浙江大学医学院附属第一医院
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Proteotypic Differences of Follicular-Patterned Thyroid Neoplasms

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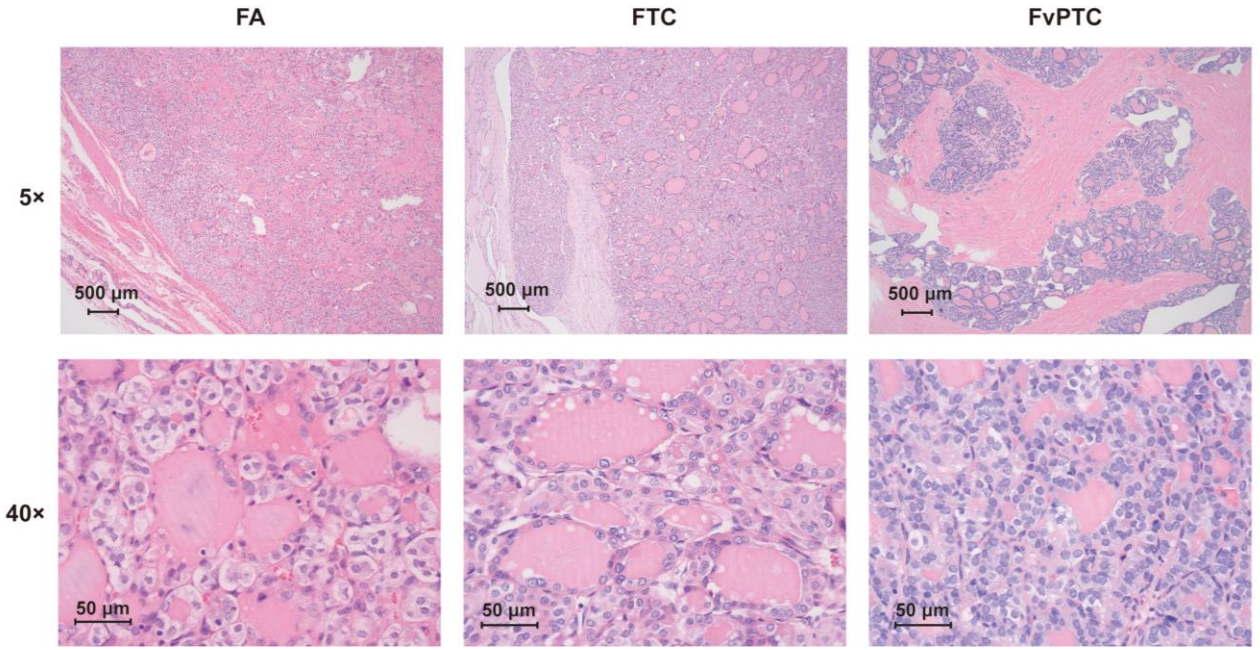
01

Background

- **Thyroid nodules, encompassing adenomatous nodules, nodular goiters, follicular thyroid adenoma (FA), follicular thyroid carcinoma (FTC), and follicular variant of papillary thyroid carcinoma (FvPTC), are a common finding in adults and exhibit follicular morphological characteristics.**

- **However, the diagnosis and identification of follicular-patterned thyroid nodules such as FA, FTC, and FvPTC have always been formidable challenges to cytopathologists because of overlapping cytological features and the lack of evidence of capsular or vascular invasion.**



- **There is still debate as to which pathological type FvPTC is closer to. Therefore, research on new potential markers for thyroid pathology, particularly markers enabling FA, FTC, and FvPTC to be distinguished, remains worth conducting.**

- **Proteomics is a promising approach for identifying biological systems and functions by quantifying and validating large numbers of proteins. Recently, pressure cycling technology (PCT) has been developed for semiautomatic processes with small-volume clinical tissues. PCT-data-independent acquisition (DIA) results in higher quantitative accuracy, provides deeper proteome coverage, and is less time-consuming than conventional approaches.**

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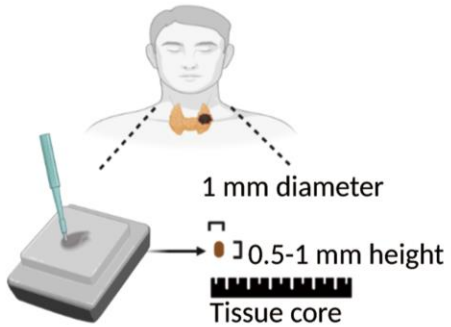
Methods

1

Patients and sampling (52 patients in the discovery group and 232 patients in the validation group)



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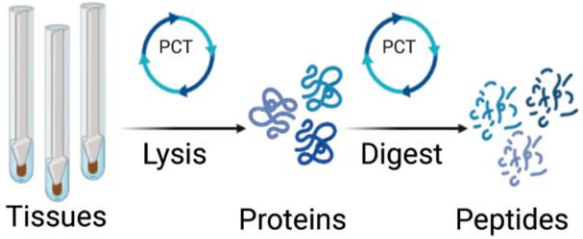
PCT-assisted proteomic sample preparation (52 samples including 18 FA, 15 FTC, 19 FvPTC)



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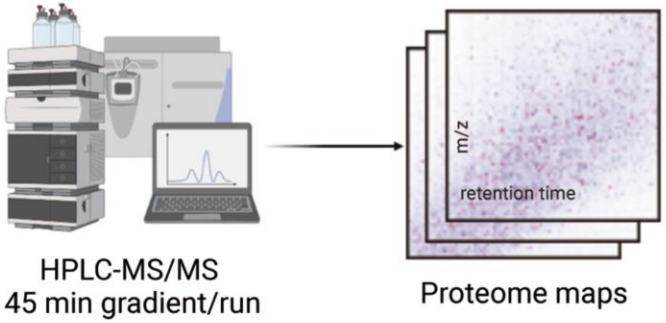
Proteomic data acquisition by DIA mode (52 samples × 2 technical replicates)



HPLC-MS/MS
45 min gradient/run

Proteome maps

4

Proteomic data analysis

104 DIA-MS files

Spectranaut™ version 13.5

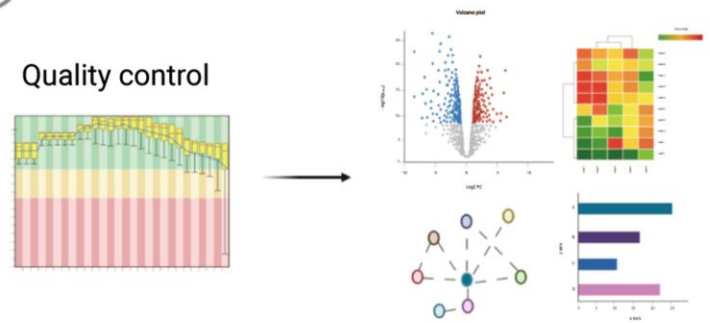
Thyroid spectral library:
157,548 peptide precursors,
121,960 peptides,
9941 proteins

Totally identied 4107 protein in the dataset of 104 DIA files

5

Bioinformatic analysis and biomarker discovery

Quality control



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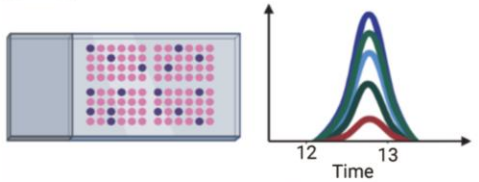
Validation study on an independent set including:



TMA validation:
101 FA
67 FTC
65 FvPTC

PRM validation:
20 FA
20 FTC
20 FvPTC

TABLE 1 | Clinical characteristics in DIA-MS, PRM-MS, and IHC analyses.

	Discovery set	Validation set	
	DIA-MS	**PRM-MS**	**IHC**
Histopathology diagnosis			
FA	18	20	101
FTC	15	20	67
FvPTC	19	20	65
Gender			
Female (%)	35 (67.3%)	41 (68.3%)	160 (68.7%)
Male (%)	17 (32.7%)	19 (31.7%)	73 (31.3%)
Age at diagnosis			
Mean	46.77	44.13	46.36
Range	33.24 - 60.3	30.13 – 58.13	32.61 – 60.11
<55 y (%)	34 (65.4%)	44 (73.3%)	157 (67.4%)
≥55 y (%)	18 (34.6%)	16 (26.7%)	76 (32.6%)
Nodule size			
Mean	2.76	2.71	2.66
Range	1.13 – 4.39	1.29 – 4.12	1.11 – 4.20
<1 cm (%)	7 (13.4%)	7 (11.7%)	33 (14.2%)
1 - 4 cm (%)	34 (65.4%)	41 (68.3%)	168 (72.1%)
>4 cm (%)	11 (21.2%)	12 (20.0%)	32 (13.7%)

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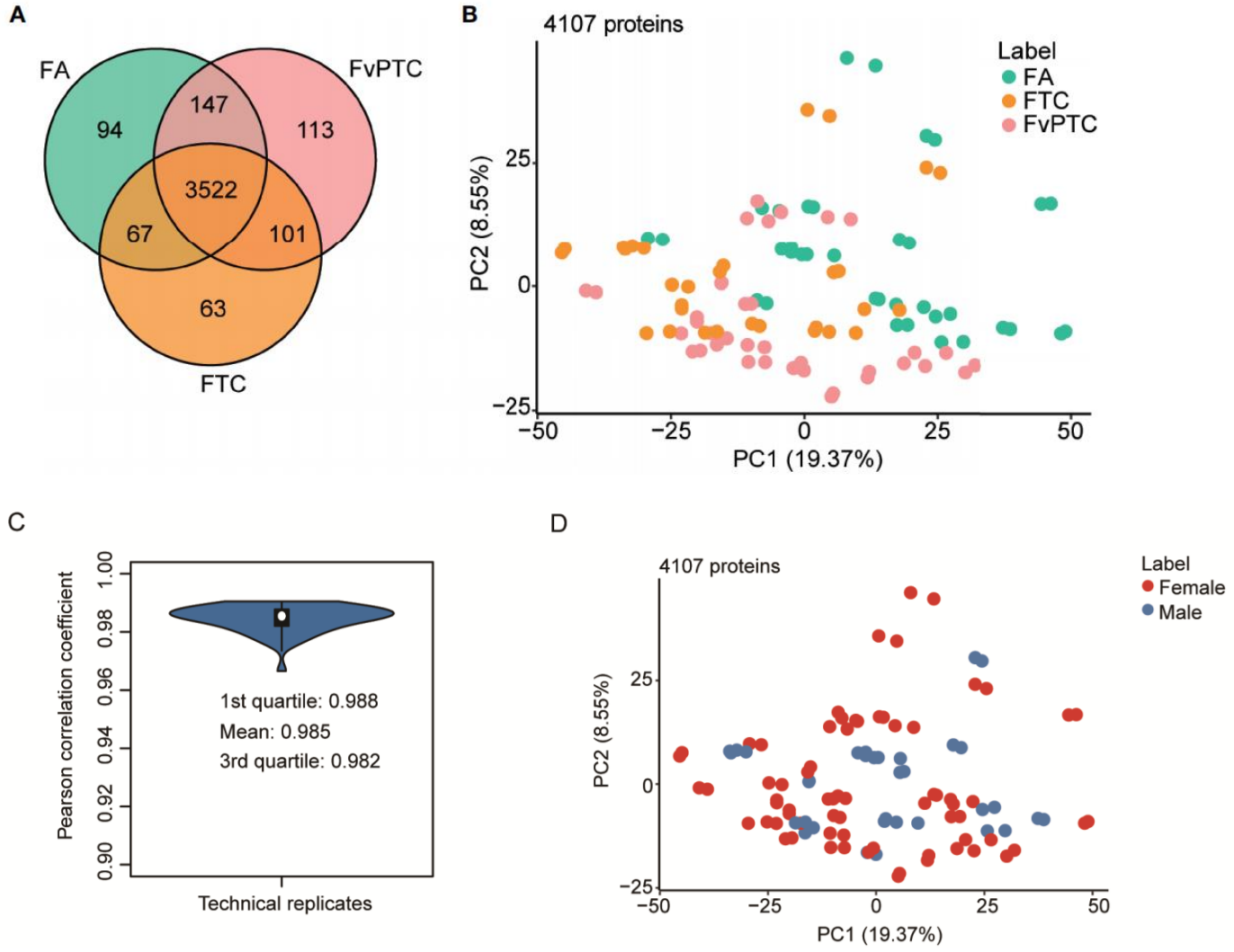
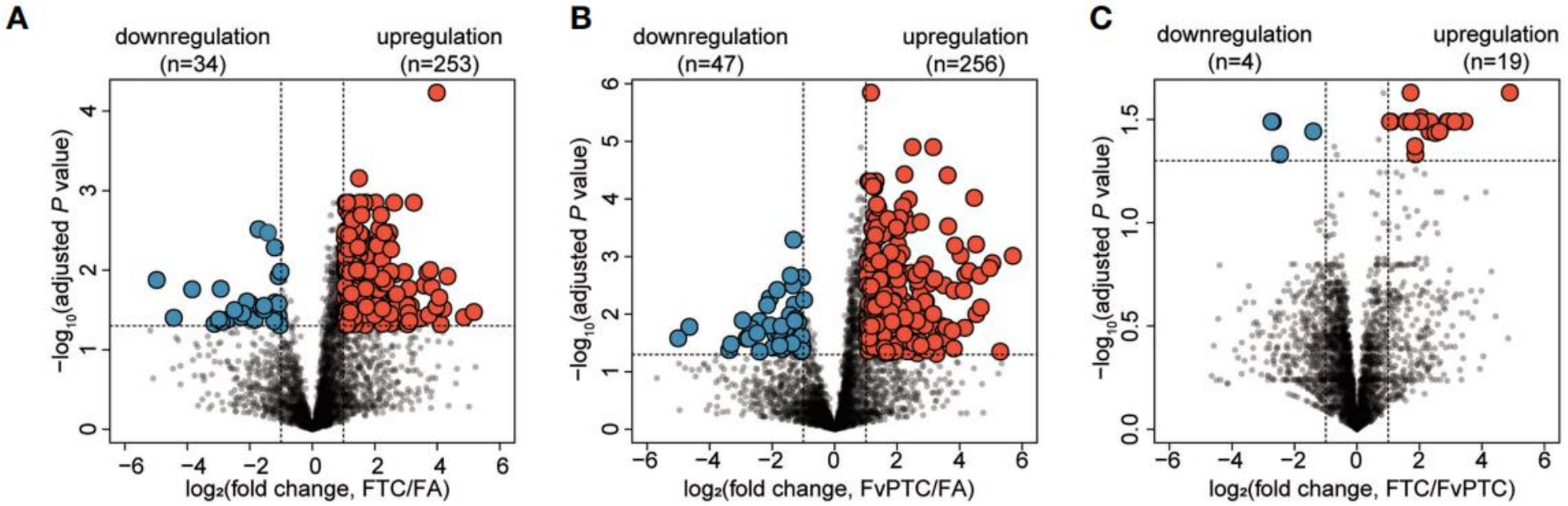
Results

Proteomic Data Analysis Based on Discovery Group



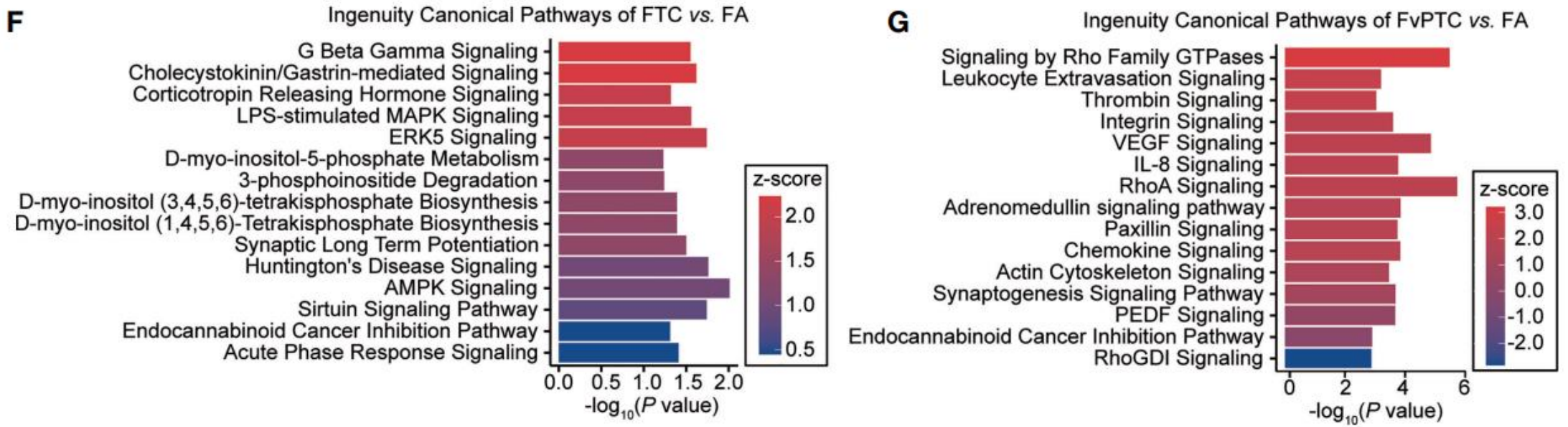
- Principal component analysis (PCA) using 4107 proteins grouped by tissue type and gender revealed that FTC is more similar to FvPTC than FA. However, there was no significant difference in gender among these three groups.

Difference Analysis of Proteomic Profifile for the Follicular Patterned Thyroid Tumors



- As the volcano plots in Figure A, by setting a cutoff value of a two-fold change and a threshold adjusted P value of less than 0.05, we identified 287 differentially expressed proteins (DEPs), specifically, 253 upregulated and 34 downregulated proteins in FTC. In addition, the comparison showed 303 DEPs in FA and FvPTC, with 256 upregulated proteins and 47 downregulated proteins in FvPTC (Figure B). Interestingly, only 23 discriminatory proteins were detected between FTC and FvPTC (Figure C), a much lower number than found in the other comparative analyses.

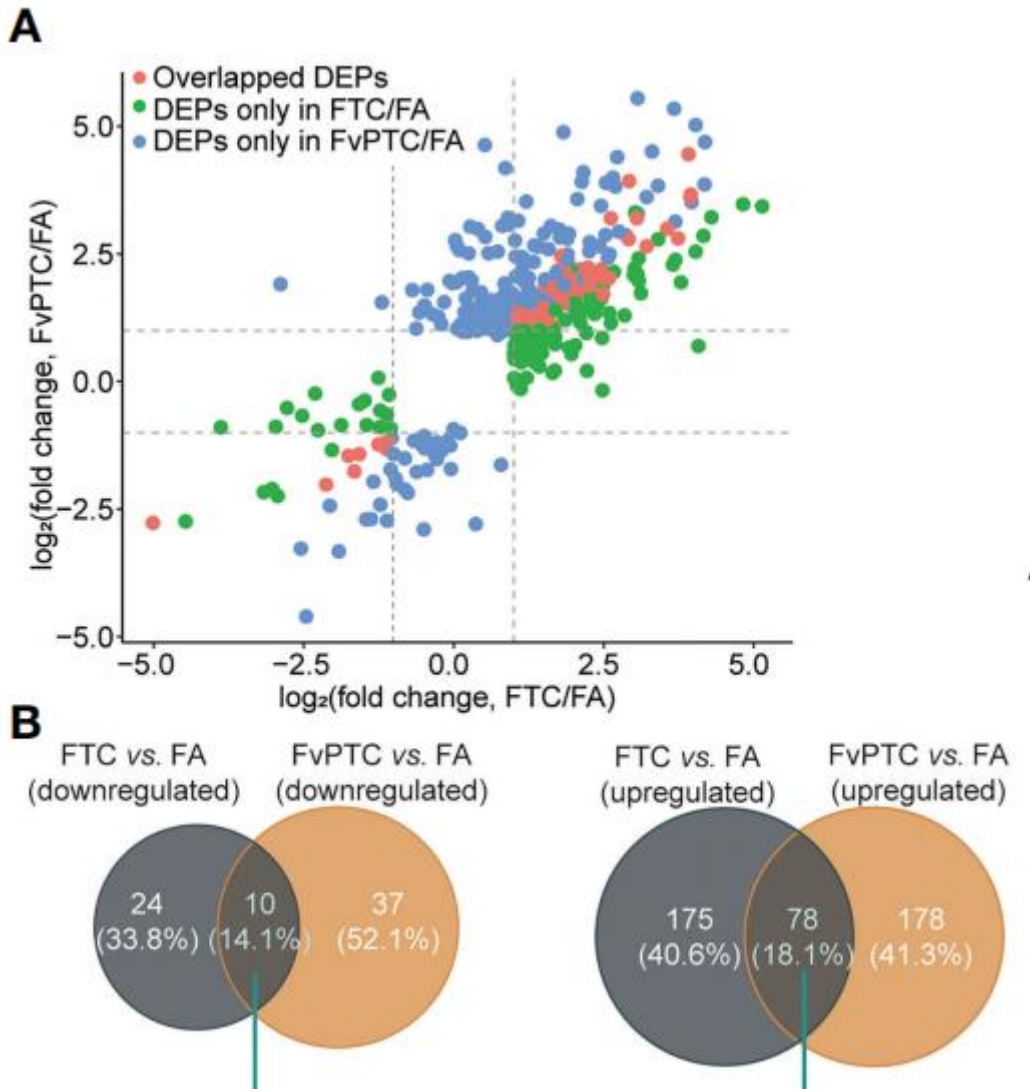
- These pairwise analyses of protein expression also showed an apparent separation of FA from FTC and FvPTC, whereas FvPTC showed no apparent distinction from FTC. These results indicated that the two malignant tumors exhibited similar proteotypes but were distinct from the benign tumor FA.



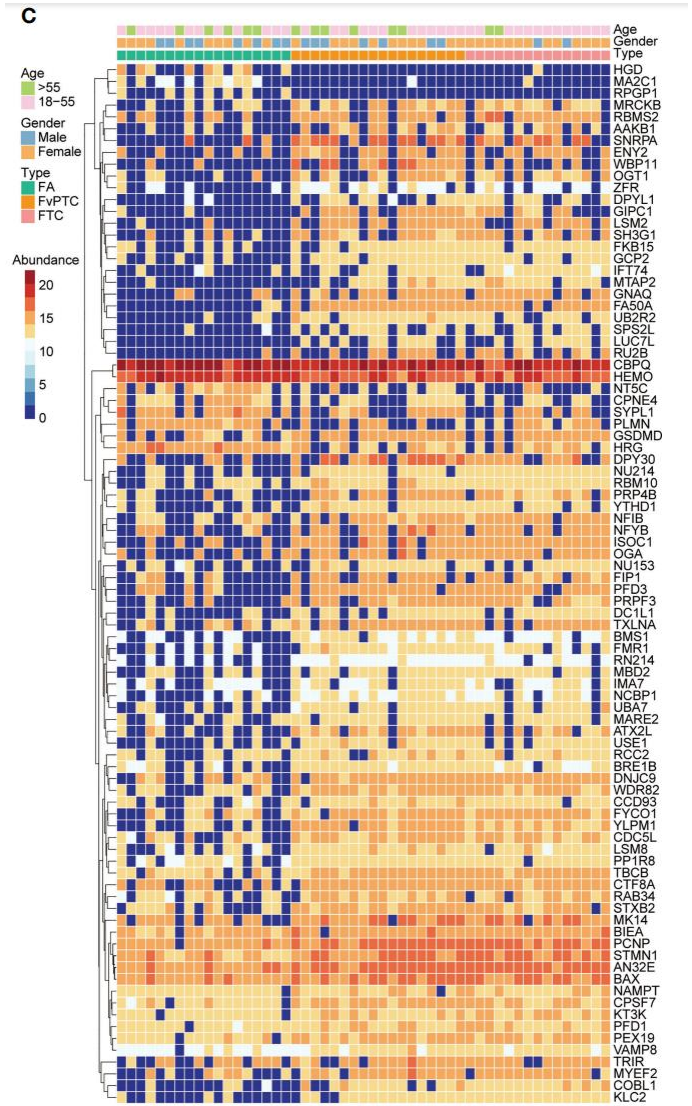
- Based on the 287 proteins identified by comparison of FTC and FA (Figure F), AMPK signaling, which participates in cell growth, autophagy, and metabolism, was the most significantly enriched pathway.

- Based on the 303 DEPs between FvPTC and FA (Figure G), RhoA signaling was substantially enriched. Signaling by Rho family GTPases was the most activated pathway, whereas RhoGDI signaling was the most inhibited pathway. In addition, the VEGF signaling pathway was significantly enriched.

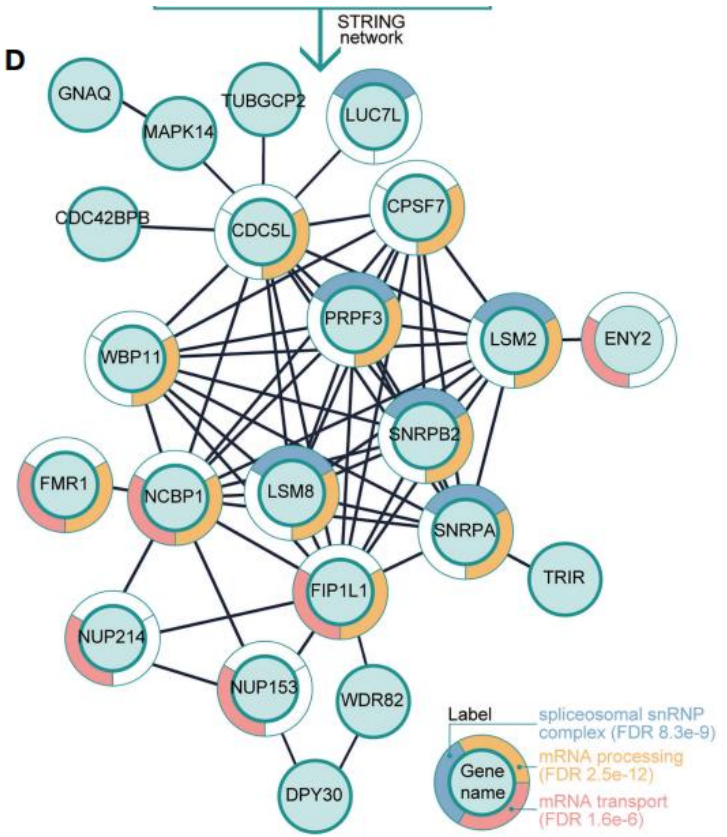
Biological Analysis for the Three Types of Tumors



- Asindicated above, 253 and 256 proteinswere upregulated but 34 and 47 proteins were downregulated in FTC and FvPTC, respectively, compared with FA. The analysis further indicated that FTC and FvPTC shared 78 upregulated and ten downregulated proteins compared with FA (Figures A, B).

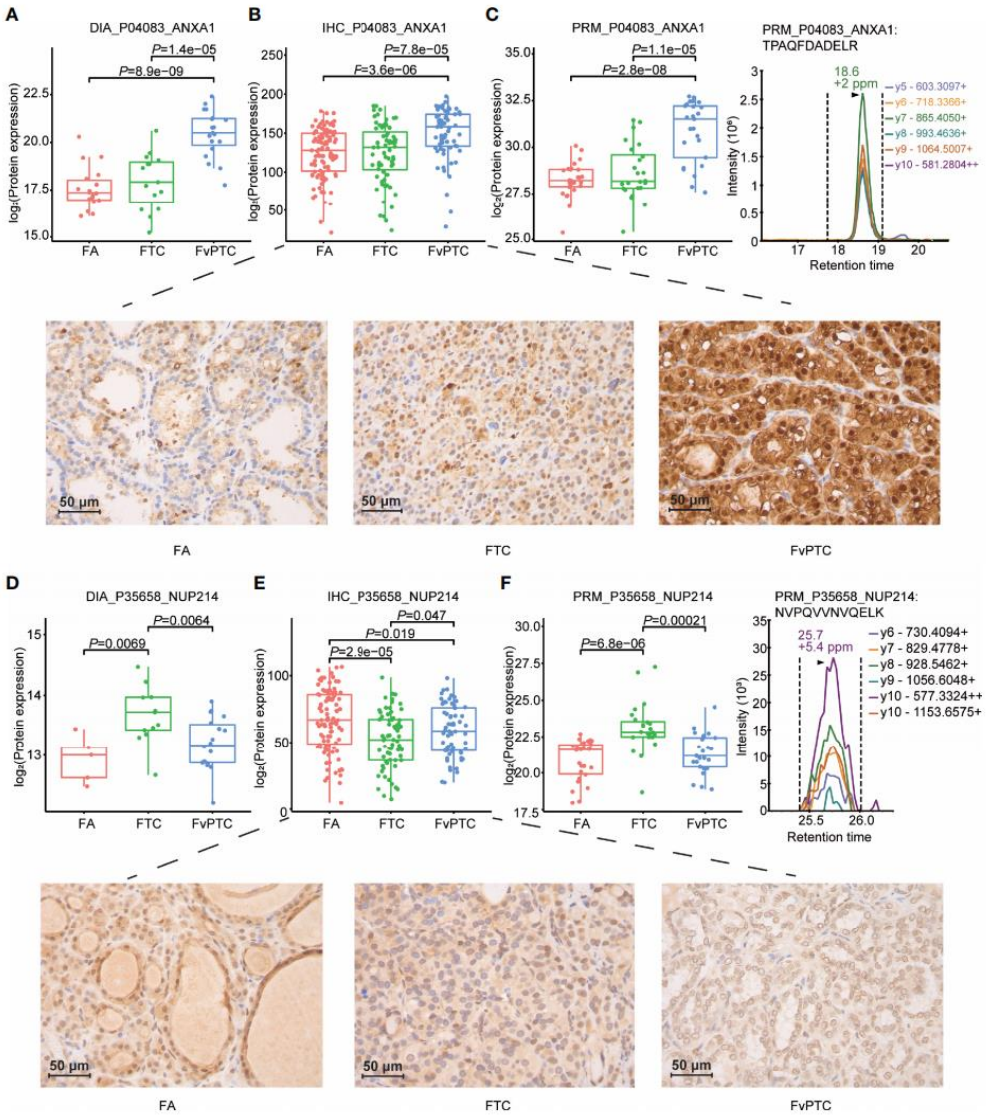


- These proteins were related to thyroid cancers and their expressions were shown in the heatmap (Figure C).



- The 88 proteins were involved in three major biological functions or processes, namely, spliceosomal snRNP complex (FDR8.3e-9), mRNA processing (FDR2.5e-12),and mRNA transport (FDR 1.6 e-6), as annotated around the nodes. The key proteins in the center of the network were PRPF3, SNRPB2, SNRPA, LSM2, LSM8, FIP1L1, NCBP1, WBP11, CDC5L, and CPSF7.

Immunohistochemistry and PRM-MS Quantification for Validation



- Furthermore, we validated the selected proteins as mentioned in the IHC and PRM-MS quantification methods in the two independent sets. Of note, the differential expression levels of ANXA1 in all three tumors were consistent across all three methods including DIA-MS, PRM-MS and IHC. In addition, interestingly, NUP214 presented higher expression as determined by DIA and PRM in FTC compared with FA and FvPTC, although IHC analysis showed different results.

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Conclusion

- **In conclusion, the current proteomic analysis of FA, FTC, and FvPTC identified certain protein signatures that can distinguish various thyroid nodules with different follicular morphological characteristics. Specifically, clusters of proteins demonstrated a marked ability to differentiate FA from FTC and FvPTC.**

- **ANXA1 is a promising biomarker for differentiating FvPTC from the other thyroid tumors.**

- **However, more research needs to be carried out to further cull and validate potential biomarker candidates. These findings may provide deeper insight for improving the diagnostic accuracy and efficiency of follicular-patterned thyroid neoplasms.**


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