A High-Throughput Proteomic Approach Provides Distinct Signatures for Thyroid Cancer Behavior

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Abstract

\textbf{Purpose:} Well-differentiated thyroid cancer (WDTC) is the most frequent form of endocrine neoplasia. One of the main challenges in the management of this disease is distinguishing low-risk patients who can be treated by surgical resection of the lesion from those with a high likelihood of recurrence who need a more extensive approach, including total thyroidectomy and radioiodine ablation.

\textbf{Experimental Design:} A tissue microarray (TMA) comprising 410 cases of WDTC was constructed with risk estimates for the following features: extrathyroidal extension, lymph node metastases, and vascular invasion. The variables examined were morphologic classification, candidate genetic, and proteomic biomarkers.

\textbf{Results:} BRAF (Raf kinase type B) mutant carriers showed increased risk of developing invasion compared with wild-type (WT) cases. However, when classified morphologically, classic papillary thyroid carcinomas (PTC) showed much higher risk estimates for invasive features compared with follicular variant PTCs (FVPTC); within these morphologic subgroups, BRAF mutational status did not provide independent risk estimates. Staining intensities for membranous galectin-3 (Gal3), HBME-1, and CK19 and nuclear Gal3 were statistically validated as markers of aggressive behavior. Estrogen receptor beta (ER\textsubscript{\beta}) was overexpressed in lesions with invasive behavior. The utility of these biomarkers remained statistically significant in the FVPTC. In contrast, a different set of biomarkers proved effective in classic PTC where upregulation of cyclin D1, loss of p27, and overexpression of ER\textsubscript{\beta} were associated with invasive behavior.

\textbf{Conclusion:} Different proteomic signatures validate the distinction of classic and FVPTC and provide a practical clinical mechanism to predict the thyroid cancer behavior and stratify patients for clinical management.

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Introduction

The most common endocrine malignancy is papillary thyroid carcinoma (PTC), a tumor that has several morphologic variants, the most common being classic PTC and follicular variant PTC (FVPTC; ref. 1). The incidence of this malignancy has been increasing recently, possibly because of increased awareness of the disease, increasingly sensitive diagnostic methods that detect ever smaller lesions, and ill-defined environmental pathogenic factors (2).

Most PTCs are diagnosed when they measure less than 3 cm; they are most frequent in women younger than 55 years. PTC is one of the most curable cancers. The main challenge for clinicians treating this condition is to distinguish high-risk from low-risk patients. This is of paramount importance, although high-risk patients require an aggressive approach with total thyroidectomy and radioiodine ablation, whereas for low-risk patients, a simple hemithyroidectomy suffices. Thus, precise categorization of these patients has profound impact on clinical management and therefore on treatment-related morbidity and cost.

Several prognostic scales have been proposed for thyroid cancer (3); although they are effective in predicting long-term mortality in patients with advanced lesions, they are poor at predicting disease recurrence and metastases in the majority of PTCs that are low-risk lesions. The relative indolence of these tumors has resulted in a paucity of large-scale clinical trials by comparing conservative and aggressive management approaches, and the majority of patients are subjected to total thyroidectomy with radioiodine ablation.

Molecular biomarkers have been sought to more accurately predict biological behavior in PTC, but have been validated in relatively small numbers of patients followed...
We analyzed the expression of this receptor as a marker characteristically show the increased nuclear staining and colony formation of well-differentiated thyroid cancer (WDTC; ref. 6–8). It is rare or absent in FVPTCs and is detectable in 10% to 20% of anaplastic thyroid carcinomas that are thought to arise by progression of well-differentiated thyroid cancer (WDTC; ref. 5). Some studies have associated this mutation with metastasis and poor prognosis, but this is controversial (6–8).

The role of BRAF (Raf kinase type B) in thyroid carcinogenesis has been increasingly recognized. The BRAF V600E mutation occurs in 29% to 83% of PTCs, with no consistent variation according to region or radiation exposure. It is rare or absent in FVPTCs and is detectable in 10% to 20% of anaplastic thyroid carcinomas that are thought to arise by progression of well-differentiated thyroid cancer (WDTC; ref. 5). Some studies have associated this mutation with metastasis and poor prognosis, but this is controversial (6–8).

The histopathological diagnosis of PTC is challenging and several immunohistochemical biomarkers have been proposed to aid in the diagnosis of malignancy. The most widely used are galectin-3 (Gal3), cytokeratin 19 (CK19), and the Hector Battifora mesothelial antigen-1 (HBME-1; refs. 9, 10). Recent studies have suggested that reduction of the sodium iodide symporter (NIS; ref. 11) and CITED-1 (12) is associated with malignancy. Other biomarkers that have been proposed as indicators of aggressive behavior include cell-cycle regulators, such as loss of p27 and upregulation of cyclin D1 (13, 14), loss of p21 (15), and immunoreactivity for p53 (16), and altered expression of adhesion molecules such as carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1; ref. 17) and its putative ligand osteopontin (OPN; ref. 18), upregulation or loss of fibronectin (FBN; refs. 19, 20), and loss of E-cadherin (E-Cadh; ref. 21). Expression of the MAGE-A (melanoma-associated antigen) is also associated with aggressive tumor behavior (22, 23).

The nuclear receptor PPARγ, a regulator of metabolism, cell growth, and differentiation, has been implicated as a partner in a rearrangement present in angioinvasive thyroid carcinomas, including PTCs. Tumors with this rearrangement characteristically show the increased nuclear staining (24). We analyzed the expression of this receptor as a surrogate marker of a Pax8-PPARγ rearrangement.

In this study, we examined a large number of PTCs by a high-throughput automated system to identify the predictors of aggressive behavior in low-risk PTC.

Materials and Methods

Cases

Patients undergoing thyroidectomy for PTC were gathered from 2 centers: Hospital de Especialidades CMN SXXI IMSS, Mexico City, Mexico, and University Health Network, Toronto, ON, Canada. Ethics Committees at both centers approved the study. Patients provided informed consent for use of their tissue for research. Clinical information at diagnosis included demographics, clinical presentation, presence of other thyroid diseases, tumor size, extrathyroidal extension (ETE), vascular invasion (VI), and lymph node metastases (LNM). All cases were reviewed after the initial diagnosis, ensuring accurate diagnosis, classification, and morphologic features of aggressive behavior by at least 2 observers and in accordance with the World Health Organization (WHO) International Classification of Diseases (1). Due to the controversial nature of encapsulated, noninvasive follicular lesions (3, 4), only unequivocal invasive FVPTCs were used for this study. Cases with obvious features of dedifferentiation were also excluded.

Nucleic acid studies

For each case, 1 to 3 sections of 10 μm of paraffin blocks were mounted, dewaxed, and air-dried by routine protocols. Areas of fibrosis, necrosis, and normal tissue were dissected away using light microscopic guidance and DNA was obtained from the remaining tumor by using phenol-chloroform extraction. All tumors were sequenced for the presence of a BRAF mutation using previously described primers (8).

Tissue microarray

Representative primary tumor and normal surrounding tissue were selected for each case and a tissue microarray (TMA) was built using the TMArrayer from Pathology Devices to include triplicate 0.6 mm cores of each type of tissue per case.

Immunohistochemistry

Immunohistochemistry (IHC) was standardized for each antibody (Supplementary Table S1). Each TMA slide provided intra-assay controls (normal versus tumor) and all TMAs were stained in the same run for each antibody to avoid interassay variability. The proteins analyzed were the following:

- Histopathologic biomarkers of malignancy: Gal3, CK 19, and HBME-1.
- Cell differentiation factors: NIS and CITED-1.
- Nuclear receptors: ERα, ERβ (estrogen receptor beta), and PPARγ.
- Adhesion molecules: CEACAM-1, OPN, FBN, and E-Cadh.

Automated analyses

Stained TMAs were scanned with the Aperio ImagescoperXT at 40× magnification and then analyzed by SpectrumXS10 software that has 3 possible algorithms: nuclear, membrane, and cytoplasmic (“colocalization;” ref. 25).
Membrane proteins were scored with a 0 to 3 scale, cytoplasmic proteins with a 0 to 300 scale, and nuclear proteins with a 0 to 100 scale as predetermined by the software algorithms.

Duplicate or triplicate measurements were obtained for each case, stain and type of tissue to derive; mean values were derived each time. Outliers or unexpected results were identified as all values outside the expected scale within the context of replicated measurements. Those showing significant deviation due to technical reasons, such as TMA mounting or staining, were excluded. A database was established with individual averaged values for each case per type of tissue and immunostain.

**Statistical analysis**

The statistical software SPSS 17.0 was used for analysis. Descriptive statistics were used according to the distribution of variables. Student’s T test and Mann–Whitney U test were used for the comparison of means according to the variables distribution. From these variables showing statistical significance in the univariate analysis, we performed multivariate analysis. Statistical significance was considered reached at \( P < 0.05 \).

**Results**

**Case characteristics**

A total of 410 cases of PTC included 325 (79.3%) women and 85 (20.7%) men. Morphologic classification based on WHO criteria identified 193 classic PTCs (53%), 153 FVPTCs (42%), and 64 (5%) other variants, including tall cell or solid variants. LNM was confirmed at the time of diagnosis in 171 (41.7%). ETE defined as invasion of adjacent organs or skeletal muscle outside the isthmus (26) was confirmed in 106 cases (25.9%), and VI was identified in 94 cases (22.9%).

Mutational analysis identified 62.1% of tumors with wild-type (WT) BRAF and 37.9% with a heterozygous BRAF V600E allele. No other BRAF mutations were identified. No wild-type (WT) BRAF and 37.9% with a heterozygous BRAF V600E allele. No other BRAF mutations were identified. No other BRAF mutations were identified. No other BRAF mutations were identified. No other BRAF mutations were identified. No other BRAF mutations were identified. No other BRAF mutations were identified. No other BRAF mutations were identified.

**Risk estimates by genotype and morphology**

Risk estimates of the entire cohort revealed that the V600E mutation was associated with increased risk of developing LNM, ETE, and VI (Fig. 1A). However, morphologic classification alone was a better predictor of these features with higher risk in classic PTC than in FVPTC (Fig. 1B). Surprisingly, the combined risk of developing BRAF V600E mutation and morphology showed higher risk values for WT BRAF in each morphologic subgroup, including classic cases (Fig. 1C), indicating that morphologic classification as classic PTC is a stronger predictor of aggressive behavior than BRAF mutation.

**Risk estimates by immunohistochemical markers**

Representative photomicrographs of the immunohistochemical markers are shown in Figure 2.

**According to features of aggressive behavior.** In tumors with ETE (Fig. 3A), membranous staining for CK19, HBME, OPN, and Gal3 was significantly increased, whereas CEACAM1 showed decreased membranous expression. In contrast, cytoplasmic staining for HBME and CK19 was significantly reduced. Among nuclear proteins, ERβ, Gal3, and p53 showed significantly increased expression in tumors with ETE, whereas cyclin D1 was significantly reduced.

Tumors with LNM (Fig. 3B) exhibited significantly stronger membranous HBME1, CK19, and Gal3. Cytoplasmic expression of both FBN and CK19 was significantly lower in tumors with LNM. Nuclear Gal3 and ERβ were significantly increased in tumors with LNM.

In tumors with VI (Fig. 3C), membranous Gal3 was significantly higher, whereas CEACAM1 was reduced. Cytoplasmic Gal3 was significantly reduced in tumors with VI.

**According to gender.** In women, tumors with ETE exhibited higher membranous staining for CK19, Gal3, HBME1, OPN, and FBN, reduced cytoplasmic CK19 and HBME1, increased nuclear Gal3, Erβ, and p53, and decreased cyclin D1. In men, reduction of membranous CEACAM1 and E-Cadh and nuclear cyclin D1 was associated with ETE (Fig. 4A).

Tumors with LNM in women exhibited significantly increased membranous staining for HBME1, Gal3, and CK19 and increased nuclear Gal3 (Fig. 4B). In men, only a reduction in membranous E-Cadh was statistically significant. Reduction in cytoplasmic FBN and CK19 was significant in both groups.

In women, VI was associated with a significant loss of membranous CEACAM1 staining and reduced nuclear PPARy (Fig. 4C).

**According to histotype.** Among FVPTCs, ETE correlated with increased membranous CK19, HBME, OPN, and Gal3 with a corresponding decrease in cytoplasmic CK19 and increase in cytoplasmic CEACAM1. Nuclear Gal3 was also significantly increased. In contrast, classic PTCs with ETE exhibited only increased nuclear ERβ (Fig. 5A).

FVPTCs with LNM exhibited increased membranous staining for HBME1, CK19, and Gal3, whereas membranous E-Cadh was significantly decreased (Fig. 5B). Cytoplasmic HBME1, CK19, and FBN showed a significant reduction, nuclear Gal3 was significantly increased, and PPARy was reduced in metastasizing FVPTC. In contrast, classic PTCs with LNM exhibited stronger membranous NIS and reduced cytoplasmic FBN. We were surprised that cyclin D1 showed lower expression in tumors with ETE but was not significantly associated with LNM or VI. Interestingly, when tumors were separated into classic and FVPTC, significantly higher expression of cyclin D1 was identified in classic PTCs with LNM, but not in FVPTCs.

FVPTC with VI showed a significant loss of membranous CEACAM1 and a significant reduction in nuclear PPARy.

The only significant correlate of VI in classic PTC was reduction in nuclear p27 (Fig. 5C).

**Combined analysis: any invasion.** Tumors with any invasion (Fig. 6A) exhibited increased membranous...
HBME1, Gal3, and CK19 and decreased membranous CEACAM1 with a corresponding decrease in cytoplasmic HBME1 and CK19. They also exhibited an increase in nuclear Gal3 and a reduction in PPARγ.

When evaluated by histotype (Fig. 6B), FVPTCs with any invasion revealed increased membranous HBME1 and Gal3 and reduced E-Cadh and membranous CEACAM-1. Cytoplasmic CK19 was reduced. Nuclear Gal3 was consistently overexpressed and PPARγ was decreased. Invasive classic PTCs showed a significant increase in nuclear cyclin D1 and a significant reduction in cytoplasmic FBN.

When evaluated by gender (Fig. 6C), invasive tumors in women displayed a significant increase in membranous Gal3 and CK19 with a corresponding reduction in cytoplasmic expression of both proteins and increase in nuclear Gal3. Cytoplasmic FBN, nuclear PPARγ, and nuclear CITED1 were also significantly decreased. In men, only membranous and cytoplasmic CK19, cytoplasmic FBN, and nuclear PPARγ showed the same pattern as shown in women. In contrast, membranous E-Cadh was significantly reduced in invasive tumors.

**Combined IHC score.** To evaluate the potential use of these markers in the clinical setting, we calculated combined IHC scores, using the most significant markers in univariate analysis. This was examined by 3 different formulas:
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IHC1 = \[\frac{[\text{Gal3Nuc} + \text{CyclD1Nuc} + (\text{HBME1Memb} + \text{CK19Memb} \times 100)]}{4}\]

IHC2 = \[\frac{[\text{Gal3Nuc} + \text{CyclD1Nuc} + \text{ERbNuc} - \text{CITED1Nuc} + (\text{HBME1Memb} - \text{CEACAM1Memb} \times 100)]}{6}\]

IHC3 = \[\frac{[\text{Gal3Nuc} + \text{CyclD1Nuc} + \text{ERbNuc} - \text{CITED1Nuc} + (\text{HBME1Memb} + \text{CK19Memb} - \text{CEACAM1Memb} \times 100)]}{7}\]

Of these, the most effective panel to assess disease aggressiveness was IHC1, showing significantly higher scores in cases with LNM (44 vs. 34, \(P = 0.002\)), ETE (46.57 vs. 34.61, \(P = 0.001\)), and any invasion (43.21 vs. 33.13, \(P = 0.001\)). The same combined score remained significant when analyzing invasive versus noninvasive FVPTC (62.8 vs. 45.1, \(P < 0.001\)).

Multivariate analysis. On multivariate analysis, considering as fixed factors gender, histotype and BRAF mutation, nuclear Gal3 \(R^2: 0.338, P < 0.001\), membranous CK19 \(R^2: 0.409, P < 0.001\), nuclear ERb \(R^2: 0.423, P < 0.001\), and membranous HBME1 \(R^2: 0.3, P = 0.001\), significantly correlated with invasive behavior.

According to individual hypothesis, when comparing FVPTC versus classic, ERb and nuclear Gal3 showed a significant difference (estimated difference: \(-13.7, P = 0.22\); and \(-4.84, P = 0.046\), respectively). Cyclin D1 showed the opposite difference (13.58, \(P = 0.043\)). Comparison with WT versus mutant BRAF showed differences for nuclear Gal3 (7.763, \(P = 0.005\), membranous CK 19 (0.556, \(P = 0.014\), and membranous HBME1 (0.634, \(P = 0.021\)). The gender hypothesis yielded no significant difference.

Reproducibility. To determine the extent to which our analyses provide reproducible findings, we performed multiple independent analyses on the same TMA sets. Detailed reanalyses of Gal3Nuc confirmed overexpression of Gal3-Nuc in tumor (7.74 vs. 1.87, \(P = 0.001\) and 4.39 vs. 1.32, \(P = 0.004\)), providing highly concordant findings between separate analyses. Reexamination of Gal3Memb, Gal3Cyt0, and Gal3Nuc in the entire cohort as a second measure of concordance yielded the following concordance: Gal3Cyto/Gal3Nuc: \(R = -0.360, P < 0.001\), Gal3Memb/Gal3Nuc: \(R = 0.561, P < 0.001\), and Gal3Memb/Gal3Cyto: \(R = -0.324, P < 0.001\).

Discussion

Genetic alterations are increasingly studied for cancer risk and prognosis. In PTC, BRAF V600E mutation has been reported to identify tumors with higher risk of developing LNM, ETE, and distant metastasis (6). However, as shown here, this risk is explained by its association with classical variant PTC (7). Within each morphologic subtype, BRAF mutation alone did not predict aggressive behavior. Thus, our data highlight the importance of detailed histotyping in concert with proteomic and mutational characterization in defining cancer behavior.

Morphology has been underestimated as an independent criterion for assessment of PTC behavior. Previous studies have described low incidences of FVPTC and have attempted mainly to link distinct genetic aberrations to the different histotypes. The inter- and intraobserver variability of thyroid cancer diagnosis has compounded the difficulty in determining the value of histologic classification (4, 5, 27).

A novel high-throughput platform for assessment of immunohistochemical markers overcomes the difficulty of subjective evaluations. We used TMAs and automated...
analysis for objective quantification with specific algorithms identifying the cell compartment of interest to establish detailed expression patterns in human tissues. Our study is the first to apply these stringent high-throughput techniques with minimal observer variation. Our data indicate that the histotypes of PTC are different not only morphologically but also by distinct proteomic signatures that portend tumor aggressiveness. Tall-cell papillary carcinoma and other less common variants were analyzed, but due to the small number of cases, we found no statistically significant differences in tumor behavior.

The protein profile of invasive tumors showed overexpression of novel molecules. The cytoskeletal molecule CK19 has been reported to be overexpressed in other highly proliferative epithelial malignancies, including pancreas. HBME-1, an uncharacterized antigen raised against mesothelial cells in culture, distinguishes aggressive behavior in mesothelioma and lung adenocarcinoma as well as thyroid (10). Interestingly, both CK19 and HBME-1 showed reduced cytoplasmic staining associated with increased membrane localization, suggesting that while expression of these markers may assist in defining malignant potential, subcellular localization is particularly relevant in determining invasive and metastatic behavior.

Previous studies have differed about the effectiveness of Gal3 as a diagnostic marker (28). Our data suggest that while membranous and nuclear staining is valuable, cytoplasmic reactivity is not. Previous investigators have iden-
tified nuclear reactivity of this antigen, but its significance as a prognostic marker has not been previously shown.

Thyroid cancer has a much higher incidence in women; however, there are no clear epidemiological or molecular data to explain this phenomenon. Reduced ER immunoreactivity has been associated with atypia, but little is known about the role of ER in thyroid carcinogenesis. Early studies did not distinguish the α and β subtypes of this receptor (29) or used ER-related peptides (30). Stimulation with estrogen promotes growth of cultured thyroid tumor cells (31). We studied both ERα and ERβ and found significant overexpression of ERβ in tumors with ETE or LNM. This was more significant in classic PTCs. As a marker of invasiveness, ERβ may in part explain some aggressive thyroid cancer cases described during pregnancy (32), and raises the potential of ER antagonist therapies for metastatic disease.

By using differential expression of proteins, our study adds to the risk assessment of WD low-risk PTC stronger evidence to support morphologic criteria, previously limited to the expert evaluation of specialized pathologists. We also validated previously recognized molecular markers, now in specific subsets of the disease and with stronger statistical evidence and as novel markers that facilitate the understanding of different morphologic variants of PTC. We also identified, with the use of nonparametric statistics, significant markers specific for gender that may explain the well-known differences in epidemiology and behavior of thyroid cancer in men compared with women.
The results we report warrant further validation in prospective studies. This initial cohort will be followed and clinical outcomes, such as recurrences, metastatic disease, or death from thyroid cancer, will be collected over the longer period of time required for true outcome analysis in this disease. Other markers that are yet to be screened, such as H- and K-RAS mutation, can also be examined. Additional studies applying the same proteomic profile should also be examined in other large groups of patients to validate these markers as determinants of clinical therapeutic management.

In summary, automated high-throughput examination provides an efficient and reproducible tool for multiple applications. It permits detailed statistical analysis, validation of molecular markers, and assessment of clinical risks in large numbers of samples from multiple population groups when annotated with detailed clinical information. The results of these studies can effectively integrate morphologic findings with function and coherently translate them into distinct signatures of disease behavior for effective clinical therapeutic or prognostic purposes.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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