showed that the differences between the two groups are independent from functional status of the thyroid nodule but due to malignant transformation. For an honest estimate of the clinical performance using all three genes together, i.e. CCND2, PCSK2, and PLAB jointly, we applied the classifier from linear discriminant analysis, which correctly identified all 12 FTC samples from the validation set, and we estimated a false-positive rate of 5.3% (exact 95% confidence interval 0.13–26.03%) (1 of 19 samples) allowing an accuracy of 96.7% (exact 95% confidence interval 83.3–99.9%) (30 of 31 samples) (Fig. 3 and Table 3).

Furthermore, we validated our data by means of IHC for the most promising combination of two genes, CCND2 and

![Fig. 2. Classification of 24 FTCs and 31 benign thyroid nodules (training set and validation set) by linear discriminant analysis. The two groups of samples (12 FTCs and 12 FAs) in the training set (red) can be distinguished perfectly based on the expression of CCND2 and PCSK2 (A). PCSK2 and PLAB have the same joint effect (B). The samples of the validation set (blue) can be classified with a sensitivity of 66.7% (exact 95% confidence interval, 34.9–90.1%) and specificity of 100% for the combination of CCND2 and PCSK2 (A). Using PLAB and PCSK2 combined, 91.7% (exact 95% confidence interval, 61.5–99.8%) of all FTCs in the validation set can be correctly identified and 94.7% (exact 95% confidence interval, 74.0–99.9%) of the benign thyroid nodules can be correctly classified as well (B). See also Table 3. The joint performance of all three genes is demonstrated in Fig. 3.](image-url)
PCSK2, in a second independent validation set of 57 FTCs and 26 benign thyroid nodules (supplemental Figs. 2 and 3). Using PCSK2 and CCND2 jointly (ROC curve in Fig. 4), we observed a sensitivity of 89.5% (exact 95% confidence interval 78.5–96.0%), specificity of 80.8% (exact 95% confidence interval 60.6–93.4%) and accuracy of this test of 86.7% (exact 95% confidence interval 77.5–93.2%) when we chose the cut-off value for identifying FTC to be category 3 or larger (Table 4). Of note, complete absence of expression of PCSK2 and/or CCND2 was only seen in FTCs but never in benign thyroid nodules (Table 4). Furthermore, only 1 of 14 minimally invasive FTCs was misclassified due to retained immunostain for both antibodies, PCSK2 and CCND2. These observations affirm the accuracy of our genes to identify even minimally invasive neoplasias.

**Genetic classification of FV-PTC**

About 10% of suspicious FNA biopsies will be classified as FV-PTC in final histology. Therefore, we employed our three-gene based classifier system on a set of seven FV-PTC (Table 5). Six of seven FV-PTC samples analyzed were correctly identified as a malignant thyroid neoplasm (85.7%). In addition, we used CITED1 and ARHI, two other markers previously described by us, to further characterize these samples. It is of note that one sample (FV-PTC_269) does not show expression of CITED1, a predictive marker for FV-PTC and PTCs. Interestingly, only in this sample we see a clear under-expression of CCND2 as seen in all other FTCs analyzed. Furthermore, sample FV-PTC_345 shows expression of CITED1, but was not identified by our three-gene profile as a malignancy. It is noteworthy that we found strong expression of the imprinted tumor suppressor gene ARHI in this sample. As we showed previously, silencing of this gene is associated with FTC carcinogenesis (21). These data might indicate that histological diagnosis of FV-PTC addresses a heterogeneous group of follicular neoplasia—an aspect that needs further elucidation. We note, by including the seven FV-PTC in our validation set, we can accurately identify 94.7% of all malignant samples (18 of 19) and 94.7% of all benign samples (18 of 19) as well.

**Discussion**

Currently, the diagnosis of thyroid nodules relies primarily on cytology (4, 8). For the majority of patients with PTC,
TABLE 5. Identified molecular alterations such as unique molecular alterations (12, 29). Other groups have identified these alterations in follicular neoplasms and tumor suppressors in others (38, 39). Thus, further clarification and it remains important to identify HCC separately.

Differences in gene expression out-weigh in FTC and HCC the similarities in gene expression out-weigh in FTC and HCC the differences. These findings and other reports support our hypothesis that FTC and some HCC may result from shared molecular alterations (21). Nonetheless, this area requires further clarification and it remains important to identify HCC separately.

Our approach has allowed us to begin to identify genetic nuances in the initiation of follicular carcinogenesis. The dysregulation of CCND2, a cell cycle regulator, is intriguing because over-expression is associated with cancer progression and malignant transformation (32, 33). However, there are emerging data that CCND2 may act in different ways beyond cell cycle control. Other reports showed that CCND2 is under-expressed in various cancers due to hypermethylation of its promoter (34, 35). Our findings might provide further insight into the biological mechanism of CCND2 inactivation. Previous reports indicated that the dysregulation of the Wnt signaling pathway might play an important role in thyroid carcinogenesis (36). The membrane-bound Frizzled receptors serve as binding targets for the Wnt proteins and subsequent activation of its intracellular Dishevelled proteins lead to transcription of targets genes such as CCND2 and CD44 (36, 37). Our data demonstrate dysregulation of this pathway from the receptor to the target genes in FTC. Corroborating our findings, a previous report identified 11 genes of the Wnt pathway, including CCND2 and CD44, under-expressed in prostate cancer (37). This seeming paradox that both over- and underexpression of the same gene can result in carcinogenesis is being explained by accumulating data showing that different signaling pathways and its downstream targets may act as oncogenes in some neoplasms and tumor suppressors in others (38, 39). Thus, fur-

TABLE 4. Distribution of CCND2 and PCSK2 expression by immunohistochemistry in 83 total follicular neoplasia samples

| Category     | 1 (+/+ +) | 2 (+/+ −) | 3 (+/−) | 4 (−/+ +) | 5 (−/−) |
|--------------|-----------|-----------|---------|-----------|---------|
| Benign nodule|           |           |         |           |         |
| FTC          |           |           |         |           |         |

* Images of samples are published as supplemental data on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org.

non-FTC, or inflammatory lesions, FNA-based cytology can make a diagnosis with high accuracy (4). However, there is a significant proportion of follicular neoplasms in which this FNA-based preoperative cytologic diagnosis fails (4–6, 8–10). Several reports show that individual skill and experience largely affect the sensitivity of this diagnostic test, ranging from as low as 57% to as excellent as 98% (10). However, an estimated 20% (ranging from 9.2–42%) of all performed FNA-based cytologies will describe a suspicious follicular neoplasia, but only 10–20% of the patients that undergo surgery based on this diagnosis will actually have a malignant thyroid nodule (4, 5, 8). Based on investigative studies, immunohistochemical analysis has been proposed as a reliable marker for differentiating between FTC and FA (26). However, most of these markers showed their limitations in clinical practice and failed to become established (4, 27). One underlying reason might be that neoplasias do not show their distinct malignant phenotype and therefore cannot be diagnosed by these methods.

Different global gene expression studies have been conducted over the last years to identify novel targets. A recent study employing serial analysis of gene expression proposed a four-gene profile to improve preoperative diagnosis of FTC, but the accuracy of 80% for the gene expression based model is not superior to other algorithms (28). In addition other microarray-based studies, that allowed the highly accurate differentiation between FTC and FA by employing a 105-genes profile, still failed to identify minimally invasive FTCs, which comprise a large proportion of all FTCs (5, 14).

Our approach overcame this problem by including diverse genes of the Wnt pathway, including CCND2, CD44, and CITED1 ARHI, proving its ability to discriminate malignant and nonmalignant samples. Expressions of the two genes CITED1 and ARHI are marked with +, whereas no detectable expression is labeled −.
The second gene we identified, PLAB, encodes a member of the TGF-β superfamily that is known to prevent apoptosis by activating the Akt pathway (25). The importance of Akt activation in follicular thyroid carcinogenesis has been previously shown by us (40). Therefore, PLAB might provide an upstream target of this pathway. Furthermore, an estimated 10% of all FNA do not result in sufficient material for a cytological diagnosis (4). Due to the lack of serum biomarkers that could identify FTCs, no preoperative noninvasive diagnosis is currently available for these patients. In this context, PLAB, a secreted protein, should be considered for further investigation to determine its feasibility as a diagnostic tool to identify thyroid malignancies from a simple blood test (41).

The third gene identified in our analysis is PCSK2. The members of this family process latent precursor proteins into their biologically active products. The mechanism by which the disruption of proprotein converting processes can promote tumorigenesis in thyroid tissue remains unknown. However, it has been shown that the inhibition of proprotein convertases enhances cell migration and metastases development of human colon carcinoma cells (42). Such a mechanism is plausible as well in thyroid carcinogenesis.

Even when we used only a combination of two of our three identified genes (CCND2 and PCSK2 or PLAB and PCSK2) we were still able to correctly classify 100% of the FTCs, including four minimally invasive ones, and all FAs. Indeed, using an independent validation series of 31 samples, we demonstrated that the combination of all three genes CCND2, PCSK2, and PLAB performed well in differentiating FTC from FA, resulting in an accuracy of 96.7% (exact 95% confidence interval of 83.3–99.9%). Furthermore, we were able to use a second validation series and a different technique, IHC, to examine a combination of only CCND2 and PCSK2, which resulted in an accuracy of 86.7%. Thus, our results appear to be superior to those reported using RT-PCR methods to detect gene expression of telomerase, galectin-3, or a number of other markers to discriminate benign from malignant follicular thyroid tumors (4, 13, 43, 44). The employment of galectin-3 IHC has been reported to reliably identify malignant thyroid lesions (26, 45). However, we and others have shown previously that this method does not succeed in improving the differentiation between FTCs and FAs in all cases (27, 43). Furthermore, analysis by means of IHC often has its limitations, not only due to variability of antibodies or interinstitutional variation but also because of nonuniform classification and interpretation. In contrast, our gene expression analysis in a total of 24 FTCs and 31 benign thyroid nodules, using the combination of three genes, resulted in 100% of FTCs being identified and 30 of 31 of benign thyroid nodules definitively identified as well. A very recent FNA-based study employing hTERT as a molecular differentiator succeeded with recognizable sensitivity and specificity (46). However, the data indicate that this test performs much better in the identification of PTC and FV-PTC compared with FTC. Indeed, a full 20% of FTCs were missed. In addition, the performance of this test in identifying minimally invasive FTCs is unclear, and the authors conclude that additional molecular-based markers need to be explored (46). The robust results from our initial testing/training set confirmed by two independent validation sets have lent confidence that our three-gene test might help to establish a new and reliable molecular adjunct for diagnosis of follicular thyroid nodules in the near future.

There exist other studies that reported accurate differentiation of thyroid carcinomas, but notably, all these models were either based on high-density gene profiles (100 or more genes), which would not work in a presurgical diagnostic setting due to limited tissue and RNA available in such a setting, or do not provide the accuracy needed (13, 14, 28, 47). Our classification model based on the limited number of genes, only three, provides the basis to pursue further evaluation. Whereas the technique to perform gene expression analysis in limited cell material has been well established (48), it needs to be shown how inadequate and/or contaminated FNA will affect the accuracy of our proposed test.

FV-PTC will be found in about 10% (range 0–22%) of inconclusive FNA cytologies (5, 6, 49, 50) and it is of note that when we employed our three-gene profile, we were able to identify FV-PTCs with an accuracy of 85.7%. Still, we need to acknowledge that FV-PTC might pose a special challenge when employing the three-gene predictor model into an FNA based setting. Our data indicate that the histological diagnosis of FV-PTC might describe a heterogeneous group of thyroid neoplasias. In this regard, it is of note that in a recent study by Lloyd et al. a concordant diagnosis of FV-PTC among 10 pathologists was made only in 39% of all cases (51). This high degree of observer variation can lead to a considerable bias of data if analysis is based on the unreviewed diagnosis of FV-PTC.

However, considering the recent studies that reported the differentiation between FV-PTC and FA using hTERT or CITED1, it may be plausible to use a four-gene test comprising CCND2, PCSK2, and PLAB plus hTERT (46, 52). Therefore, there is accumulating molecular evidence that suggest that, in the near future, the majority of, if not all, thyroid malignancies can be targeted for definitive surgery, abolishing the requirement of a completion surgery (46, 53, 54). More importantly, most of the FAs that currently would have gone to unnecessary surgery would have been spared an extensive operation.

In summary, we have demonstrated that genetic classification of follicular thyroid neoplasia with a minimal number of three genes is highly accurate and may provide a tool to overcome the difficulties in today’s preoperative diagnosis of follicular malignancies. It is hoped that the quantitative nature of such a test will be a useful gene-based objective adjunct to the preoperative diagnosis of a disease that currently relies solely on cytology.

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References

1. Kinder BK 2003 Well differentiated thyroid cancer. Curr Opin Oncol 15:71–77
2. Welker MJ, Orlov D 2003 Thyroid nodules. Am Fam Physician 67:559–566
3. Ross DS 2002 Nonpalpable thyroid nodules—managing an epidemic. J Clin Endocrinol Metab 87:1938–1940
4. Segev DL, Clark DP, Zeiger MA, Umbricht CB, Conrad GT, Clark DP, Westra WH, Smith DC, Zahurak M, Saji M, Hardy E, Kruhlak M, Larin A, Savchenko V, Miyakawa M, Torosian H, Waters M, Dugas ME, Leung RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
5. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
6. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
7. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
8. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
9. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
10. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
11. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
12. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
13. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
14. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
15. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
16. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
17. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
18. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
19. Regev-Rudensky A, Edelstein D, Franza BR, Friedman N, Groudine M, Venter JC, Fairhead JF, Hall PN, Perry A, Zubovets Y, Gyorke C, Ghosh SK, Nowell PC, Ebert MP, Leong RW, Tse PC, Chan MW, Bai AH, To KF, Malfertheiner P, Sung J 2003 Absence of cyclin D1 expression is associated with poor prognosis in gastric cancer patients. Br J Cancer 88:1560–1565
transcriptase gene expression and the surgical management of suspicious thyroid tumors. Clin Cancer Res 10:5762–5768

47. Finley DJ, Zhu B, Barden CB, Fahey 3rd TJ 2004 Discrimination of benign and malignant thyroid nodules by molecular profiling. Ann Surg 240:425–436; discussion 436–7

48. Giannini R, Faviana P, Cavinato T, Elisei R, Pacini F, Berti P, Fontanini G, Ugolini C, Camacci T, De Ieso K, Miccoli P, Pinchera A, Basolo F 2003 Galectin-3 and oncofetal-fibronectin expression in thyroid neoplasia as assessed by reverse transcription-polymerase chain reaction and immunocytochemistry in cytologic and pathologic specimens. Thyroid 13:765–770

49. Kesmodel SB, Terhune KP, Canter RJ, Mandel SJ, LiVolsi VA, Baloch ZW, Fraker DL 2003 The diagnostic dilemma of follicular variant of papillary thyroid carcinoma. Surgery 134:1005–1012; discussion, 1012

50. Bakshi NA, Mansoor I, Jones BA 2003 Analysis of inconclusive fine-needle aspiration of thyroid follicular lesions. Endocr Pathol 14:167–175

51. Lloyd RV, Erickson LA, Casey MR, Lam KY, Lohse CM, Asa SL, Chan JK, DeLellis RA, Harach HR, Kakudo K, LiVolsi VA, Rosai J, Sebo TJ, Sobrinho-Simoes M, Wenig BM, Lae ME 2004 Observer variation in the diagnosis of follicular variant of papillary thyroid carcinoma. Am J Surg Pathol 28:1336–1340

52. Aldred MA, Huang Y, Liyanarachchi S, Pellegrata NS, Gim O, Jhiang S, Davuluri RV, de la Chapelle A, Eng C 2004 Papillary and follicular thyroid carcinomas show distinctly different microarray expression profiles and can be distinguished by a minimum of five genes. J Clin Oncol 22:3531–3539

53. Finley DJ, Arora N, Zhu B, Gallagher L, Fahey 3rd TJ 2004 Molecular profiling distinguishes papillary carcinoma from benign thyroid nodules. J Clin Endocrinol Metab 89:3214–3223

54. Mazzanti C, Zeiger MA, Costouros N, Umbricht C, Westra WH, Smith D, Somervell H, Bevilaqua G, Alexander HR, Libutti SK 2004 Using gene expression profiling to differentiate benign versus malignant thyroid tumors. Cancer Res 64:2898–2903