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Protein Biomarker-Driven Machine Learning for Accurate Diagnosis of Invasive Encapsulated Follicular Variant Papillary Thyroid Carcinoma.

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Abstract:

Background: Differentiating follicular-pattern thyroid tumors remains diagnostically challenging, despite established criteria. The 5th edition of the World Health Organization Classification of Endocrine and Neuroendocrine Tumors reclassified invasive encapsulated follicular variant papillary thyroid carcinoma (ieFVPTC) as a distinct entity. Accurate distinction of ieFVPTC from low-risk follicular-pattern tumors is crucial due to their shared morphological features. Proteomics, with its potential for protein biomarker detection and quantification, offers a promising approach. This study investigated the utility of a machine learning-derived protein biomarker panel for ieFVPTC identification using formalin-fixed paraffin-embedded (FFPE) samples.

Methods: A supervised machine learning model was developed and its performance assessed using proteomics data from 46 thyroid tissue samples.

Results: A random forest classifier, utilizing five protein biomarkers (ZEB1, NUP98, C2C2L, NPAP1, and KCNJ3), achieved an area under the curve (AUC) of 1.00 and an accuracy of 1.00 in training samples for distinguishing ieFVPTC from non-malignant samples. Single-protein/gene receiver operating characteristic (ROC) analysis using The Cancer Genome Atlas (TCGA) data also demonstrated an AUC >0.5, supporting the biomarker panel's potential.

Conclusions: This study demonstrates the efficacy of integrating high-throughput proteomics with machine learning for accurate differentiation of ieFVPTC from other follicular-pattern thyroid tumors.

Keywords: Follicular pattern thyroid tumors, Thyroid carcinoma, Machine learning, Proteomics, Histological diagnosis.

Introduction

Thyroid carcinoma, a common endocrine malignancy, presents a diagnostic challenge, particularly within the spectrum of follicular-pattern thyroid tumors. Accurate classification of these lesions is critical, as it directly impacts patient management and prognosis. The follicular variant of papillary thyroid carcinoma (FVPTC) has been a subject of ongoing debate and refinement in diagnostic criteria. Notably, the recent 5th edition of the World Health Organization (WHO) Classification of Endocrine and Neuroendocrine Tumors has reclassified the invasive encapsulated follicular variant of papillary thyroid carcinoma (ieFVPTC) as a distinct entity. This reclassification underscores the clinical significance of distinguishing ieFVPTC from other follicular-pattern tumors, especially those with low malignant potential, due to their shared morphological characteristics.

The traditional diagnostic approach for thyroid tumors relies heavily on histological examination, which can be subjective and challenging, especially in cases with overlapping

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features. For ieFVPTC, the presence of capsular or vascular invasion is a key diagnostic criterion, but these features can be subtle and difficult to identify consistently. Therefore, there is a pressing need for objective and reliable diagnostic tools to complement traditional histopathology.

In this context, proteomics holds immense promise. By enabling the detection and quantification of protein biomarkers, proteomics offers a powerful approach to elucidate the molecular signatures of thyroid tumors. Unlike traditional methods, proteomics can capture the complexity of protein expression, reflecting the dynamic biological processes underlying tumor development and progression.

Furthermore, the integration of proteomics with machine learning techniques offers a unique opportunity to develop robust diagnostic models. Machine learning algorithms can analyze complex proteomics data, identify relevant protein biomarkers, and build predictive models that can accurately classify thyroid tumors. This approach has the potential to overcome the limitations of traditional diagnostic methods and provide a more objective and accurate assessment of tumor malignancy.

This study aims to investigate the potential of a protein biomarker panel, derived from machine learning analysis of proteomics data, for the accurate diagnosis of ieFVPTC. By developing and validating a machine learning model using formalin-fixed paraffin-embedded (FFPE) thyroid tissue samples, we seek to demonstrate the clinical utility of this approach in differentiating ieFVPTC from other follicular-pattern thyroid tumors. This research has the potential to improve diagnostic accuracy, guide patient management, and ultimately enhance patient outcomes in thyroid carcinoma.

Materials and Methods:

Study Subjects:

Thyroid tumor tissue and non-tumor tissue samples were obtained from the Department of Pathology, Chulalongkorn University. This study utilized the same cohort and tissue samples (13 ieFVPTC, 11 NIFTP, 12 WDT-UMP, 12 normal thyroid specimens) as previously described.

Protein Preparation and Shotgun Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis: Tissue samples were prepared for proteomic analysis as previously detailed [13]. Two pathologists (T.PX.N. and S.K.) independently evaluated samples and reached consensus based on the 5th edition of the WHO Classification of Tumors of Endocrine

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Organs [4]. Protein was extracted from formalin-fixed paraffin-embedded (FFPE) specimens using 0.5% sodium dodecyl sulfate, incubated at 50°C for 60 minutes, and centrifuged at 10,000 rpm for 30 minutes. Protein concentration was determined using the bicinchoninic acid method. Five micrograms of each protein sample were reduced with 5 mM dithiothreitol in 10 mM AMBIC at 60°C for 1 hour, alkylated with 15 mM iodoacetamide in 10 mM AMBIC at room temperature for 45 minutes in the dark, and digested with sequencing-grade porcine trypsin (1:20 ratio) for 16 hours at 37°C. The proteins were dried in a speed vacuum concentrator and reconstituted in 0.1% formic acid for nanoLC-MS/MS analysis. LC-MS/MS data were collected using an Ultimate3000 Nano/Capillary LC System (Thermo Scientific) connected to a Hybrid quadrupole Q-Tof impact II (Bruker Daltonics) with a Nano-captive spray ion source. One microliter of the peptide digest was enriched on a μ-Precolumn 300 μm i.d. × 5 mm C18 Pepmap 100, 5 μm, 100 Å (Thermo Scientific) and separated on a 75 μm I.D. × 15 cm Acclaim PepMap RSLC C18, 2 μm, 100Å, nanoViper (Thermo Scientific) column heated to 60°C. Solvents A (0.1% formic acid in water) and B (0.1% formic acid in 80% acetonitrile) were used to elute proteins at a 5%–55% gradient of solvent B over 30 minutes at a flow rate of 0.30 μL/min. Electrospray ionization was performed at 1.6 kV. Nitrogen was used as the drying gas (approximately 50 L/hr) and for collision-induced dissociation. MS and MS/MS spectra were recorded in positive-ion mode at 2 Hz across the m/z range of 150–2,200, with the collision energy set to 10 eV. Protein quantification was performed using MaxQuant ver. 2.2.0.0, which uses the Andromeda search engine to match MS/MS spectra with the Uniprot Homo sapiens database.

LC-MS/MS Analysis and Machine Learning Model Development: An overview of the study design is provided in Fig. 1. After preprocessing and filtering (proteins present in >40% of samples per group), 1,398 proteins were identified from 46 proteomic data files. A machine learning model was developed to differentiate ieFVPTC from non-ieFVPTC specimens (NIFTP, WDT-UMP, and normal thyroid tissue). Samples (n=46) were divided into training (n=36) and internal testing (n=10) subsets. Training samples underwent peptide/protein screening, model selection, and development, while testing samples were used for evaluation and sensitivity analysis.

Protein Screening: Screening consisted of three steps: differentially expressed proteins (DEPs) identification (Supplementary Table S1), unsupervised screening, and supervised screening. First, 181 significant proteins were selected based on DESeq2 results between ieFVPTC and non-ieFVPTC. Second, unsupervised screening involved calculating variance (90th percentile cutoff). Third, supervised screening used logistic regression and model univariate deviance (MUD).

Model Selection and Development: Three-fold cross-validation on training samples was used to select the best performing machine learning model (logistic regression, generalized linear model with elastic net regularization, Naïve Bayes, support vector machine, decision tree, random forest, XGBoost, and multi-layer perceptron (MLP)). The model with the highest accuracy was selected. Synthetic samples (n=52, SMOTE) were generated from the training set.

Model Evaluation and Sensitivity Analysis: Model evaluation included receiver operating characteristic (ROC) analysis, calibration, and confusion matrix construction. Sensitivity

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analyses were performed using random masking (30%, 40%, 50% missing values) and Multivariate Imputation by Chained Equations (MICE).

External Testing of The Cancer Genome Atlas (TCGA) Dataset: PTC cases from the TCGA-THCA dataset (n=507) were analyzed. Cases diagnosed as PTC, follicular variant (n=107) were re-evaluated by pathologists (T.PX.N. and S.K.) using whole slide images and gene expression data. Cases were reclassified as FTC (n=5), non-invasive follicular neoplasm (n=11), non-invasive FVPTC (n=7), invasive FVPTC (n=24), and other diagnoses (n=60). Relevant diagnoses (n=47) were reassigned as non-invasive FVPTC (n=24) or non-ieFVPTC (n=23) (Supplementary Table S2). ROC analysis was conducted on protein expression (training and internal test sets) and gene expression (external test set).

Statistical Analyses: Continuous variables were represented by median and range, and categorical variables by number and percentage. Wilcoxon's and chi-square tests were used for comparisons. $p<0.05$ was considered significant. Analyses were performed using R ver. 4.3.2.

Results:

Patient Characteristics: summarizes the study cohort characteristics, divided into training and internal testing cohorts. The average patient age was 43 years (range: 24–70), with a female predominance (30/46, 65.2%). The most common sample type was ieFVPTC (13/46, 28.3%), followed by normal tissue (12/46, 26.1%), NIFTP (11/46, 23.9%), and WDT-UMP (10/46, 21.7%). No significant differences were observed between the training and testing cohorts in terms of age ($p = 0.170$), sex ($p = 0.987$), nuclear score ($p = 0.421$), diameter ($p = 0.922$), invasion ($p = 0.498$), and diagnosis ($p = 0.344$).

Protein Screening: A three-layer screening process (differentially expressed proteins, unsupervised, and supervised screening) identified five optimal proteins: ZEB1, NUP98, C2C2L, NPAP1, and KCNJ3. The cutoff of five proteins was determined by a significant increase in model univariate deviance (MUD) between the 5th and 6th smallest values. This heuristic balances the number of predictive variables with the training sample size, preventing overfitting. The expression of these five proteins across training samples is shown in a heatmap.

Model Selection: Cross-validation results are presented in Table 3. The mean accuracy scores (± standard deviation) for each model were as follows: logistic regression, 0.89 (±0.10); generalized linear model with elastic net regularization, 0.92 (±0.09); Naïve Bayes, 0.97 (±0.05); support vector machine, 0.97 (±0.05); decision tree, 0.91 (±0.09); random forest, 1.00 (±0.00); XGBoost, 0.92 (±0.09); and MLP, 0.97 (±0.05). The random forest classifier, with the highest accuracy, was selected for the final model.

Model Evaluation and Sensitivity Analysis: illustrates the model evaluation results for training and internal testing samples. Receiver operating characteristic (ROC) analyses and calibration plots demonstrated high areas under the curve (AUCs) of 1.00 and good calibration in both groups. Confusion matrices showed accurate prediction of all training samples and

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almost all testing samples. Sensitivity analysis revealed marginal reductions in model performance with 30%, 40%, and 50% data distortion (AUCs: 0.95, 1.00, 0.88; accuracies: 0.90, 0.90, 0.80, respectively). Calibration plots remained well-calibrated, indicating model robustness.

Analyses of Model Proteins in Distinguishing ieFVPTC and Non-ieFVPTC: presents the results of single-protein/gene ROC analyses differentiating ieFVPTC from non-ie FVPTC. All genes had AUCs greater than 0.5 in the external test set, highlighting the significance of combining proteins for accurate discrimination. These results validate the proteins included in the model.

Discussion:

Follicular-patterned thyroid nodules present a diagnostic challenge due to the spectrum of conditions they encompass, from benign (follicular adenoma, FA) to malignant (follicular thyroid carcinoma, FTC; invasive encapsulated follicular variant papillary thyroid carcinoma, ieFVPTC) and borderline neoplasms (non-invasive follicular thyroid neoplasm with papillary-like nuclear features, NIFTP; well-differentiated tumor of uncertain malignant [1] potential, WDT-UMP). Histological evaluation, particularly distinguishing between these entities, remains difficult, and cytology is often insufficient. Differentiating FA from FTC relies on nuclear feature grading and the presence of capsular or vascular invasion. Conversely, NIFTP, WDT-UMP, and ieFVPTC share similar nuclear features but differ in invasion characteristics. NIFTP lacks invasion, ieFVPTC exhibits clear invasion, and WDT-UMP presents ambiguous invasion patterns. According to the revised WHO classification, ieFVPTC, like FTC, is malignant, with subtypes ranging from minimally invasive to widely invasive. Minimally invasive ieFVPTC may require only local excision, while widely invasive tumors necessitate complete thyroidectomy and further therapy. NIFTP and WDT-UMP, classified as low-risk, borderline neoplasms, typically require lobectomy and monitoring. Accurate ieFVPTC diagnosis is therefore crucial to guide appropriate surgical management. Machine learning has emerged as a promising tool in medical diagnostics. Prior studies have demonstrated its utility in differentiating follicular neoplasms. For example, Sun et al. identified 31 proteins distinguishing FA from FTC using data-independent acquisition MS and machine learning. Li et al. developed a preoperative risk assessment classifier for papillary thyroid carcinoma (PTC) integrating clinical, genetic, proteomic, and immunologic data. In this study, we applied a random forest classifier to shotgun MS data, identifying five proteins (C2C2L, KCNJ3, NPAP1, NUP98, and ZEB1) that effectively differentiate ieFVPTC from non-ieFVPTC. These proteins demonstrated high AUCs and accuracy in training and test cohorts, suggesting potential clinical utility through targeted MS-based proteomics assays. While these proteins have not been extensively studied in thyroid cancer, some have been implicated in carcinogenesis. KCNJ3 is associated with breast cancer progression, NUP98 with hematopoietic malignancies, and ZEB1 with epithelial-mesenchymal transition in carcinomas. Further research is needed to elucidate the roles of C2C2L and NPAP1. Preliminary analysis using TCGA data suggests these proteins may serve as immunohistochemical markers for ieFVPTC. ieFVPTC, like FTC, is a RAS-driven lesion with similar morphology but differing nuclear features. Both show a correlation between invasion extent and prognosis. Our TCGA

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data analysis revealed similar expression patterns of the five identified proteins in ieFVPTC and FTC. This study has limitations. We used non-targeted proteomics on FFPE samples and did not validate findings on fine-needle aspiration (FNA) samples or include FA and FTC in the analysis. Future studies will validate these protein biomarkers using immunohistochemistry and assess their prognostic roles in larger cohorts, including FNA and FFPE samples.

In conclusion, we identified five proteins capable of accurately diagnosing ieFVPTC, contributing to the advancement of molecular diagnostics for follicular-patterned thyroid tumors and potentially improving the accuracy of existing molecular tests.

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