Molecular Markers in Thyroid Cancer Diagnostics

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Approximately 37,340 cases of thyroid cancer were projected for 2008 with resultant 1590 deaths, making it the most common endocrine malignancy in the United States. Thyroid cancer accounts for 2.5% of cancer incidence, but only 0.28% of cancer deaths. Clinically evident thyroid nodules are found in approximately 4.2% of the general population, but the number found on autopsy is much higher, ranging from 8% to 65%. Nodules are more common in women and in patients with a history of radiation exposure. The incidence of thyroid cancer has more than doubled in the last 30 years. This increase is largely due to better radiographic detection of subclinical nodules rather than a truly increased incidence of the disease.

Fine-needle aspiration biopsy (FNA) was adopted into widespread use in the 1970s and 1980s and greatly enhanced the ability to diagnose thyroid nodules preoperatively. Indeed, preoperative FNA reduced the number of thyroid surgeries by at least 25% and doubled the yield for cancer.

Unfortunately, thyroid nodules present clinicians with a diagnostic conundrum as cytologic interpretations of FNAs are limited. Diagnostic outcomes of FNAs fall into 3 broad categories: benign, malignant, and indeterminate. The indeterminate group includes lesions with cellular atypia, suspicious lesions, and follicular lesions, all of which have some concerning features, but fail to meet criteria for diagnosis of cancer. Between 60% and 80% of FNAs are benign, 4% to 7% are malignant, and 10% to 20% are indeterminate. The standard of care for indeterminate lesions is surgery to enable a histologic diagnosis. Unfortunately, only 4% to 30% are found to be malignant on histopathology, ultimately rendering many of these operations unnecessary. Furthermore, considerable interobserver variability is reported in the cytologic interpretation of FNA specimens.

One of the greatest challenges in thyroid cancer research is to develop an adjunct to FNA to clarify the indeterminate lesions as benign or malignant. Such a marker would...
improve preoperative prognostication and prevent risks associated with surgery in patients with benign lesions. Unfortunately, no one marker has proven the panacea. Nonetheless, several areas of research show great promise in the molecular diagnosis of these lesions.

**IMMUNOCYTOCHEMICAL MARKERS**

**Galectin-3**

Galectins are a family of animal lectins defined by their ability to bind β-galactosides and by their consensus amino acid sequences. Galectins have an affinity for a wide variety of glycoproteins and glycolipids. These lectins exert effects in the cytoplasm and at the cell surface where they mediate interactions among cells, and between the cells and extracellular matrices. Some members can induce apoptosis whereas others, including galectin-3, transform cells in culture to a malignant phenotype. Galectins modulate the behavior of cells including cell adhesion and migration, and thus affect metastatic capabilities of tumors.13

Galectin-3 seems to play a large role in the pathogenesis of papillary thyroid cancer (PTC). Yoshii and colleagues transfected a PTC cell line with antisense galectin-3 cDNA and saw a significant suppression of anchorage-independent cell proliferation. These investigators concluded that galectin-3 was necessary to maintain the malignant phenotype in the PTC cell line.14 In another study, the same group transfected human fetal follicular cells with plasmids containing galectin-3 and transformed them into a malignant phenotype. The transformation was demonstrated by increased saturation density, serum-independent growth and clonogenic potential.15 Several groups established galectin-3 expression in follicular cell malignancies but not in benign lesions or normal thyroid tissue. Expression was detectable in specimens from tissue as well as by FNA, and separated benign from malignant lesions of the thyroid.16–20 This work opened the door to the possibility of galectin-3 being used as a diagnostic marker for thyroid cancers.

Saussez and colleagues looked at the differential serum levels of galectin-3 in patients with benign lesions and malignant tumors versus controls. The median serum level of galectin-3 was significantly different between the control and malignant groups, between control and benign groups and, most importantly, between the benign and malignant groups. Using a cutoff level of 3.2 ng/mL, the investigators calculated 74% sensitivity, 73% specificity, a positive predictive value of 57%, and a negative predictive value of 85% for detecting a thyroid malignancy among multinodular goiters.21 However, another group led by Inohara did not detect differences in serum galectin-3 between patients with benign and malignant lesions.22

The greatest hope for galectin-3 is as an adjunct to traditional cytopathology of FNA biopsies. Several groups have looked at the diagnostic feasibility of using immunocytochemistry for galectin-3 in FNA specimens.17,23–27 Sensitivities in these studies ranged from 75% to 100% with specificities from 75% to 100%. In the largest study to date, a prospective multicenter trial, Bartolazzi and colleagues looked at galectin-3 levels in 465 FNA cytology specimens and compared them to final pathology of the surgical specimens. These investigators found a 78% sensitivity and 93% specificity. Of note, 29 of 130 (22%) cancers were missed using the galectin-3 test.23

Several investigators issue caveats about using galectin-3 in thyroid nodule diagnostics. Mehrotra and colleagues28 did not find that galecin-3 distinguished benign from malignant tissue samples, but were sharply criticized for their methods.29 In a systematic review of the available data on FNA and galectin-3, Sanabria and colleagues30 concluded that many of the studies had important methodological
weaknesses and were not designed to properly evaluate galectin-3 as a diagnostic test. Those that they considered appropriate did not suggest that galectin-3 was a powerful enough marker to be used as a single entity.

Whereas it probably does not have utility as a single marker for thyroid carcinoma, available data suggest that galectin-3 does have potential when used in conjunction with cytology and other markers currently under study (see later discussion).

**HBME-1**

Hector Battifora mesothelial cell antibody (HBME-1) is a mouse monoclonal antibody originally developed to stain malignant mesothelioma. Early studies found that it also stained thyroid cancers of follicular origin, particularly PTC, with a much greater affinity than benign thyroid lesions.

Subsequent work established that the antibody also stained cells from FNA biopsies. Van Hoeven and colleagues evaluated HBME-1 as a diagnostic test and found 100% sensitivity and 76% specificity for thyroid malignancy in a small patient sample ($n = 29$). Larger-scale studies found sensitivities of 80% to 90%, but specificities varied widely, from 60% to 96%. Although encouraging, HBME-1 has not demonstrated utility as a single test for thyroid nodule diagnostics. Many investigators have looked at HBME-1 in combination with other markers as part of a panel.

**IMMUNOCYTOCHEMICAL COMBINATIONS**

**HBME-1 and Galectin-3**

Perhaps the greatest potential for these markers is when they are used in combination with each other. Several groups looked at HBME-1 and galectin-3 in concert. Using tissue microarray, Wiseman and colleagues assembled a panel of 7 immunohistochemical markers, including HBME-1, galectin-3, and CK-19, and calculated 87.9% sensitivity and 94% specificity for cancer. Rossi and colleagues looked at the combination of HBME-1 and galectin-3 in tissue samples and found 95% sensitivity and 100% specificity for cancer. Similar results were found on testing FNA samples. Saggiorato and colleagues calculated a sensitivity of 97% and specificity of 91% for cancer after testing 125 FNA specimens.

**HBME-1 and Cytokeratin-19**

Scognamiglio and colleagues found that HBME-1 together with CK-19 had 83% sensitivity and 100% specificity for malignancy when staining tissue samples. Nga and colleagues applied HBME-1 and CK-19 to FNA specimens and found a 100% sensitivity and 100% specificity, but the sample population was small ($n = 22$).

**hTERT and Telomerase**

DNA polymerases are unable to replicate the complete sequence of a chromosome. To ensure that functional components of the genome are replicated, the ends of chromosomes are capped with telomeres, repeats of the nonsense sequence TTAGGG. With each cycle of DNA replication, the telomeres at the end of chromosomes shorten. Telomerase is a ribonucleoprotein that functions to lengthen the telomeres and ensure seamless duplication of DNA. Without it, chromosomes can become unstable and enter senescence; with it, cells can gain immortality. Telomerase is not found in healthy somatic cells, except stem cells, lymphocytes, and germine cells. Telomerase activity is also sharply increased in most malignancies.

Many groups have looked at activity of telomerase in thyroid tissues. Whereas most groups detected higher levels in malignant thyroid tumors compared with benign lesions, the authors’ group did not. Higher activity correlated to higher stage
on diagnosis, tall-cell variant of PTC, undifferentiated cancers, extrathyroidal extension, recurrence, distant metastasis, and older age. Not surprisingly, high activity levels were found in specimens with background thyroiditis and in one case of thyroid lymphoma.

The catalytic subunit of telomerase is hTERT. Some groups have looked into hTERT expression as a potential marker for thyroid malignancy. As with telomerase activity, they found higher expression levels of hTERT in malignant specimens than benign ones. Wang and colleagues looked at splice variants of hTERT and found that malignant tumors exhibited a greater proportion of the active variant when compared with benign lesions.

Whereas every study save one found higher activity or expression levels in malignant nodules, nearly every one also found some activity in benign lesions, such as follicular adenomas and thyroiditis. This result limits the diagnostic potential for these markers by lowering specificity. Nonetheless, several groups have explored the diagnostic usefulness of telomerase and hTERT in FNA. Two groups concluded that these markers had no diagnostic utility. Lerma and colleagues looked at FNAs with suspicious findings but which lacked criteria for a malignant diagnosis. Telomerase activity was positive in 6 of 18 specimens, but only 5 of those 6 were malignant on histology. Of note, they excluded samples with an abundance of lymphocytes. In contrast, Guerra and colleagues found that telomerase testing clarified the diagnosis in 5 of 11 FNAs with indeterminate cytology. Despite these findings, the variation of expression within a given tumor type and the consistent positivity among benign lesions renders telomerase and hTERT short of the sensitivity and specificity needed for them to be useful diagnostically.

GENETIC MARKERS

BRAF

The RAF protein is a serine-threonine kinase along the MAP kinase cascade. The RAF protein has 3 isoforms, ARAF, BRAF, and CRAF, with BRAF being the most common found in thyroid follicular cells. All forms of RAF activate MEK in the MAP kinase cascade, but BRAF does so much more avidly than the other forms. The most common BRAF mutation, occurring in more than 95% of cases, is the BRAF1799A. Here an A/T transversion at position 1799 results in a valine to glutamate substitution at residue 600 (V600E). This occurrence destabilizes the inactive form of the protein, resulting in its constitutive action.

The BRAF mutation is the most common mutation in PTC, occurring in 35% to 69%. This mutation is specific to classic and tall-cell variants of PTC; it is rarely found in other types of differentiated thyroid cancer. The BRAF mutation occurs early and plays an important role in the pathogenesis of PTC. In one study, Knauf and colleagues showed thyroid-specific expression of the mutation in mice induced invasive PTC. The mutation confers a worse clinical prognosis as it is associated with older age at diagnosis, tall-cell histology, extrathyroidal extension, lymph node metastasis, recurrence, and advanced tumor stage.

Many groups have looked into adding BRAF testing as an adjunct to cytology on FNAs. Knowing BRAF status preoperatively could be reassuring for lesions with benign cytology and could indicate more aggressive surgery in malignant ones. Because of its prevalence and specificity in PTC, testing FNA samples for the BRAF mutation could theoretically classify some indeterminate or follicular lesions as malignant. The majority of studies show that BRAF testing of FNA specimens was highly concordant with BRAF results derived from tissue. Most found BRAF mutations...
among the indeterminate group of FNAs, but the numbers varied widely and tended to be small; BRAF status clarified between 5% and 43% of indeterminate lesions. Of note, 2 studies found no BRAF mutations among their indeterminate lesions.

BRAF is an extremely important marker in PTC, and there may be a role for preoperative testing to prognosticate and to guide surgical decisions. BRAF testing in FNA specimens is highly specific. However, it lacks the sensitivity necessary to clarify a large proportion of indeterminate or follicular lesions and is unlikely to come into widespread use for this purpose.

**RET/PTC**

Another derangement along the MAP kinase cascade associated with differentiated thyroid cancer is the RET/PTC rearrangement. RET is the signaling subunit of the receptor for the glial-derived neurotrophic factor family of genes. Although it is normally expressed in c-cells of the thyroid, it is not highly expressed in follicular cells. In the rearrangement, the 3' end of RET fuses with the 5' end of an unrelated gene. There are at least 11 RET/PTC rearrangements described to date, the 2 most common being RET/PTC1 and RET/PTC3. The resulting RET/PTC oncogene constitutively activates the MAP kinase cascade.

RET/PTC oncogene is found in approximately 20% of PTC and is not commonly seen in other thyroid cancers. The rearrangement is associated with a younger presentation, classic PTC histology, and an increased risk for lymph node metastasis. Early studies showed that transgenic mice expressing the RET/PTC1 or RET/PTC3 oncogene developed papillary carcinoma. RET/PTC rearrangements are associated with childhood and radiation induced PTC. Among post-Chernobyl children with PTC, RET/PTC1 was found in 16% and RET/PTC3 in 58%. In sporadic childhood PTC, RET/PTC1 was found in 47% and RET/PTC 3 in 18%. RET/PTC rearrangements were significantly more frequent among Japanese adult survivors of the atomic bombings during World War II compared patients not exposed to radiation. Further studies conducted in Japan explored the direct relationship between radiation and PTC. Ito and colleagues induced RET/PTC rearrangements in undifferentiated thyroid cells by exposing them to 50 Gy of radiation. Mizuno and colleagues induced PTC in human thyroid tissue transplanted into nude mice. This group found that the rearrangement was present as early as day 2 of exposure to 50 Gy.

As with BRAF mutations, RET/PTC rearrangements have been explored as a possible adjunct to FNA cytology. In most of the studies, RET/PTC testing helped to classify few if any indeterminate lesions. Cheung and colleagues tested 45 indeterminate or insufficient FNA specimens for RET/PTC 1, 2, and 3, and reached a malignant diagnosis in 11 of them or 24%. As a caveat, however, Elisei and colleagues found RET/PTC rearrangements in the benign lesions of 52% of post-Chernobyl, 38% medically irradiated and sporadic patients. Domingues and colleagues found rearrangements in FNA specimens from benign lesions as well. Like BRAF, RET/PTC rearrangements offer opportunities to study the pathogenesis of PTC, but offer little in the way of diagnostics.

**PAX8-PPARγ**

The PAX8-PPARγ oncogene is the result of the interchromosomal translocation t(2,3)(q13;p25) joining paired box 8 (PAX8) and peroxisome proliferator-activated receptor gamma (PPARγ). The PAX8-PPARγ oncogene was originally thought to be unique to follicular carcinoma of the thyroid (FTC), but has since been detected in follicular adenoma as well. The oncogene is found in 29% to 56% of FTC and in
4% to 13% follicular adenomas, and is not seen in PTC, anaplastic thyroid carcinoma (ATC), goiters, Hurthle cell adenomas, Hurthle cell carcinomas, or poorly differentiated thyroid cancers.\textsuperscript{100–103} One study found the oncogene in 55% of adenomas, but sampled only 11 cases.\textsuperscript{102} Another study in Japan found no rearrangements in FTC, suggesting possible regional variation.\textsuperscript{104} This oncogene is associated with more favorable prognostic indicators such as female sex, improved tumor differentiation, and lower risk of metastasis.\textsuperscript{105}

The mechanism of the PAX8-PPAR\textsubscript{\textgamma} oncogene is incompletely understood. The authors do know that PAX8-PPAR\textsubscript{\textgamma} is near mutually exclusive with RAS mutations and thus is likely to constitute a distinct molecular pathway.\textsuperscript{106} The PAX8-PPAR\textsubscript{\textgamma} fusion oncoprotein transforms human thyrocytes in vitro to a malignant phenotype and likely exerts its action by interfering with the transcriptional function of the wild-type PPAR\textsubscript{\textgamma} protein.\textsuperscript{107,108} The wild-type PPAR\textsubscript{\textgamma} is upregulated in tumors with the PAX8-PPAR\textsubscript{\textgamma} rearrangement.\textsuperscript{109} Several groups have used gene expression microarray to better characterize transcriptional changes specific to PAX8-PPAR\textsubscript{\textgamma} mutants. Functions of differentially expressed genes run the gamut from lipid/glucose/amino acid metabolism, tumorigenesis, angiogenesis, signal transduction, cell growth, and translational control.\textsuperscript{110,111} Of note, Giordano and colleagues\textsuperscript{112} found that PAX8-PPAR\textsubscript{\textgamma} mutants had upregulated expression of known wild-type PPAR\textsubscript{\textgamma} targets.

Only one group has pursued PAX8-PPAR\textsubscript{\textgamma} testing as a diagnostic adjunct. Sahin and colleagues\textsuperscript{105} found that performing PPAR\textsubscript{\textgamma} immunohistochemistry, as a surrogate for PAX8-PPAR\textsubscript{\textgamma}, improved the sensitivity of intraoperative frozen section from 84% to 96%, but lowered specificity from 100% to 90%.

**MicroRNA**

First described by Lee and colleagues in 1993, microRNAs (miRNA) are short sequences of noncoding RNA that negatively regulate posttranscriptional gene expression. MiRNAs are 19 to 25 base pairs long and are relatively few in number; there are currently 695 listed on the miRBase database maintained by the Sanger Institute (http://microrna.sanger.ac.uk/sequences/). Changes in the expression levels of miRNAs are seen in every cancer studied to date, including lymphomas, colorectal carcinoma, breast cancer, lung cancer, hepatocellular carcinoma, and thyroid cancer. MiRNA regulation has been implicated in the control of metastasis, invasion, proliferation, cell cycle, and apoptosis.\textsuperscript{29} As such, they have generated much interest in the diagnosis, prognosis, and treatment of cancer.

Several groups have explored the expression of miRNA in PTC. Early studies used array techniques to test a broad range of known miRNAs in benign and malignant thyroid specimens.\textsuperscript{113,114} This early work revealed that miR-221, miR-222, and miR-146b were upregulated in PTC samples in several studies.\textsuperscript{113–115} Pallante and colleagues\textsuperscript{114} observed that overexpression of miR-221 caused increased proliferation and knockdown caused growth arrest. In the same study, the group transfected a normal differentiated rat thyroid cell line with mutations implicated in PTC such as RAF and RET/PTC, and saw a corresponding increase in miR-221 with each new malignant phenotype. Using bioinformatic analysis they identified p27\textsuperscript{kip1}, an important regulator of cell cycle, as the target for miR-221 and miR-222.\textsuperscript{116} Nikiforova and colleagues showed there was a strong relationship between miRNA expression and mutation status in thyroid tumors. These investigators found that miRNA expression levels could accurately cluster tumor specimens by mutation.\textsuperscript{113}

Work into other cancers of the thyroid is under way as well. Weber and colleagues found that both miR-197 and miR-346 were overexpressed in follicular thyroid cancer when compared with follicular adenomas. In vitro overexpression induced proliferation...
whereas knockdown led to growth arrest. This group showed that the likely targets for these miRNAs were the genes ACVR1 and TSPAN3 for miR-197 and EFEMP2, and CFLAR for miR-346. Of note, they were able to accurately identify FTC in 87% of thyroid specimens based on the expression profile of ACVR1, TSPAN3, and EFEMP2. Visone and colleagues looked at the miRNA expression in anaplastic thyroid cancer and found decreased levels of miR-30d, miR-125b, miR-26a, and miR-30a-5p.

The distinct patterns of expression noted among the miRNAs have led some groups to explore the use of miRNA in thyroid nodule diagnostics. Chen and colleagues used reverse transcription-polymerase chain reaction (RT-PCR) to determine the expression levels of miR-221, miR-222, and miR-146b in thyroid FNAs from PTC and benign lesions. All 3 miRNAs were upregulated in malignant specimens, and miR-222 and miR-146b were statistically significant between malignant and benign samples. Nikiforova and colleagues compiled a panel of 7 miRNAs differentially expressed between differentiated cancers of the thyroid and benign lesions and then tested its utility against 13 FNAs. This group found that when one marker was overexpressed more than 2-fold, the sensitivity for malignancy was 100% with a specificity of 94% and accuracy 94%. When 3 or more markers were overexpressed the sensitivity dropped to 88%, but the specificity and accuracy jumped to 100% and 98%, respectively.

**MICROARRAY**

As applicability of single gene or single protein markers in the diagnosis of thyroid nodules has proven to be disappointing, many groups have turned to high-throughput methods such as microarray. Distinct expression profiles have been identified for many diseased and normal tissues, including thyroid. This technology has been used to explore tumor biology and pathogenesis as well as tumor diagnostics.

A microarray chip contains probe sets to measure quantitatively the expression profiles of thousands of genes. Powerful statistical tools are able to separate accurately the expression profiles according to disease state, and produce a list of genes differentially expressed between tissues carrying various diagnoses. Differential expression of specific genes in an unknown sample can be used to make a diagnosis. Many academic and commercial groups are currently working to create and test gene lists with utility in the clinical realm.

The authors’ group has explored microarray technology in thyroid disease for the last 7 years. Early on it was shown that microarray accurately separated benign from specific malignant lesions including PTC, FTC, and Hurthle cell carcinoma. In hopes of separating malignant from benign lesions in a broader sense, the authors compared expression between a mix of malignancies and a mix of benign lesions. The resulting expression profile was able to detect malignancy with a sensitivity of 90.9% and a specificity of 96.2%. Encouraged by these results, the authors explored the application of microarray in FNA specimens. Clustering analysis of the expression profiles generated from a mix of benign and malignant FNA specimens resulted in 3 distinct groups, malignant (n = 10), benign (n = 7), and indeterminate (n = 5; 3 benign, 2 FVPTC). The classification of malignant or benign was 100% concordant with the histologic diagnosis. In contrast, the cytologic diagnosis of these 17 FNA specimens was only 76% concordant (13 out of 17). All 5 in the indeterminate cluster had suspicious findings on cytologic evaluation.

Others have explored the application of microarray to distinguish benign from malignant thyroid lesions as well. In one of the earliest studies, Mazzanti and
Table 1
Comparison of molecular markers for thyroid carcinoma currently under study

<table>
<thead>
<tr>
<th>Method</th>
<th>Marker Description</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>References</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocytochemistry</td>
<td>HBME-1 and Galectin-3</td>
<td>95</td>
<td>100</td>
<td>Rossi(^{36})</td>
<td>Experiments done on paraffin-embedded tissue blocks, no validation set (n = 95)</td>
</tr>
<tr>
<td>Immunocytochemistry</td>
<td>HBME-1 and Galectin-3</td>
<td>97</td>
<td>91</td>
<td>Saggiorato(^{26})</td>
<td>Experiments done on preoperative FNAs classified as follicular neoplasms, no validation set (n = 125)</td>
</tr>
<tr>
<td>Immunocytochemistry</td>
<td>HBME-1 and CK-19</td>
<td>83</td>
<td>100</td>
<td>Scognamiglio(^{37})</td>
<td>Experiments done on tissue from paraffin-embedded blocks, only follicular adenomas and PTC, no validation set (n = 127)</td>
</tr>
<tr>
<td>Immunocytochemistry</td>
<td>HBME-1 and CK-19</td>
<td>100</td>
<td>100</td>
<td>Nga(^{38})</td>
<td>Validation done on FNAs collected ex vivo (n = 22)</td>
</tr>
<tr>
<td>MicroRNA</td>
<td>Three of the following more than 2-fold overexpressed: miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224, miR-197</td>
<td>88</td>
<td>100</td>
<td>Nikiforova(^{113})</td>
<td>Validation done on RNA from preoperative FNA (n = 13)</td>
</tr>
<tr>
<td>Microarray</td>
<td>627 genes more than 2-fold differentially expressed</td>
<td>91</td>
<td>96</td>
<td>Finley(^{124})</td>
<td>Validation done on RNA from snap-frozen tissue (n = 14)</td>
</tr>
<tr>
<td>Microarray/Multigene assay</td>
<td>Differential expression of G3PDH, SYNGR2, LSM7, KIT, Hs.296031, c21orf4 and Hs24183</td>
<td>75</td>
<td>100</td>
<td>Rosen(^{135})</td>
<td>Validation done on RNA from snap-frozen tissue (n = 10)</td>
</tr>
<tr>
<td>Multigene assay</td>
<td>Differential expression of MCM5, MCM7 and RAD9</td>
<td>98</td>
<td>66</td>
<td>Kebebew(^{141})</td>
<td>Experiments done on RNA from snap frozen tissue, no validation set (n = 95)</td>
</tr>
<tr>
<td>Multigene assay</td>
<td>Differential expression of ECM1, TMPRSS4, ANGPT2 and TIMP1</td>
<td>91</td>
<td>95</td>
<td>Kebebew(^{144})</td>
<td>Validation done on RNA from intraoperative FNAs (n = 31)</td>
</tr>
</tbody>
</table>
colleagues used a teaching set of 63 samples to generate a gene list to define benign and malignant within the group. These investigators built 6-gene and 10-gene models from this list and tested them using 10 unknown samples. Using the analysis of variance test with Bonferroni correction and the leave-one-out method of cross-validation, both the 6- and 10-gene models separated benign from malignant with 100% accuracy. In a subsequent study, the group used expression from 41 tumor specimens to build an expression-ratio model based on the 6 genes. The model was tested using 10 unknown samples, and 75% sensitivity and 100% specificity for malignancy were calculated. In one of the largest studies, Prasad and colleagues generated a 75 gene list from 94 samples (50 benign, 44 malignant) and validated it using RT-PCR and Western blot on a new set of 31 thyroid tumors. This group documented differential expression of 6 of the 75 genes between benign and malignant as predicted by microarray.

**Multigene Assays**

An interesting offshoot from microarray studies is the development of gene panels to distinguish benign from malignant thyroid lesions. The group led by McMillan and Kebebew has been especially active in this area. This group developed several smaller arrays with 96 genes to determine expression of a more targeted group of genes. One array tested the differential expression of cell cycle regulatory genes and isolated 3 (MCM5, MCM7, and RAD9), which separated malignancies with a 98.2% sensitivity and a 65.7% specificity. In similar studies, Kebebew and colleagues put extracellular matrix and adhesion molecule and angiogenesis-modulating gene probes on 2 separate 96-gene arrays. In this study they used the expression profiles of at least 100 tumors on each array to generate a list of 4 differentially expressed genes with diagnostic potential (ECM1, TMPRSS4, ANGPT2, and TIMP1). In a separate study, Kebebew and colleagues tested the panel against 31 FNA specimens and developed a scoring model for expression. The panel had a sensitivity of 91% and a specificity of 95%.

**SUMMARY**

Although FNA biopsy is an important preoperative test for thyroid nodules, it does not provide a diagnosis in up to 20% of patients with indeterminate lesions. These patients undergo surgery with its incumbent risks, but a low percentage of them carry a diagnosis of cancer. Early explorations into single markers showed promise, but ultimately lacked either the sensitivity or specificity necessary for wide adoption. Assays testing several markers show the greatest promise in this conundrum (Table 1). Immunocytochemistry testing for 2 or more markers has shown a great improvement on single-marker tests. The recent application of miRNAs and the powerful analysis enabled by microarray are also exciting. Future work will determine how many markers (panels of 6, 10 or 100?) measured by what technique (immunocytochemistry, microarray, RT-PCR, enzyme-liked immunosorbent assay?) will ultimately solve this diagnostic dilemma.

**REFERENCES**


23. Bartolazzi A, Orlandi F, Saggiorato E, et al. Galectin-3-expression analysis in the surgical selection of follicular thyroid nodules with indeterminate fine-needle


