
17. DIAGNOSTIC MOLECULAR MARKERS IN THYROID CANCER

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INTRODUCTION

Thyroid cancer is the commonest classical endocrine tumor, accounting for approximately 1% of all cancers. The diagnosis of thyroid cancer is typically made on cytopathologic features on fine needle aspiration or histological features on surgical samples. Following treatment of patients with thyroidectomy, and in some cases radioiodine, patients are monitored for disease recurrence using a variety of scanning modalities and serum thyroglobulin. The accuracy of both the preoperative testing and postoperative monitoring is excellent in many cases; however, there are some important deficiencies that have led to the development new tools for clinical use. Specifically, the application of molecular methods to the analysis of pathology and blood samples has led to the development of highly sensitive markers for the diagnosis of new cases of thyroid cancer, and in the evaluation of patients for recurrent disease. In this review, the molecular analysis of thyroid nodules, lymph nodes and peripheral blood as adjunctive tests for thyroid cancer will be discussed.

PREOPERATIVE EVALUATION OF THYROID NODULES

Thyroid nodules are extremely common with prevalence rates approaching 50–60% of adults under 60 years old. Because only approximately 5% of thyroid nodules are malignant, accurate pre-operative characterization of thyroid nodules is critical in selecting patients appropriate for surgical thyroidectomy. Fine needle aspiration (FNA) is the single most important diagnostic procedure in the evaluation of thyroid nodules.

Table 1. Molecular markers for thyroid nodules and lymph nodes

Potential diagnostic markers for thyroid nodules and lymph nodes	
Telomerase	GLUT-1
Galectin-3	CA 19-9
Thyroid peroxidase	CD 15
Thyroglobulin	HBME-1
Oncofetal fibronectin	CD 30
ret/PTC oncogenes	CD 57
Pax8/PPAR γ oncogene	CD 97
B-Raf mutations	Leu-7
Nm23	Epithelial Membrane Antigen
High mobility Group I (Y) Protein	Cyclooxygenase 2
Ceruloplasmin	Cytokeratin 19
Survivin	Cytokeratin 20
	TSH Receptor Hypermethylation

For small, solid nodules, experienced cytopathologists can accurately distinguish most benign nodules and papillary cancers. However, cytological features do not distinguish benign from malignant follicular neoplasms, and cystic papillary thyroid cancers are a common cause of false negative results. Importantly, only 15% of cytological follicular neoplasm will ultimately be follicular carcinomas; therefore, 85% of individuals that undergo surgery for these nodules will have done so unnecessarily. Finally, by its nature, cytopathologic interpretation of FNA samples is subjective. For these reasons, the application of molecular analysis to better characterize thyroid nodule cytologic samples has been an area of intense interest.

With the advent of methods, such as reverse transcriptase-polymerase chain reaction (RT-PCR), in which tiny amounts of samples are suitable for analysis, and increases in the number of antibodies suitable for immunocytochemistry, the possibility of improving FNA-based characterization of thyroid nodules is now possible. In the initial section of this review, several of the most carefully studied molecular markers (Table 1) for thyroid FNA will be discussed.

Telomerase

Telomeres are chromosomal end structures, consisting of tandem repeats of TTAGGG that play a critical role in the protection of chromosomes during cell division and are important in chromosome positioning during replication (1). Chromosomes typically lose about 50 to 200 nucleotides of telomeric sequence from chromosomal ends per cell division because DNA polymerase is unable to replicate the ends of linear DNA. The resultant progressive shortening of chromosomes as cells divide has been described a cellular “biological clock”; once the chromosomes are shortened to a critical length through telomeric loss, cell growth stops and apoptosis is induced. Therefore, preservation of chromosomal end length during division would be expected to retard this natural “aging” of cells and result in continuous cell growth.

Telomerase is an enzyme that extends telomeres, thereby preserving chromosomal length. Structurally, telomerase is a ribonucleic acid-protein complex containing a catalytic component, the human telomerase reverse transcriptase (hTERT) (2). Telomerase expression and activity have been identified in immortalized human cell lines and cells and in bone marrow cells that normally divide, but not in normal human adult epithelial cells. However, expression of telomerase and demonstration of telomerase activity using a PCR-based assay (TRAP; Telomeric Repeat Amplification Protocol) has been described in a variety of malignancies and other dividing cells, such as germinal cells of the ovary and testis (1). Based on these results, detection of telomerase activity and expression of hTERT have been explored as potential distinguishing markers for the presence of malignant thyroid cells.

Saji et al. (3) evaluated surgical pathology samples from thirty papillary thyroid cancers, three benign nodules and ten normal thyroid specimens for telomerase activity by TRAP and found that 67% of the malignant tissues had telomerase activity compared to 0% of the benign nodules. In this study, 64% of the papillary thyroid cancers that had a non-diagnostic preoperative FNA were positive for telomerase activity. Haugen et al. (4) and Onoda, et al. (5) obtained similar results when they investigated surgical thyroid specimens.

The ability of telomerase help differentiate follicular adenoma from carcinoma was reported by Umbricht et al. (6) who studied frozen tissue samples from patients undergoing thyroidectomy for follicular neoplasm on FNA. TRAP assays were performed on 44 follicular thyroid tissue specimens and 22 normal thyroid tissue samples. The authors reported a sensitivity of 100% and a specificity of 76% for detecting follicular carcinoma. The false positive samples occurred mostly in tumors that also had lymphocytic infiltration, as lymphocytes are known to have detectable telomerase activity. However, it is possible that the presence of telomerase activity in histologically benign specimens may represent an early step in the development of an invasive tumor. This issue of potentially creating an assay that is more sensitive than the clinical gold standard is a problem for many RT-PCR-based assays.

De Deken et al. (7) demonstrated a decrease of telomere length as well as increased variability in the telomere size in benign nodules without measurable telomerase activity, as compared to normal thyroid tissues. These data suggest that the benign nodular cells may have progressed through more mitotic divisions than the adjacent normal tissue and therefore may be closer to their limit for further growth, consistent with benign tumor growth.

The cloning of the human telomerase reverse transcriptase cDNA allowed for RT-PCR analysis of expression of its mRNA in clinical samples. This created an opportunity to design a more user-friendly telomerase assay that could be applied to FNA samples. Saji et al. (8) studied 19 malignant and 18 benign thyroid surgical samples for evidence of hTERT gene expression by RT-PCR. hTERT mRNA was detected in 15 (79%) of the malignant and 5 (28%) of the benign tumors; all 5 benign lesions with demonstrable hTERT gene expression had lymphocytic infiltration on final pathology. hTERT mRNA was not detected in any of the normal thyroid specimens. The

results correlated with TRAP assay results from the same samples. Similar results were reported by the same authors using thyroid FNA samples (9).

Fine needle aspiration specimens have also been investigated for telomerase activity using TRAP assay. Sebesta et al. (10) failed to show any additional usefulness of measuring telomerase activity in a small study of FNA samples. It is likely that RT-PCR of hTERT is more sensitive than TRAP assay due to the logarithmic amplification inherent in RT-PCR, thus accounting for a greater sensitivity when used to analyze FNA samples.

The frequency of telomerase-positive results, either by RT-PCR or TRAP assay for papillary thyroid cancer varies between studies. For example, some report that as many as 67% of papillary thyroid cancers are positive (3), while others indicate a much lower percentage of about 20% (11). The small number of cases in many of these studies makes interpretation quite difficult. Similarly, results regarding the association between telomerase activity and tumor aggressiveness also vary; some studies demonstrate a correlation between telomerase activity and tumor progression (5, 12) while others do not (3). It is clear that larger, more extensive studies are needed before telomerase can be considered an effective diagnostic tool.

Galectin-3

Galectin-3 is a member of the lectin family that regulates the functions of its protein targets by interacting with attached galactose-containing glycoprotein side chains. As a group, galectins regulate cell growth and differentiation, intercellular recognition and adhesion, as well as malignant transformation. Galectin-3 levels have been directly correlated with metastatic potential in fibrosarcoma and melanoma cell lines. *In vitro* studies have indicated that expression of galectin proteins is elevated in thyroid cancer cell lines and microarray analysis has demonstrated increased levels of galectin-3 in papillary thyroid cancers (13) This led to their investigation as molecular markers used to distinguish between benign and malignant thyroid tissues.

Xu, et al. (14) evaluated protein derived from 41 surgical thyroid specimens for galectin-1 and galectin-3 expression by Western blot. Elevated levels of both proteins were demonstrated in thyroid cancer compared to normal thyroid tissue. Similarly, normal thyroid tissue did not express galectin 1 or 3 by immunohistochemical analysis, but high levels of both proteins were detected in both papillary and follicular thyroid cancers, and in regional nodal metastases. A second group evaluated 41 malignant and 35 benign thyroid tissue specimens (15) for both galectin-3 protein and RNA levels. Galectin-3 expression was identified in 18 of 18 of papillary cancer samples, 4 of 8 follicular cancers, 2 of 3 poorly differentiated cancers, 5 of 5 anaplastic cancers, 3 of 6 medullary, and 1 of 1 Hurthle cell cancers. By contrast, none of the normal or benign nodular tissues expressed galectin-3, other than those with lymphocytic infiltration. Levels of galectin-3 mRNA appeared to correlate with protein levels in papillary cancers and normal tissue (15).

To determine if galectin-3 immunocytochemistry could be applied to FNA samples, Orlandi, et al. (16) evaluated FNA and surgical pathology specimens from 64 patients who had undergone thyroidectomy whose preoperative diagnosis was

malignant (n = 15), indeterminate (n = 37), and benign (n = 12). The final histologic diagnosis included 18 papillary and 17 follicular cancers, as well as 29 follicular adenomas. All papillary thyroid cancers expressed galectin-3 in both FNA and surgical specimens. For the follicular cancers, immunoreactive galectin-3 was detected in all surgical specimens in a heterogeneous pattern, and in all but 3 FNA samples. By contrast, only 3 of 29 benign follicular adenomas expressed galectin-3.

In a more recent study (17), different antibodies against human galectin-3 were used in an immunohistochemical study of thyroid surgical specimens (13 benign and 62 malignant). Immunoreactive galectin-3 was most prevalent in the papillary thyroid cancers (33 of 45), but some benign lesions were 3 of 8 benign adenomas demonstrated immunoreactive galectin-3.

Finally, Bernet, et al. (18) applied quantitative RT-PCR to galectin-3 analysis to determine if a particular "cut-point" of galectin-3 gene expression correlated best with malignancy. In this study, markedly elevated levels of galectin-3 mRNA were identified in papillary cancers compared with normal tissue. There was no difference between the galectin-3 mRNA levels in follicular adenomas and carcinomas.

Based on the above results, galectin-3 immunocytochemistry seems to be a promising new marker of thyroid cancer that could be applied to FNA analysis. It appears that classic molecular approaches, such as quantitative RT-PCR may not be helpful for the conundrum of follicular neoplasm FNA results. However, additional studies are still required.

Thyroid peroxidase

Thyroid peroxidase (TPO) is a thyroid-specific enzyme that catalyzes iodide oxidation, thyroglobulin iodination, and iodothyronine coupling. Reduced expression of TPO impairs thyroid follicular cell function correlates with a loss of differentiated thyroid function and has been well described in thyroid cancer cell lines and tumor samples. Thus, immunohistochemical staining for TPO expression and molecular analysis of the TPO gene have been studied for use as diagnostic tools for thyroid cancer.

DeMicco, et al. reported a retrospective study of 150 FNA samples including 125 benign tissues (19), and demonstrated that 113 of 125 benign lesions were characterized by immunoreactive TPO in more than 80% of cells while <80% of the cells expressed TPO in all 25 malignant lesions. Thus, using this level of TPO-expressing cells as a positive, they reported a sensitivity of 100% and a specificity of 90%.

Christensen, et al. reported their prospective experience using this method in 124 consecutive FNAs using the same anti-TPO primary antibody (20). In their hands, TPO immunohistochemistry (>80% cut-off) correctly identified all cases of cancer. Only one benign follicular adenoma was identified as malignant by this immunohistochemical criterion. These investigators concluded that TPO immunohistochemistry of FNA samples using the 80% cut-off values has a sensitivity of 100% and a specificity of 99%. These results are obviously subjective and may be antibody dependent.

Because germline mutations of the TPO gene that cause functional loss of TPO activity cause of congenital hypothyroidism, loss of heterozygosity (LOH) at the TPO gene locus has been implicated as a cause of the organification defect typical of benign

and malignant thyroid tumors. However, in a study of 40 hypoactive thyroid nodules (21), LOH of the TPO gene was noted in only 6, making this an unlikely method for evaluating thyroid nodules preoperatively.

Thus, it appears that immunostaining for thyroid peroxidase may be a valuable addition to the analysis of FNA samples. Studies with additional available antibodies may be useful from a practical standpoint.

Oncofetal fibronectin

Fibronectins are high-molecular-weight glycoproteins found in the extracellular matrix. Oncofetal fibronectin is characterized by the presence of the oncofetal domain (IIICS domain), which is absent in normal fibronectin. Overexpression of this variant of fibronectin has been demonstrated in many epithelial cancers and it has been studied as a molecular marker of malignancy. Several investigators have evaluated the utility of the oncofetal fibronectin mRNA as a marker of thyroid malignancy.

Higashiyama, et al. (22) evaluated 19 malignant and 33 benign surgical thyroid specimens by competitive RT-PCR and demonstrated elevated levels in papillary and anaplastic cancers versus benign tissues. Levels were variable in follicular carcinomas and were not clearly different from follicular adenomas. The same group also reported detection of oncofetal fibronectin mRNA on surgical samples using in situ hybridization and reported similar results (23).

Takano et al. (24) examined 72 FNA samples (23 normal, 14 adenomatous goiters, 13 follicular adenomas, 3 follicular carcinomas, 18 papillary carcinomas and 1 anaplastic cancer) for expression of oncofetal fibronectin mRNA using RT-PCR. 95% of the papillary or anaplastic carcinomas by cytology also expressed oncofetal fibronectin mRNA compared to only 4% ($n = 109$) of benign specimens. In contrast, none of the 6 follicular tumors expressed oncofetal fibronectin. Fifty of these patients underwent surgery, based on the results of the surgical histology, oncofetal fibronectin RT-PCR was 97% sensitive and 100% specific. These results are similar to Higashiyama, et al. as all but one cancer sample included in this study was papillary. These results suggested that oncofetal fibronectin mRNA amplification was an accurate marker of papillary, but not follicular carcinoma. A potential cause of false positive results is the expression of oncofetal fibronectin in thyroid fibroblasts (25). Despite the fibroblast data, the results of the immunohistochemical and molecular studies suggest that measurement of oncofetal fibronectin expression may be useful as an adjunctive test for identifying papillary thyroid carcinoma.

Ret/PTC

Ret/PTC oncogenes are genomic rearrangements that couple the tyrosine kinase domain of the Ret receptor to different 5' regions leading to aberrant expression and activation of Ret. To date, there are 8 Ret/PTC proteins, however, the prevalence is greatest for Ret/PTC 1, 2, and 3. Translocations involving Ret are particularly prevalent in papillary carcinomas that develop following exposure to radiation. Because

these rearrangements are largely limited to thyroid carcinomas, the expression of PTC oncogenes has been studied as molecular markers for thyroid malignancy.

In a study of 73 thyroid specimens from which both FNA and surgically obtained tissue was available, Cheung, et al. (26) evaluated the presence of PTC1-5 by RT-PCR. Only Ret/PTC 1, 2 or 3 were detected in the samples; Ret/PTC translocations were not detected on FNA and surgical samples from 39 benign tissue samples, including 11 follicular adenomas, 25 nodular hyperplasia's and 3 Hashimoto's thyroiditis cases. In contrast, Ret/PTC1, 2, or 3 expression was detected in 17 FNA samples and 21 surgical specimens derived from 33 malignant thyroid tumors. Of importance, this molecular method was more accurate than routine cytopathology in these samples.

Conflicting results were reported by Elisei, et al. (27) who studied 154 patients referred to surgery for FNA-characterized benign nodules (n = 65) or papillary thyroid cancer (n = 89). Expression of Ret/PTC-1 and Ret/PTC-3, the most common Ret/PTC oncogenes, was identified in both benign and malignant nodules. RET protein expression has been evaluated by immunohistochemistry in papillary thyroid cancers (28). Overall, expression of Ret was heterogenous and was demonstrated in regions of cellular atypia in both malignant and benign lesions. Thus, based on these data, it appears that Ret/PTC may not be helpful in pre-operative diagnosis due to a relatively low prevalence in many populations with papillary thyroid cancer and potential issues with specificity. However, more studies are needed to clarify a role for Ret/PTC rearrangement or Ret overexpression in the diagnosis of thyroid nodules.

Pax8-PPAR γ

Kroll, et al. (29) identified a chromosomal translocation t(2;3)(q13;p25) causing a fusion gene between Pax8 and the peroxisome proliferator activated receptor gamma (PPAR γ) in follicular thyroid carcinomas. Specifically, 5 of 8 follicular cancers expressed the fusion gene, while all of the 20 follicular adenomas, 10 papillary thyroid carcinomas and 10 other benign nodules did not express the rearranged gene, suggesting that detection of Pax 8-PPAR γ fusion gene expression might accurately identify follicular carcinomas preoperatively.

The specificity of the Pax 8-PPAR γ may not be complete, as other groups (30, 31) have reported expression of PAX8-PPAR γ in benign follicular adenomas, albeit at a lower frequency than follicular carcinomas. The importance of expression of Pax8-PPAR γ in follicular adenomas on malignant transformation is uncertain. It has been speculated that overexpression of PPAR γ alone, even in the absence of a defined chromosomal rearrangement, may be a marker of malignant transformation. Detection of PPAR γ overexpression by immunohistochemistry appears to be more sensitive, but also, less specific for detection of follicular carcinoma (31).

B-Raf

Mutations in the serine-threonine kinase, B-Raf have been described in 35–70% of papillary thyroid carcinomas, with almost no overlap with other known oncogenes

or other benign or malignant thyroid lesions (32–34). Because this mutation appears quite specific for papillary thyroid cancer, and it is limited to two specific mutations, detection of the mutations has been proposed as an adjunctive test for FNA analysis (32). This method would likely be useful only for papillary thyroid cancer detection, however.

Nm23

Re-expression of the Nm23 tumor suppressor gene has been demonstrated to reduce the metastatic potential of malignant cells *in-vitro* and reduced expression of Nm23 occurs in aggressive forms of breast cancer (35). In thyroid tissues, the interesting finding of increased expression has been demonstrated, primarily in stage IV papillary cancers and anaplastic carcinomas (36). Farley et al. (37) also evaluated 34 thyroid tumors, including 4 follicular adenomas, 19 papillary carcinomas, 6 follicular carcinomas and 5 medullary carcinomas for Nm23 mRNA levels. In this study, overexpression of Nm23 was noted in follicular and medullary cancers, although there was overlap between benign and malignant samples. Similarly, Berthau, et al. (38) reported that immunocytochemical analysis of Nm23 protein expression did not accurately distinguish between benign and malignant lesions. Mechanistically, the finding that overexpression of nm23, rather than reduction or loss of nm23 expression were demonstrated suggests an alternative function for this protein in thyroid cancer (39).

High mobility group I(Y) protein—HMGI(Y)

The high mobility group I (HMGI) proteins are nuclear proteins that regulate chromatin structure and function. HMGI(Y) is particularly highly expressed during embryogenesis, and its reexpression has been described in cancers, but not in normal adult tissues. Chiappetta et al. (40) reported evaluated expression of HMGI(Y) protein by immunohistochemistry on 358 thyroid tissue samples. HMGI(Y) was detected in 18 of 19 follicular carcinomas, 92 of 96 papillary tumors and 11 of 11 anaplastic cancers, but in only 1 of 20 hyperplastic nodules, 44 of 200 benign follicular adenomas and 0 of 12 normal thyroid tissue samples. HMGI(Y) mRNA was detected in 4 of 4 malignant tumors while eight benign FNA samples (6 follicular adenomas and 2 normal thyroid tissue) were negative. Thus, HMGI(Y) may be a potentially useful diagnostic tool for thyroid cancer that warrants further identification.

Ceruloplasmin

Because ceruloplasmin, a copper transport protein that shares homology with lactoferrin (a molecular marker for several tumor types), it has been investigated as a tumor marker in thyroid cancer. Tuccari et al. (41) evaluated 56 surgical thyroid specimens for ceruloplasmin expression by immunohistochemistry. None of the 15 follicular adenomas expressed ceruloplasmin, while two of two Hurthle cell tumors, all 21 follicular, and all 6 papillary carcinomas were positive. All of the medullary thyroid cancers were negative for ceruloplasmin, as was the normal thyroid tissue surrounding the thyroid cancers. The functional role of ceruloplasmin in thyroid tumors as its potential role as a marker for malignancy require further clarification.

Cytokeratins

Cytokeratins are structural proteins found in all epithelial cells; several types of keratins have been identified with altered expression patterns in malignancies. In thyroid cancer, immunocytochemical expression for prekeratin was detected in papillary thyroid cancer but not normal thyroid tissues, follicular adenomas and follicular thyroid carcinomas (42). With the development of more specific antibodies that identify cytokeratin subtypes, a more comprehensive evaluation was able to be performed. Schelfhout et al. (43) used monoclonal antibodies against cytokeratin 8, 18 and 19 to characterize cytokeratin expression in different thyroid histologies. Of these, cytokeratin-19 was overexpressed 12 of 12 papillary cancers, while follicular cancers, follicular adenomas, colloid nodules and normal thyroid tissue were negative or had only weak staining. The authors concluded that staining with antibodies against cytokeratin 19 is a useful diagnostic tool for papillary thyroid cancer. However, these promising results were not able to be confirmed. Sahoo, et al. (44) evaluated 35 surgical thyroid specimens for cytokeratin 19 expression. Although papillary cancers tended to display more intense staining than other tumors, the presence or absence of immunoreactive cytokeratin 19 did not distinguish the tumor histologic subtypes. Technical issues could account for the discrepant results and further studies are needed. Cytokeratin 20 has also been evaluated in lymph nodes and peripheral blood of patients with medullary and follicular cell-derived thyroid cancer (see below).

GLUT 1

Because malignant cells typically are characterized by an increased rate of glucose utilization, overexpression of glucose transporters has been identified in malignancies, particularly overexpression of Glut-1. In thyroid cancer, Haber, et al. (45) reported the absence of immunoreactive Glut-1 in 38 benign thyroid tissues, but its presence in 9 of 17 papillary, 2 of 6 follicular and 2 of 2 anaplastic cancers. These results suggest that Glut-1 could be potentially useful marker of malignancy. These results concur with clinical studies that demonstrate enhanced glucose uptake using [18F]-2-fluoro-deoxyglucose (FDG) PET in aggressive thyroid tumors with a worse prognosis (46). Thus, determination of Glut-1 expression levels may be important both diagnostically and prognostically in thyroid cancer.

CA 19-9 and CD15

CA 19-9 and CD 15 (Leu-M1) are have markers for a variety of epithelial tumors and Hodgkin's disease, respectively, that have been evaluated in thyroid cancer. Immunohistochemical expression of both CA19-9 and CD15 were identified in benign thyroid tumors and in papillary carcinomas, suggesting these would not be useful markers in the clinical setting (47).

HBME-1

In contrast to CA 19-9 and CD 15, HBME-1, a tumor suppressor gene whose product is involved in signal transduction, has been reported to have a pattern of expression

suggesting it would be a potential marker of papillary thyroid cancer (48). Of importance is a recent report that demonstrated that papillary and follicular cancers with apocrine or Hurthle cell features, respectively, have distinctly lower levels of HBME-1 expression than more typical papillary and follicular tumors (49). The biological impact of this finding is uncertain. Mase, et al. recently published data demonstrating that HBME-1 expression was detected in 23% of follicular adenomas, 27% of benign goiters, but in 85% of follicular and 97% of papillary cancers (50). Based on these results, HBME-1 is a potentially useful marker FNA samples, although follicular carcinomas require initial evaluation and the papillary cancer data require confirmation.

CD30

The CD30 antigen (Ki-1) is a cytokine receptor that is expressed in activated B and T lymphocytes, but not normal adult epithelial cells. Its expression has been demonstrated in Hodgkin's disease and Burkitt's lymphoma. The presence and distribution of both CD30 and the CD30 ligand in the thyroid were investigated using immunohistochemistry in 131 thyroid specimens and 6 normal thyroid glands (51). Normal thyroid tissue did not express CD30 or the CD30 ligand including tissue adjacent to benign nodules or follicular cancer did not express either molecule, while tissue adjacent to papillary and medullary cancer expressed CD30 ligand. Of thyroid tumors examined, 20% of follicular adenomas showed coexpression of CD30 and CD30L, while 7% of the follicular, 33% of the anaplastic, 76% of the papillary and 67% of the medullary cancers expressed both proteins. The overlap in expression between benign and malignant thyroid tissues may ultimately limit the use of this marker in identifying thyroid cancer, however, the regulation of these proteins may be very interesting for thyroid cancer biology.

Epithelial membrane antigen and Leu-7 (CD57)

Epithelial membrane antigen (EMA) is a glycoprotein that is expressed by malignant epithelial cells, while Leu-7 is an antigen expressed by immune cells whose expression has been demonstrated in a variety of tumors. Cheifetz et al. (52) evaluated the expression of these proteins in 40 benign and malignant nodules by immunohistochemistry of surgical specimens. For EMA, 16 of 22 malignant (73%) and 5 of 18 benign (28%) tumors were positive, and Leu-7 expression was detected in 20 of 22 malignant tumors and 6 of 18 benign tumors, both of which were significantly different statistically. Leu-7 expression as a marker of thyroid malignancy was also evaluated by Khan, et al. (53) who found that 95% of 83 malignant and 21% of 77 benign surgical specimens were positive. This results in an overall sensitivity of 98% and a specificity of 82%, but, as with other immunohistochemical markers, differences in the intensity and distribution of the staining were noted.

Cyclooxygenase-2

Cyclooxygenase type 2 (Cox-2) is a highly inducible enzyme in the phospholipase A2 pathway that appears to be involved in carcinogenesis. Cox-2 mRNA and protein

levels are upregulated in many epithelial cell-derived malignancies. Similarly, in thyroid cancer, Cox-2 gene and protein expression are also elevated both in surgical and FNA samples (54). These data suggest that in addition to being a treatment target, Cox-2 mRNA and/or protein levels could distinguish benign from malignant thyroid tumors.

Hypermethylation of the TSH receptor

Gene silencing can occur through a variety of mechanisms. One of the most common is hypermethylation of CPG islands in promoter regions that cause reduced expression of genes. This phenomenon has been shown to occur in thyroid cancer. Xing, et al. (55) demonstrated that detection of TSH receptor gene methylation by PCR was an accurate adjunct in the evaluation of thyroid tumors. Further work in this area is required to determine if this method is useful in a clinical setting.

MOLECULAR MARKERS OF TUMOR RECURRENCE OR PROGRESSION

The use of highly sensitive molecular tests to identify recurrent or progressive disease using tissue and/or tumor-specific markers have been used to detect metastases in bone marrow, lymph nodes, peripheral blood, and other sites. Methods employed include RTT-PCR amplification of tissue or tumor-specific transcripts or isolation of cancer cells directly using cell sorting. These approaches are particularly attractive for thyroid cancer because, in comparison to other solid tumors, initial therapy of thyroid cancer frequently results in the removal and ablation of all thyroid tissue, making both tumor and tissue-specific markers useful for early diagnosis. Several markers have been applied to nodes (Table 1) and peripheral blood (Table 2).

Lymph node recurrence

The most common sites of tumor metastases in thyroid cancer are local-regional lymph nodes, particularly for papillary cancer. These metastases are frequently present at diagnosis and can be difficult to isolate and eradicate. Standard approaches to diagnosis of local nodes include the level of elevation of serum thyroglobulin concentrations, the presence of abnormally sized or appearing nodes on anatomic imaging often with abnormal cytology on FNA, or iodine uptake in an extrathyroidal location. The diagnosis of metastatic thyroid cancer within a node frequently is confirmed by FNA, but

Table 2. Published diagnostic peripheral blood markers for thyroid cancer

Marker	References
Thyroglobulin	(66–77, 82–85, 87,88)
Thyroid Peroxidase	(67, 74, 84)
Ret/PTC Oncogenes	(67)
Cytokeratin 20	(58)
TSH Receptor	(76)
Human Kallikrein 2	(86)

this method is difficult for small nodes in the neck bed where the amount of aspirated tissue may be small. To enhance diagnostic sensitivity, there has been an interest in developing RT-PCR based approaches to amplify thyroid-specific transcripts from node FNA for both thyroid cancer derived from for papillary and follicular thyroid cancer and for medullary thyroid cancer.

Arturi, et al. (56) reported their experience using RT-PCR amplification of thyroglobulin and TSH-receptor mRNAs from nodal tissue obtained by FNA of 46 lymph nodes and compared them to cytopathology, thyroglobulin immunoassay of the aspirate fluid, and final histopathology. RT-PCR detected thyroid transcripts in 41 of 41 histopathologically confirmed metastatic tumor samples, including 45% that were inadequate or false negative by standard cytopathology. Similar results were obtained by Gubala, et al. (57) who reported their experience in 70 nodes aspirated from 60 patients with suspected thyroid cancer recurrence. Taken together, these data confirm that thyroid-specific mRNAs can be amplified from nodes in patients with metastatic thyroid cancer, that false positives from ectopic transcription in lymphocytes appears to be uncommon using these particular primers, and the overall accuracy may be adequate for clinical use. Weber, et al. (58, 59) used a slightly different approach, amplifying cytokeratin 20, an epithelial cell tumor marker, mRNA using RT-PCR from nodes suspected of harboring metastatic differentiated thyroid cancer. In comparison to cytokeratin 20 immunohistochemistry and cytology, the molecular diagnostic approach was more sensitive.

This group has also reported similar data for patients suspected to have recurrent medullary thyroid cancer in cervical nodes. The report that amplification of cytokeratin 20 and preprogastrin mRNA, a marker of neuroendocrine tumors, by RT-PCR demonstrated enhanced sensitivity and specificity over routine cytology (60, 61). These results, in combination with detection of medullary cancer-related mRNAs in peripheral blood of patients suggest this approach may be useful for patients with medullary cancer (62).

The importance of detecting metastases earlier has not been clarified in thyroid cancer, a disease that typically follows an indolent course. However, for patients with malignant melanoma, amplification of tyrosinase mRNA from sentinel lymph node tissue removed at surgery correlates with development of metastatic melanoma and subsequent prognosis (63, 64). With time, it is likely that early detection will result in better prognosis. The development of markers of aggressiveness, such as p53 mutation analysis, may provide additional predictive data that will help clinicians stratify patients for appropriate treatment paradigms. Other markers derived from cDNA array analysis may also be particularly useful in the future.

Detection of distant metastases

The most frequently employed tests for monitoring patients with thyroid cancer for tumor recurrence are measurements of circulating serum thyroglobulin concentrations and radioiodine scanning, both of which rely on thyroid-specific gene transcription or function. Non-thyroid specific monitoring methods include ultrasound, magnetic resonance imaging, computed tomography, positron emissions tomography,

and physical examination. Thyroid-specific monitoring, rather than tumor-specific monitoring is particularly useful for patients treated with thyroidectomy and radioiodine ablative therapy who are, theoretically, devoid of all thyroid tissue, benign or malignant.

The development of more sensitive and specific thyroglobulin assays has led to increased dependence on this test in monitoring paradigms. The ease of a simple blood test and the lack of exposure to radiation are two advantages of this method. However, there are several important limitations of serum thyroglobulin monitoring; 1) circulating autoantibodies directed against thyroglobulin (anti-thyroglobulin antibodies) interfere with clinical assays in approximately 20% of patients, and 2) stimulation of thyroglobulin transcription and release with either endogenous or exogenous thyrotropin (TSH) is required for adequate clinical sensitivity (65). There has therefore been an interest in developing new assays for thyroid cell detection that are not altered by antibodies and are sensitive enough to not require TSH stimulation.

Qualitative thyroid mRNA assays

Ditkoff, et al. (66) reported results from 100 individuals including 87 with thyroid cancer, 6 with benign thyroid disease (nontoxic goiters), and 5 normal subjects following total thyroidectomy (except normal subjects). Total RNA was isolated from the macrophage layer of peripheral blood, and, using RT-PCR amplification of thyroglobulin mRNA, they detected thyroid transcripts in blood from 9 of 9 patients with metastatic thyroid cancer, but from only 7 of 78 patients thought to be free of disease, and no patients having surgery for benign disease or normal control subjects. Detailed clinical information was not included regarding the clinical status of the patients and TSH levels were not reported. However, these investigators clearly demonstrated that thyroglobulin mRNA could be amplified from peripheral blood and that its presence appeared to correlate with stage of disease.

Tallini, et al. (67) subsequently reported data using different RT-PCR assays for detection of thyroid transcripts from peripheral blood. In this study, the investigators evaluated 44 patients including 24 with thyroid cancer (16 with metastases and 8 free of disease), either pre-operatively, postoperatively, or at both time points for peripheral blood expression of thyroglobulin, thyroid peroxidase, and the RET/PTC1 thyroid oncogene. 56% of the patients with either local or distant metastases had positive assays, compared to 63% of those thought to be free of disease. Of those thought to be free of disease that had positive assays, 80% had cervical adenopathy at diagnosis and were felt to be at high risk of tumor recurrence. Of the patients with benign disease, 2 of 20 patients had a positive mRNA assay, both of which reverted to negative after surgery. The *in vitro* sensitivities of this assay were approximately 50 cells/ml of blood. Technically, these authors isolated total RNA from whole blood drawn into EDTA-containing tubes and did not isolate a buffy coat layer.

Ringel, et al. (68) also developed a thyroglobulin mRNA assay designed for detection of circulating thyroid cells. The method employed in this study used whole blood placed directly into an RNA-stabilization solution and resulted in a more sensitive assay. In this study, 87 individuals with thyroid cancer were evaluated. Thyroglobulin

mRNA was detected in all 14 cervical or distant metastases during L-T4 therapy, while 65% of patients with thyroid bed uptake and 20% of patients with no uptake had detectable thyroglobulin mRNA. These data suggested both a high sensitivity and lower specificity of the assay than the prior studies. Of concern was that similar to the patients with multinodular goiter analyzed by Tallini et al. circulating thyroglobulin mRNA was detectable in all of the normal subjects evaluated and in 20% of athyreotic patients. These results raised the possibility that thyroglobulin may not represent a truly thyroid-specific transcript and that this more sensitive assay detected ectopically transcribed thyroglobulin in non-thyroid cells. Alternatively, the assay could have been detecting very early minimal residual or recurrent disease.

Additional data have been published from many groups using similar qualitative approaches to amplify thyroglobulin and other mRNA transcripts from peripheral blood. The results have been remarkably variable, with some groups demonstrating excellent correlation between tumor stage and results (69–72), while others demonstrate no correlation with tumor stage (73–75). Several have concluded that the assay is more useful for papillary rather than follicular cancer (69), while others have demonstrated optimal screening by combining thyroglobulin mRNA with new highly sensitive thyroglobulin immunoassays (71). Taken together, nearly all groups have confirmed the presence of circulating thyroglobulin mRNA in peripheral blood of normal subjects, and in a subset of athyreotic patients, suggesting that ectopic transcription of thyroglobulin or splice variants of thyroglobulin can be detected.

The importance of assay methodology has been highlighted in several recent studies. Bojunga, et al. (73) reported data using low and high sensitivity qualitative thyroglobulin mRNA assays in patients with thyroid cancer. Using a lower sensitivity assay, they detected circulating thyroglobulin mRNA in 69% of patients with metastatic disease, 46% of patients with thyroid cancer thought to be free of disease, 25% of patients with benign thyroid disease and 18% of control patients. The more sensitive assay increase sensitivity modestly, but resulted in the complete loss of specificity. Gupta, et al. (76) created PCR primers designed to carefully avoid amplification of all known splice variants of thyroglobulin and the TSH receptor. Using these PCR primers, these authors reported detection of thyroid transcripts in 83% of thyroid cancer patients with positive compared to 5% of patients with negative radioiodine scans. All normal volunteers were negative. The specificity was slightly greater for TSH mRNA detection rather than thyroglobulin mRNA detection. Similarly, Savagner, et al. (77) designed thyroglobulin primers that amplified known splice variants and others that did not. They determined that the splice variants account for approximately 1/3 of the total amplified thyroglobulin mRNA, and that when the primers that do not amplify the region are used, the results correlated with the volume of thyroid tissue and TSH concentration. Taken together, these data clearly demonstrate the importance of methodology in performing these assays, and in proper evaluation of the published data. Differences in sensitivity could be due to the method of sample collection, storage of samples between the phlebotomy and RNA isolation, the specific method for reverse transcription and the PCR primers employed.

Quantitative thyroid mRNA assays

Due to the subjective nature of PCR and the apparent discrepancy in the results of studies using qualitative RT-PCR systems, there has been interest in attempting to quantify peripheral blood RT-PCR assays in order to define a clinically relevant level of detection. The advent of real-time quantitative PCR has enabled testing of this approach in clinical trials. Similar to quantitative RT-PCR, the methodological issues are considerable, particularly when attempting to detect very rare transcripts within a particular sample. Other major issues when considering quantitation of RNA is normalization to a control transcript. Traditionally, normalization to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) or beta actin has been employed; however, tremendous variability in these control transcripts has been reported (72–74). An alternative is normalization to total RNA (18S), while others have chosen not to normalize transcripts at all and normalize to the original blood volume (78–80). This also may not be an accurate method and the use of a “geometric” panel of markers has recently been suggested (81). Thus, it is apparent that normalizing to different control transcripts clearly will alter the reported results and, to date, no standard method has been applied by all laboratories; however, it appears clear that normalizing to a single “housekeeping” gene such as GAPDH or beta actin is likely not appropriate for these samples (81).

Wingo, et al. (82) reported the first quantitative thyroglobulin mRNA assay. In this study, total RNA was derived from peripheral blood samples and the assay was extensively tested. Calibration assays revealed interassay variability of 17–22% due primarily to RNA stability, RNA handling and the reverse transcriptase reaction. The assay displayed reproducible results over a three log concentration range. Ringel, et al. (83) subsequently used this assay to analyze peripheral blood RNA from 107 patients with thyroid cancer; including 84 during L-T4 therapy, 14 following L-T4 withdrawal, and 9 before and after thyroxine withdrawal. Twenty-three patients had circulating anti-thyroglobulin antibodies. Using an arbitrary cut-point to identify patients as either positive or negative for detection (36 PCR cycles), thyroglobulin mRNA measurement assay was more sensitive than thyroglobulin immunoassay, but was less specific at detecting the presence of local and distant metastases. In addition, while there was a statistical correlation between the level of thyroglobulin mRNA and the presence of thyroid tissue on scan, the level of thyroglobulin mRNA did not correlate well with stage of disease. Importantly, the assay appeared to be unaffected by circulating anti-thyroglobulin antibodies, suggesting that perhaps Thyroglobulin mRNA could be used as an adjunctive test to identify patients with recurrent or residual thyroid tissue in the presence of anti-thyroglobulin antibodies. However, the authors cautioned that there was significant overlap between the patients with positive results without definable disease and those with disease, a factor which may limit the usefulness of this particular assay method in clinical practice. Thus, for individual patients, the absolute value of thyroglobulin mRNA did not appear to be diagnostically useful, but the presence or absence of thyroglobulin mRNA might be useful. In addition, similar to other studies, even using a cut-point, a significant minority (38%) of patients with no evidence of

disease had positive results and many had detectable values below the cut-point. The relevance of an isolated thyroglobulin mRNA level is uncertain as it might reflect a false positive result from ectopic expression, or the presence of bona fide residual thyroid tissue.

Savagner, et al. (77) developed a quantitative assay for measurement of thyroglobulin mRNA in peripheral blood. In this study, the cut point of a positive or negative assay was determined to be the amount of circulating prostate specific antigen mRNA as a control transcript, no internal normalization was performed and results were reported per total RNA amount. The results in this study were similar to those of Ringel, et al. in that using a mean value, there was a statistical correlation with the absence or presence of residual or recurrent thyroid tissue, but there was significant overlap between all groups for individual data.

Similar to the experience with qualitative thyroglobulin mRNA assays, variable results have also been reported with the quantitative approach. Some of these differences are methodological (different primers, use of DNase I, normalization), inherent in the assay method (instability of RNA), while others may be interpretive. Takano, et al. (84) performed a study evaluating thyroglobulin mRNA from peripheral blood and similar to Ringel, et al. identified this transcript in all patients. Unlike the prior study, they were not able to correlate levels with stage of disease. However, in this study, the normalization was performed in a different manner (GAPDH), different PCR primers were utilized, and DNase I treatment was not performed, all different from Ringel, et al. Takano, et al. (84) also report similar data amplifying thyroid peroxidase (TPO) as a tumor marker, results that did not agree with those of Roddiger, et al. (74) who reported a better correlation using TPO mRNA amplification than thyroglobulin mRNA in patients with thyroid cancer. Eszlinger, et al. (85) also did not demonstrate correlation between thyroglobulin mRNA levels and the presence or absence of thyroid tissue. They evaluated several different methods of blood collection and also describe important differences in results depending on the types of tubes used for phlebotomy and the time between the sample collection and RNA isolation. These authors used a new set of primers and normalized to beta actin, factors that distinguish their method from others. To further clarify the importance of recognition of assay differences between groups, Span, et al. (75) used the same thyroglobulin PCR primers as earlier reports and were not able to confirm a relationship between stage of disease and level of thyroglobulin mRNA. However, distinct from those reports, the authors used a different method of RNA isolation and normalize their results to beta actin, both important differences in assay methods that can alter results.

Tumor-specific mRNA assays

Additional markers, such as cytokeratin 20 and human kallikrein 2 mRNA amplification have recently been reported to have potential diagnostic benefit for thyroid cancer patients (58, 86). These are not thyroid-specific, but may be cancer-specific. These preliminary data require confirmation, but may be an interesting alternative approach to molecular diagnosis of metastatic disease.

Thus, based on these data, it seems that there is clear evidence of ectopic expression of thyroglobulin, or at least splice variants of thyroglobulin in non-thyroid tissues. Assay quantitation to “subtract out” this amplification is of uncertain value due to differences in the reported methods and the challenges of normalization of results. Further study and clarification of these issues, in particular, the use of primers that do not amplify splice variants, determination of the best processing protocol for blood RNA isolation, and whether an appropriate form of normalization exists are required before a clear assessment regarding the clinical usefulness of this approach to molecular diagnosis can be made.

SUMMARY

The use of molecular assays to analyze clinical tissues in the diagnosis and management of thyroid cancer, similar to other tumors, will likely allow for more accurate characterization of the aggressiveness of individual tumors and may allow for the early diagnosis of recurrence. The application of these methods to thyroid nodules and nodal metastases is less encumbered by difficulties arising from amplification of transcripts in non-thyroid cells. For these tissues, these assays are likely to be used clinically in the near-future. New data arising from cDNA arrays identifying novel markers of malignancy or tumor aggressiveness make this a growing area of interest. The use of molecular assays in diagnosing distant metastases is more problematic due to issues with ectopic expression of either full length or splice variants of genes thought to be thyroid-specific. Assay quantitation is a complex problem owing to variability in the level of expression of “housekeeping” genes and the variety of phlebotomy and RT-PCR methods reported. Additional research in this area is clearly required before a recommendation can be given regarding clinical applicability of these tests.

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