Neuro-Oncology

24(7), 1126–1139, 2022 | https://doi.org/10.1093/neuonc/noac050 | Advance Access date 25 February 2022

Detection of tumor-specific DNA methylation markers in the blood of patients with pituitary neuroendocrine tumors

Grayson A. Herrgott[†], Karam P. Asmaro[†], Michael Wells[†], Thais S. Sabedot, Tathiane M. Malta, Maritza S. Mosella, Kevin Nelson, Lisa Scarpace, Jill S. Barnholtz-Sloan, Andrew E. Sloan, Warren R. Selman, Ana C. deCarvalho, Laila M. Poisson, Abir Mukherjee, Adam M. Robin, Ian Y. Lee, James Snyder, Tobias Walbert, Mark Rosenblum, Tom Mikkelsen, Arti Bhan, John Craig, Steven Kalkanis, Jack Rock, Houtan Noushmehr[‡], and Ana Valeria Castro[‡]

Department of Neurosurgery, Hermelin Brain Tumor Center, Henry Ford Health System, Detroit, Michigan, USA (G.A.H., K.P.A., M.W., T.S.S., T.M.M., M.S.M., K.N., L.S., A.C.C., A.M.R., I.Y.L., J.S., T.W., M.R., T.M., S.K., J.R., H.N., A.V.C.); Department of Neurosurgery, Omics Laboratory, Henry Ford Health System, Detroit, Michigan, USA (G.A.H., K.P.A., M.W., T.S.S., T.M.M., M.S.M., J.S., H.N., A.V.C.); Department of Population and Quantitative Health Sciences, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA (J.S.B.); Department of Neurological Surgery, University Hospitals of Cleveland, Cleveland, Ohio, USA (A.E.S., W.R.S.); Case Comprehensive Cancer Center, Cleveland, Ohio, USA (A.E.S.); Department of Biostatistics, Henry Ford Health System, Detroit, Michigan, USA (L.M.P.), Department of Pathology, Henry Ford Health System, Detroit, Michigan, USA (A.M.), Department of Endocrinology, Henry Ford Health System, Detroit, Michigan, USA (A.B.); Department of Otolaryngology, Skull Base, Pituitary and Endoscopy Center, Henry Ford Health System, Detroit, Michigan, USA (J.C.)

[†]These authors are co-first authors.
[‡]These authors are co-senior authors.
Moved to the National Cancer Institute, Bethesda, Maryland, USA (this work was completed while at Case Western Reserve University School of Medicine, Cleveland, Ohio, USA) (J.S.B.)

Corresponding Author: Ana Valeria Castro, MD, PhD, Department of Neurosurgery, Hermelin Brain Tumor Center, Omics Laboratory, Henry Ford Health System, 2799 West Grand Blvd, E&R 3096, Detroit, MI 48202, USA (acastro1@hfhs.org; dra.anavaleria@gmail.com).

Abstract

Background. DNA methylation abnormalities are pervasive in pituitary neuroendocrine tumors (PitNETs). The feasibility to detect methylome alterations in circulating cell-free DNA (cfDNA) has been reported for several central nervous system (CNS) tumors but not across PitNETs. The aim of the study was to use the liquid biopsy (LB) approach to detect PitNET-specific methylation signatures to differentiate these tumors from other sellar diseases.

Methods. We profiled the cfDNA methylome (EPIC array) of 59 serum and 41 plasma LB specimens from patients with PitNETs and other CNS diseases (sellar tumors and other pituitary non-neoplastic diseases, lower-grade gliomas, and skull-base meningiomas) or nontumor conditions, grouped as non-PitNET.

Results. Our results indicated that despite quantitative and qualitative differences between serum and plasma cfDNA composition, both sources of LB showed that patients with PitNETs presented a distinct methylome landscape compared to non-PitNETs. In addition, LB methylomes captured epigenetic features reported in PitNET tissue and provided information about cell-type composition. Using LB-derived PitNETs-specific signatures as input to develop machine-learning predictive models, we generated scores that distinguished PitNETs from non-PitNETs conditions, including sellar tumor and non-neoplastic pituitary diseases, with accuracies above ~93% in independent cohort sets.

Conclusions. Our results underpin the potential application of methylation-based LB profiling as a noninvasive approach to identify clinically relevant epigenetic markers to diagnose and potentially impact the prognostication and management of patients with PitNETs.

Key Points

- PitNETs release biologically and clinically relevant DNA methylation markers in the circulation that are detectable in serum and plasma.
- Machine-learning predictive models using serum- and plasma-derived tumor-specific methylation markers distinguish PitNETs from other pituitary and CNS diseases.

Importance of the Study

This study encompasses the analysis of circulating cell-free (cf) DNA methylome in the serum and plasma from patients with pituitary diseases (PitNETs and other sellar conditions), other CNS diseases or conditions, and controls. Our results contain 2 novel elements: (1) the discovery of biologically and clinically relevant PitNET-specific methylation signatures suitable to develop machine-learning prediction models to distinguish PitNETs from other CNS tumors or conditions with an accuracy above ~93%, and (2) both serum and plasma cfDNA methylome are useful and, possibly, complementary to providing molecular information specific to PitNETs. Finally, profiling cfDNA in liquid biopsy specimens could complement imaging to differentiate challenging cases of PitNETs, particularly the nonfunctioning subtype, from other sellar tumors and diseases, using presurgical noninvasive techniques which could, ultimately, optimize therapeutic plans.

Pituitary neuroendocrine tumors (PitNETs) comprise the second most common neoplasm of the central nervous system (CNS) (~17%).[1,2] Stratified by endocrine status these tumors are classified as functioning and nonfunctioning (or silent) pituitary tumor subtypes (46%-64% and 36%-54% frequency, respectively).[3] The diagnosis and classification of these tumors are based on a variety of workup procedures, including blood hormonal profiling, imaging and immunostaining for adenohypophyseal hormones (eg, prolactin, growth hormone, etc.), and cell lineage-deriving transcription factors (eg, mainly SF1, PIT1,TPIT),[4] which requires surgical removal of the tumor. In rare cases, the preoperative differential diagnosis of silent PitNETs from other pituitary diseases (OPD) may be challenging via imaging alone (eg, PitNET vs histiocytosis, supra-sellar meningiomas, low-grade gliomas or pituicytomas, etc.).[5–7] Therefore, the differential diagnosis of these controversial cases could benefit from other noninvasive presurgical assessment methods to assure the most appropriate management.

Liquid biopsy (LB) is a method used to detect molecular elements, such as circulating cell-free DNA (cfDNA), shed by tumors into biofluids (eg, blood, cerebrospinal fluid, etc.). Despite the shielding effect of the blood-brain barrier (BBB), the feasibility to detect cfDNA, particularly the tumor fraction, in the bloodstream of patients has been reported in many CNS neoplasms, including PitNETs.[8–12] For instance, a recent study has demonstrated the ability to detect somatic gene variants using plasma cfDNA in PitNETs, despite the rarity of somatic mutations in these tumors.[12] In contrast, genome-wide methylation abnormalities are pervasive across PitNET tissue[13–18] and yet have not been reported via the profiling of blood-derived cfDNA. Methylation patterns are tissue- and tumor-specific providing opportunity to infer the origin and differentiate tumor types.[10,11,16,19–21] Indeed, studies involving CNS tumor tissue and LB specimens have shown that specific methylome signatures distinguish PitNETs from other CNS tumors[16] or from non-neoplastic tissue[22] and are useful for diagnostic, prognostic, and predictive applications in CNS tumors.[11,16,23–25] Here we aimed to differentiate PitNETs from OPD through analysis of LB specimens which has not been reported in these tumors. We found that both serum and plasma cfDNA methylomes were useful and complementary in providing molecular information specific to PitNETs which also recapitulated tumor tissue findings. By combining PitNET-specific epigenetic signatures derived from both serum and plasma sources, we were able to develop machine-learning predictive models that differentiated PitNETs from other pituitary or CNS diseases and conditions with high accuracy and reliability.

Our results pave the way for the potential clinical application of liquid biopsy as a noninvasive approach to identify relevant epigenetic markers and to shift paradigms in the differential diagnosis and management of PitNETs.

Methods

Patients

We conducted an analysis of methylome data from a cohort composed of archival serum or plasma samples collected from patients who underwent transsphenoidal surgery for the resection of sellar masses at: (1) the Henry Ford Health System (HFHS), Detroit, Michigan—PitNETs (n = 37); OPD (craniopharyngiomas [n = 4 serum, 4 matching plasma, 1 additional duplicate plasma], pituicytomas [n = 1 serum, 1 matching plasma], histiocytosis [n = 1 serum], cysts [n = 1 serum, 1 plasma], chordoma [n = 1 serum]) and other CNS

diseases (OCD, lower-grade gliomas [n = 18], skull-base meningiomas [n = 16]; paired PitNET serum and tissue were available for 13 patients; and (2) Case Western Reserve University/University Hospitals of Cleveland, Cleveland, Ohio (CWRU/UH, plasma, n = 24, PitNETs). As controls, we profiled serum derived from patients with other CNS non-neoplastic diseases (HFHS, n = 7) and plasma from 4 healthy donors were used as controls (publicly available source, n = 4)[26] (cohort described in detail in Table 1). The project was approved by the Institutional Review Board of each Institution (HFHS IRB# 10963; University Hospitals IRB # CC296 (CASE 1307)) and patients consented to have their specimens used for research purposes. For tissue, we have previously compiled methylome data from the non-neoplastic pituitary gland (n = 15) and PitNET specimens available in public repositories (n = 164) and generated at the Hermelin Brain Tumor Center (HBTC) (n = 13), namely the Panpit cohort (n = 179)[22]; additionally, we profiled the tissue of OPD (n = 9, craniopharyngiomas, Rathke cleft cyst, rhabdoid tumor, and histiocytosis). We also harnessed tissue methylome data of lower-grade gliomas (n = 100) and skull-base meningiomas (n = 65) from publicly available data (https://www.cancer.gov/tcga) or generated in house[9,22] and paired methylome and transcriptome data from PitNET, namely the Neou cohort.[18] We used TCGAbiolinks, an open access R/Bioconductor package for integrative analysis of included TCGA data.[27]

Serum and Plasma Collection and Processing

Peripheral blood (15 mL) was drawn from each subject at the time of surgical procedure before the tumor excision. Plasma and serum were obtained within 1 hour from the collection (details in Supplementary Methods).

DNA Isolation, Quantification, Quality Control, and DNA Methylation Data Generation

Extracted cfDNA or DNA from serum/plasma and tissue samples, respectively, were profiled using an Illumina Human EPIC array (HM850K) as described in our previous manuscript.[9] Data quality assessment of the LB methylome data was assessed with shinyMethyl (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4176427/) (details in Supplementary Methods). Methylation array data were processed with the minfi package in R as previously described.[28] Before analysis, we removed probes with missing values and any masked probes, as provided through comprehensive characterizations conducted by Zhou et al[29] (details in Supplementary Methods). Prior to analysis involving methylomes profiled through 850K and 450K Illumina platforms, we aligned all serum/plasma and tissue methylomes from serum/plasma or tissue to identify common probes between the arrays (n = 393K common probes).

DNA Methylation Exploratory Analysis

Unsupervised analysis. We generated a three-dimensional (3D) genome-wide principal component analysis (PCA) of the mean methylome levels across all serum and plasma samples from patients with distinct tumor types and non-neoplastic brain diseases, using the function *prcomp* (version 3.6.0). We also compared the 1K most variably methylated probes across serum and plasma samples (n_{serum} = 59 and n_{plasma} = 41 samples). CpGs with the highest variance across multiple cohorts and PitNET similarity (tissue and LB) were used for an unsupervised clustering through t-Distributed Stochastic Neighbor Embedding (t-SNE) dimensionality reduction for visualization (details in Supplementary Methods).

Supervised analysis. In order to identify serum or plasma-derived PitNET-specific differentially methylated probes (DMPs), we performed supervised analysis between PitNETs and non-PitNET methylomes and selected probes which presented significant adjusted *P*-values and mean methylation differences across pairwise comparisons (Wilcoxon rank-sum test). For the development of predictive models described below, we input probes that presented less than 5% methylation differences between serum and plasma and to increase PitNET-probe specificity, less than 0.5% difference between PitNET serum and tissue, namely PitNET Epigenetic Liquid Biopsy (PeLB) probes. Similar comparisons were performed to select nonfunctioning PitNET-specific DMPs (NF-PeLB). Each CpG probe was mapped to their genomic location as CpG islands (CGI), shores, shelves, and open sea regions as previously defined.[29]

Machine-learning prediction modeling—Random Forest. To investigate the potential application of PeLB as a diagnostic tool to differentiate sellar masses and other diseases, independently of the LB source (serum or plasma), we used a random forest machine-learning approach to generate a model for binary classification (PitNET and non-PitNET or NF-PitNETs and non-PitNET), using PitNET-specific methylation signatures concomitantly identified in serum (n = 59) and plasma (n = 41) specimens (details in Supplementary Methods).

Identification of tissue-derived methylation markers in the serum/plasma specimens. In order to investigate whether probes that differentiate groups in tissue specimens also differentiate similar groups in LB specimens (serum or plasma) we performed the following analyses: (1) supervised analysis between the following groups to detect tissue-specific DMPs: PitNETs vs non-neoplastic controls; F-PitNET vs NF-PitNET; PitNET vs other CNS tumors, PitNET vs OPD, using publicly available or in house generated tissue methylome data from PitNETs and pituitary controls and other CNS tumors (lower-grade glioma/skull-base meningioma) (Supplementary File S2); (2) alignment of the significant tissue-derived DMPs with serum or plasma methylomes; and (3) selection of DMPs which retained differential methylations between concordant groups in serum or plasma specimens.

Integrative analysis of PitNET liquid biopsy-derived probes with respective putative target genes and associated pathways. We mapped each differentially methylated CpG probe derived from the different supervised

Table 1. Demographic and Clinicopathological Information for the Serum- and Plasma-Based Cohorts

Features	Serum (N = 59)		Plasma (N = 41)	
	Median	(Q1, Q3)	Median	(Q1, Q3)
Age (years)	56	(42.0, 65.0)	51	(42.0, 61.0)
	n	%	n	%
Sex				
Females	30	50.85	18	43.9
Males	27	45.76	23	56.1
NI	2	3.39	—	—
Race/ethnicity				
African American	11	18.64	8	19.51
Caucasian	40	67.8	27	65.85
Other	1	1.69	2	4.88
Unknown	7	11.86	4	9.76
Source/WHO Classification (2017)				
PitNET	13	22.03	24	43.9
Corticotroph	1	7.69	2	8.33
Gonadotroph	4	30.77	4	16.67
Lactotroph	2	7.69	1	4.17
Mammosomatotroph	—	—	1	4.17
Null cell	5	38.46	4	16.67
Plurihormonal	—	—	4	16.67
Plurihormonal PIT1	1	7.69	2	8.33
Somatotroph	—	—	1	4.17
Thyrotroph	—	—	3	12.5
Unknown	—	—	3	12.5
Non-PitNET	46	77.97	17	41.46
Control (nontumor)	7	11.86	—	—
Control (healthy)	—	—	4	9.76
Skull-base meningioma	16	27.12	—	—
Lower-grade glioma	12	20.34	6	14.63
Brain metastatic carcinoma—other CNS diseases (OCD)	1	1.69	—	—
Other pituitary diseases (OPD)	10	16.95	7	17.07
Craniopharyngioma	5	50	5	71.43
Colloid cyst	1	10	1	14.29
Pituicytoma	1	10	1	14.29
Histiocytosis	1	10	—	—
Rhabdoid teratoma	1	10	—	—
Chordoma	1	10	—	—
Functioning status				
Functioning	4	30.77	6	25
Nonfunctioning	9	69.23	15	62.5
Unknown	—	—	3	12.5
Tumor size				
Giant	2	15.38	—	—
Macroadenoma	10	76.92	20	83.33
Microadenoma	1	7.69	2	8.33
Unknown	—	—	2	8.33

Table 1. Continued

Features	Serum (N = 59)		Plasma (N = 41)	
	Median	(Q1, Q3)	Median	(Q1, Q3)
Tumor invasion				
Invasive	7	53.85	23	95.83
Noninvasive	6	46.15	—	—
Unknown	—	—	1	4.17
Knosp grade				
0	4	30.77	—	—
1	2	15.38	—	—
2	3	23.08	—	—
3	2	15.38	—	—
4	2	15.38	—	—
NI	—	—	24	100
Last report status				
Alive	12	92.31	14	58.33
Dead	1	7.69	4	16.67
Lost follow-up	—	—	6	25

Abbreviations: NI, not informed; PitNET, pituitary neuroendocrine tumors; WHO, World Health Organization.

comparisons listed above with their putative target gene using EPIC manifest (hg38) and conducted gene set enrichment analyses using ingenuity pathway analysis (IPA) and DAVID Functional Annotation in efforts to explore associated canonical and disease-relevant processes.[29–31]

In silico functional validation of serum- or plasma-derived DMPs. In efforts to assess the biological relevance of serum- and plasma-derived DMP-putative target gene pairs obtained from the various PitNET comparisons, we harnessed publicly available matching methylomic and transcriptomic data from tumor tissue PitNETs (Neou et al,[18] n = 82). Correlational analyses and differential expression of genes were established through the ELMER tool,[32] in addition to broad-scale literature searches (details in Supplementary Methods; Supplementary File S5).

Deconvolution. In order to assess whether serum or plasma samples presented methylation signatures that are representative of circulating tumor- or non-neoplastic cell-specific and are differential across cohorts, we applied previously described DNA methylation-based methodologies to deconvolute the relative contribution of cell types to a given serum or plasma sample (MethylCIBERSORT and python-based).[26,33] We included available methylation signatures from immune (B cells, CD4T, CD8T, natural killer cells, monocytes, neutrophils), and non-immune cells (neuron, glial, and vascular endothelial cells).[26,33] We generated our own methylation signatures from non-neoplastic pituitaries obtained from cadavers and followed the steps for defining the signatures as previously described by Moss et al[26] and Chakravarthy et al[33] (details in Supplementary Methods).

Data Availability

Data supporting the findings of this study are available within the article and Supplementary information, and from the European Genome-Phenome Archive (accession EGA#####). Remaining data are available from the authors upon request.

Statistical Analysis

All processing and statistical analyses were done in R (4.1.2). Non-parametric Kruskal-Wallis and Wilcoxon rank-sum test and multiple testing adjustments (eg, false discovery rate [FDR]) were used to identify group-specific DMPs as stated in the previous sections and across discrete variables: cfDNA concentration (ng/mL), deconvoluted cell proportions (%) and sample mean methylation levels (β-values). Relationships between discrete variables were explored through the utilization of Spearman's correlation coefficient (ρ) and corresponding *P*-values. We utilized random forest analyses as the machine-learning method for prediction and classification of the samples as PitNET or non-PiNET. Performance and clinical utility of both models in the respective independent cohorts were evaluated through Mathew's correlation coefficient (MCC) and the clinical utility index (CUI)[34,35] (details in Supplementary Methods).

Results

Characterization of Pituitary Cell-Free DNA Methylome

cfDNA quantification and methylome data quality. Our serum or plasma methylome samples met the quality

control for all parameters used in the quality control assessment (eg, bisulfite conversion), independently of concentration of cfDNA (Supplementary Figure S1C and D). Methylation quality was not correlated with pre-analytical features, such as date of collection. Total extracted serum cfDNA quantities in patients with PitNET were significantly higher compared to controls in serum and lower compared to OPD in both serum and plasma specimens (Supplementary Figure S1A; Supplementary File S1). The concentration of plasma cfDNA in PitNET patients was higher and not significantly different compared to their glioma counterparts (Supplementary Figure S1A; Supplementary File S1). PitNET serum or plasma cfDNA concentrations were higher in nonfunctioning compared to functioning PitNET and were not correlated with size and invasion status (Supplementary Figure S1A and B; Supplementary File S1). PitNET cfDNA mean concentrations were significantly lower across serum cohorts compared to plasma counterparts.

Methylome levels detected in serum or plasma specimens segregate PitNETs from non-PitNET samples—unsupervised analysis. The 3D PCA of the genome-wide mean methylation levels of either serum or plasma cfDNA samples showed separation between PitNETs and non-PitNETs, and partial segregation between functioning vs nonfunctioning PitNET more evident in serum (Figure 1A). Differential methylation patterns of the 1K most variant methylated probes across groups were more evident in plasma than serum specimens (Figure 1B).

Differential immune and non-immune cell compositions across groups were observed in serum and plasma. Using MethylCIBERSORT[33] to estimate cell composition we observed that the proportion of whole pituitary methylation signatures in PitNET serum or plasma were significantly different compared to controls and OPD; however, the direction of estimated proportion differences was discordant between both sources, that is, the proportion of this cell type in PitNETs was higher in serum, and lower in plasma specimens than comparison groups (Figure 1C). Neuron proportions were lower in PitNET in relation to OPD. Differential proportions of specific immune cell-type signatures, such as monocytes and neutrophils, were concordant with plasma results; however, for CD4 and CD8 T-cell signatures, only appreciable in plasma samples (Figure 1C). Comparisons of estimated proportions across different sources derived from the same patient with OPD (ie, serum, plasma, or tissue) showed significant and positive correlations across multiple cell types, that is, CD4T, neutrophils, natural killer cells (details in Supplementary File S5). Except for glial cells that were not estimated by MethylCIBERSORT, the immune and non-immune cell-type distribution estimated through both deconvolution algorithms were significantly and positively correlated between the 2 deconvolution methods more frequently in plasma than in serum samples (Supplementary Figure S2B).

Tumor-specific signatures distinguish PitNETs from non-PitNET in serum or plasma samples—supervised analysis. The supervised analysis between PitNETs and non-PitNETs yielded the identification of 110 serum (mean methylation difference [diff.mean]; –0.13 > diff.mean > 0.16, $P\text{-value}_{FDR} < .05$) and 112 plasma DMPs (–0.23 > diff.mean > 0.25, $P\text{-value}_{FDR} < .01$) (Figure 2A; Supplemental Files S3 and S4). Differential mean methylation levels of plasma-derived DMPs across most comparison groups, including PitNET vs OPD, were more evident in plasma samples, while the serum DMPs only distinguished PitNETs from lower-grade gliomas (Figure 2A). We further filtered both PitNET-specific DMP sets to probes that explicitly differentiated nonfunctioning PitNET from OPD and mapped them to their putative target genes depicted in Figure 2D.

Mean methylation levels of DMPs resulting from the comparison of PitNET and OPD ($n_{serum} = 91$; $n_{plasma} = 115$) significantly differentiated PitNET and OPD in serum but not in plasma (Supplementary Figure S3C), while the mean methylation of DMPs derived from the comparison between nonfunctioning and functioning PitNETs ($n_{serum} = 49$; $n_{plasma} = 56$) differentiated these groups only in plasma specimens (Supplementary Figure S2B).

In all supervised analyses, the identified DMPs which distinguished the respective groups were mostly located in intergenic regions, overlapping enhancers, and mainly annotated in open sea regions (Supplementary Files S2–S4).

PeLB and NF-PeLB scores accurately discriminate PitNETs from other groups—Random Forest Model. We developed and validated predictive models which involved DMPs derived through compounded analysis of both serum and plasma methylomes (details in Supplementary Methods). PeLB and NF-PeLB scores above 0.57 and 0.37, respectively, predicted whether a LB specimen originated from a patient with PitNET or a non-PitNET condition or disease in an independent cohort, with 100% and ~93% accuracies, respectively (SE: 100%/87.5%, SP: 100%/94.7%) with satisfactory reliability and clinical utility as evaluated via values of the MCC (100% and ~77%, respectively) and CUI+ (100% and ~82%, respectively) (Figure 2B and C; Supplementary Figures S3B and S5A and B).

PitNET liquid biopsy-derived probes mapped to genes involved in relevant pathways associated with tumorigenesis. We compiled genes putatively targeted by DMPs derived from different supervised comparisons. Serum-, plasma-, and compounded source-derived gene sets showed enrichment for relevant pathways involved in tumor behavior, immune response, and cell metabolism, among others (details in Supplementary Figure S2A–C).

DMPs derived from tissue comparisons are present in the serum or plasma cfDNA methylome from patients with PitNETs. t-SNE depicting the 250 most variant CpG probes showed that serum and plasma cfDNA from PitNET patients clustered with PitNET and OPD and segregated from skull-base meningiomas and lower-grade gliomas (Figure 3A).

Among the tissue DMPs derived from the supervised analysis between controls vs PitNETs (n = 1544 DMP), functioning vs nonfunctioning (n = 187 DMP), PitNET vs other CNS tumors (n = 287 DMP), and PitNET vs OPD (n = 147 DMP), 133 and 250 DMPs were captured in serum

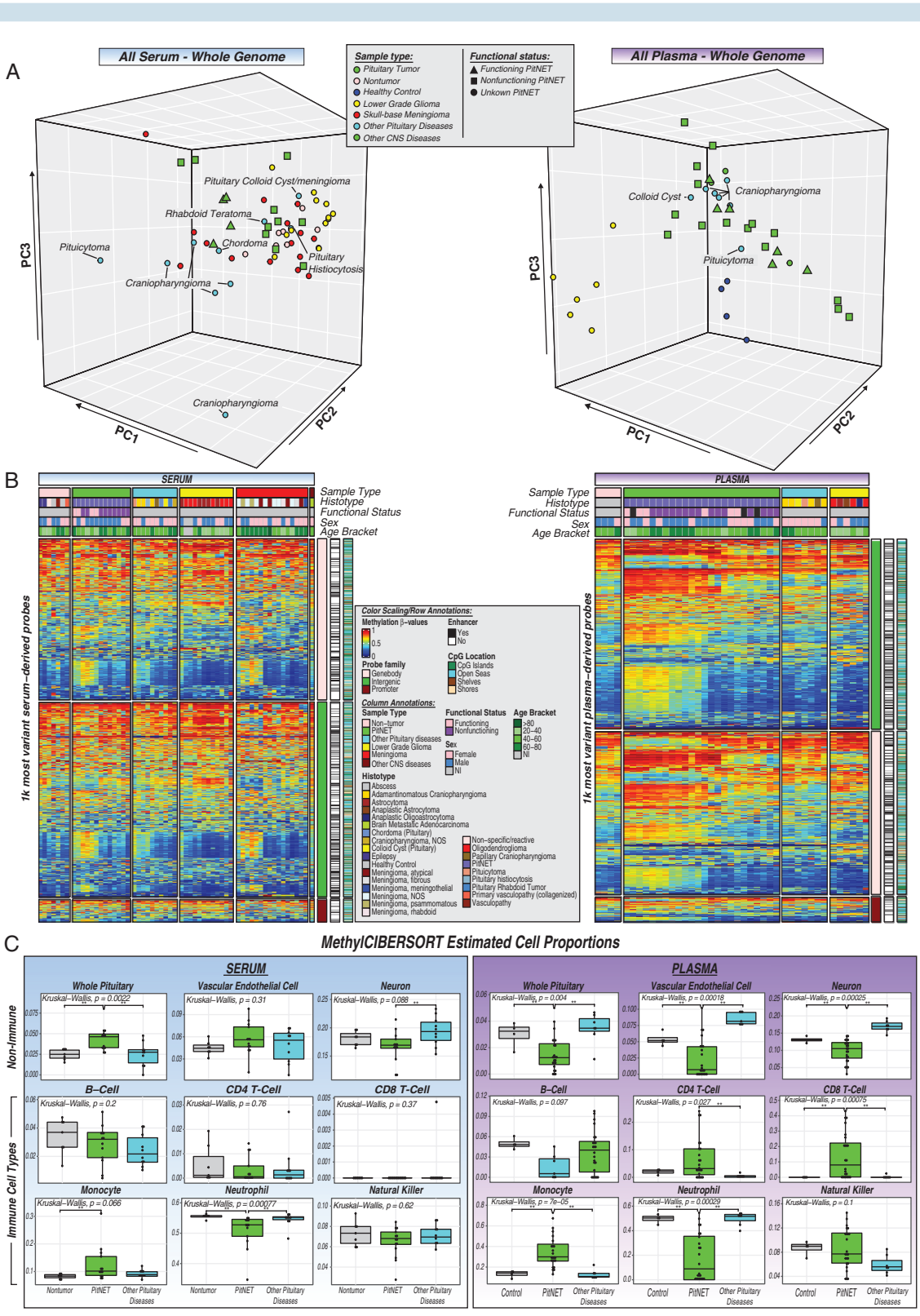

Fig. 1 Exploratory analysis of the liquid biopsy-derived cfDNA methylome. (A) Principal component analysis of the genome-wide mean methylation of serum (n = 59) or plasma (n = 41) cfDNA cohorts; (B) Heatmap of the methylation levels (β-values) of the 1K most variably methylated probes across liquid biopsy-based sample cohorts; (C) Boxplots depicting the estimated cell proportions of liquid biopsy specimens using MethylCIBERSORT. Comparisons are provided across immune and non-immune cell types between PitNETs, other pituitary diseases, and control specimens (Kruskal-Wallis and Wilcoxon rank-sum means; **Wilcoxon *P*-value < .05). Abbreviations: cfDNA, cell-free DNA; PitNETs, pituitary neuroendocrine tumors.

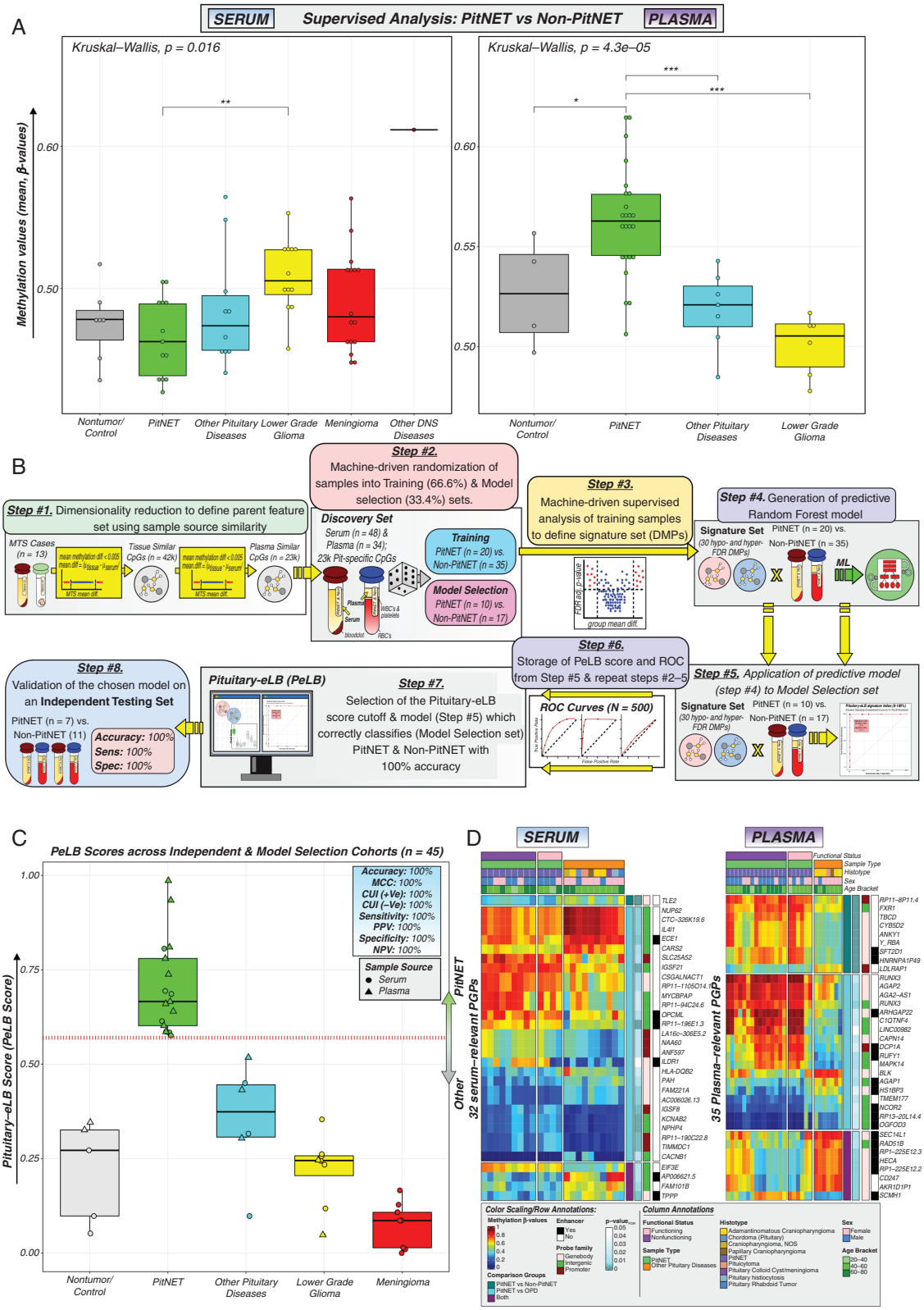

Fig. 2 Supervised analysis across liquid biopsy samples. (A) Mean methylation levels across DMPs resulting from comparison of PitNET and non-PitNET liquid biopsy samples (DMP: n_{serum} = 110; n_{plasma} = 112; Wilcoxon rank-sum test and Kruskal-Wallis; **P*-value < .05, ***P*-value < .01, ****P*-value < .001); (B) Schematic outline of steps to developing a machine-learning-based model to differentiate across PitNET from other sellar and CNS diseases, using liquid biopsy specimens—the PitNET epigenetic liquid biopsy (PeLB) model; (C) PeLB score distributions across model

or plasma, respectively, and also discriminated the corresponding comparison groups.

These DMPs were associated with genes reportedly relevant to pituitary diseases and involved in cell growth and hormonal signaling (eg, Notch pathway, protein kinase A, and HOTAIR signaling), cell metabolism, and tumor progression (eg, PFKFB4), among others (Figure 3D; Supplementary Figure S4D).

PitNET-specific DMP detected in serum or plasma specimens are functionally relevant in PitNET tissue. In overlapping serum- or plasma-derived DMPs obtained from several PitNET-focused comparisons with the Neou PitNET tissue paired transcriptome and methylation datasets, we were able to identify multiple putative target genes whose expression levels were significantly and negatively correlated with the methylation levels of CpG probes located in gene regulatory regions (enhancers or promoters) (Figure 4A and B). Additionally, we showed that putative target genes associated with serum/plasma DMPs derived from the comparison between functioning and nonfunctioning presented differential expression and methylation between these groups in PitNET tissue (Figure 4C). We also observed that in functioning PitNET, probes associated with HDAC4, TRIM5, and CAMK2N1 genes, involved in transcription factor and protein kinase binding activities, presented lower methylation levels and higher gene expression compared to nonfunctioning PitNET (Figure 4C). Literature reports on the biological or clinical importance of these probe-gene pairs for tumorigenesis are displayed in Supplementary File S5. Briefly, they include genes related to pituitary gland development, cell proliferation, gene expression, metal ion binding, and others.

Discussion

Methylation profiling of cfDNA circulating in biofluids, such as blood (serum or plasma), has been useful for the early detection, prognostication, and surveillance of intra- and extracranial neoplasms, as shown by our group and others.[10,11,36,37] Herein, our results suggest that similar to other CNS tumors, PitNETs release tumor-related information in the blood that allows the identification of clinically relevant methylation signatures specific to patients with PitNETs (Figure 1B; Supplementary Figure S3C).

Standard approaches, including clinical features, hormonal assessment using blood/urine, imaging of the pituitary gland, and pathological assessment obtained by surgery are, in most cases, sufficient to the diagnosis and classification of PitNETs.[38] However, there are challenging sellar disease cases, such as rare primary or secondary sellar tumors or non-neoplastic diseases that may be misdiagnosed as PitNETs, in particular as NF-PitNETs,[5–7] that could benefit from a presurgical and noninvasive diagnostic approach to better guide the appropriate management. Here, the unsupervised analysis of methylome in serum or plasma specimens shows that distinct mean genome-wide methylation levels separate PitNET from non-neoplastic specimens, other CNS tumors and pituitary diseases, and PitNET functional subtypes; differences that are more appreciable in plasma-derived samples (Figure 1A and B). We also observed that serum- or plasma-derived probes clustered together with PitNET/OPD but not with lower-grade glioma and meningioma tissue (t-SNE) (Figure 3A). Altogether, these findings suggest that serum or plasma cfDNA from patients with PitNETs contain methylation fingerprints specifically related to these tumors. Capitalizing on these observations, we developed prediction models using a combination of serum- and plasma-derived PitNET or NF-PitNET-specific DMPs that accurately classified independent CNS cohorts into their respective memberships with 100% and ~93% accuracy, respectively, alongside commendable measurements of reliability and clinical utility (Figure 2B and C; Supplementary Figure S5A and B). Although compelling, these results warrant validation in a larger cohort of LB samples, particularly among conditions that mimic NF-PitNET.[5–7]

To complement the evidence of the presence of tumor-specific features in the LB samples, we also investigated the cell composition of serum and plasma samples in our cohort. In the absence of standard methods, such as flow cytometry, we applied and contrasted 2 DNA methylation-based deconvolution methods to estimate cell-type composition in serum and plasma samples.[26,33] These methods have shown to reliably deconvolute cell types in tissue or plasma samples of different CNS tumors or other conditions.[26,33] Through MethylCIBERSORT deconvolution,[33] we observed differential immune and non-immune cell-type proportions across patients with PitNETs compared to controls and OPD (eg, whole pituitary, vascular endothelial, and immune cells) which was more apparent in plasma specimens compared to serum counterparts (Figure 1C). Notably, these results were highly correlated with the python-based method[26] (Supplementary Figure S2B), suggesting the consistency in results of DNA methylation-based deconvolution methods. Interestingly, some immune signatures detected in serum or plasma recapitulated the findings from tissue deconvolution in a subset of our cohort (Supplementary File S5); but these results warrant further confirmation through standard methods, such as flow cytometry.

We also showed that PitNET methylation signatures identified in tissue could be captured in serum or plasma cfDNA and distinguished similar PitNET groups (eg, PitNET vs non-PitNETs, vs control, functioning vs nonfunctioning). We attempted to explore the biological and functional roles of the putative target genes by aligning DMP derived from different group comparisons in serum or plasma

Fig. 2 Continued
selection and independent cohorts, with performance parameters (*y*-axis: PeLB score; PeLB score ≥0.57 = PitNET; <0.57 = non-PitNET); (D) Heatmap displaying the methylation levels of DMPs resulting from the comparison of PitNETs and other pituitary diseases across serum (n = 23) or plasma (n = 31) specimens and their putative target genes; sorted by sample type. Abbreviations: DMPs, differentially methylated probes; PitNETs, pituitary neuroendocrine tumors.

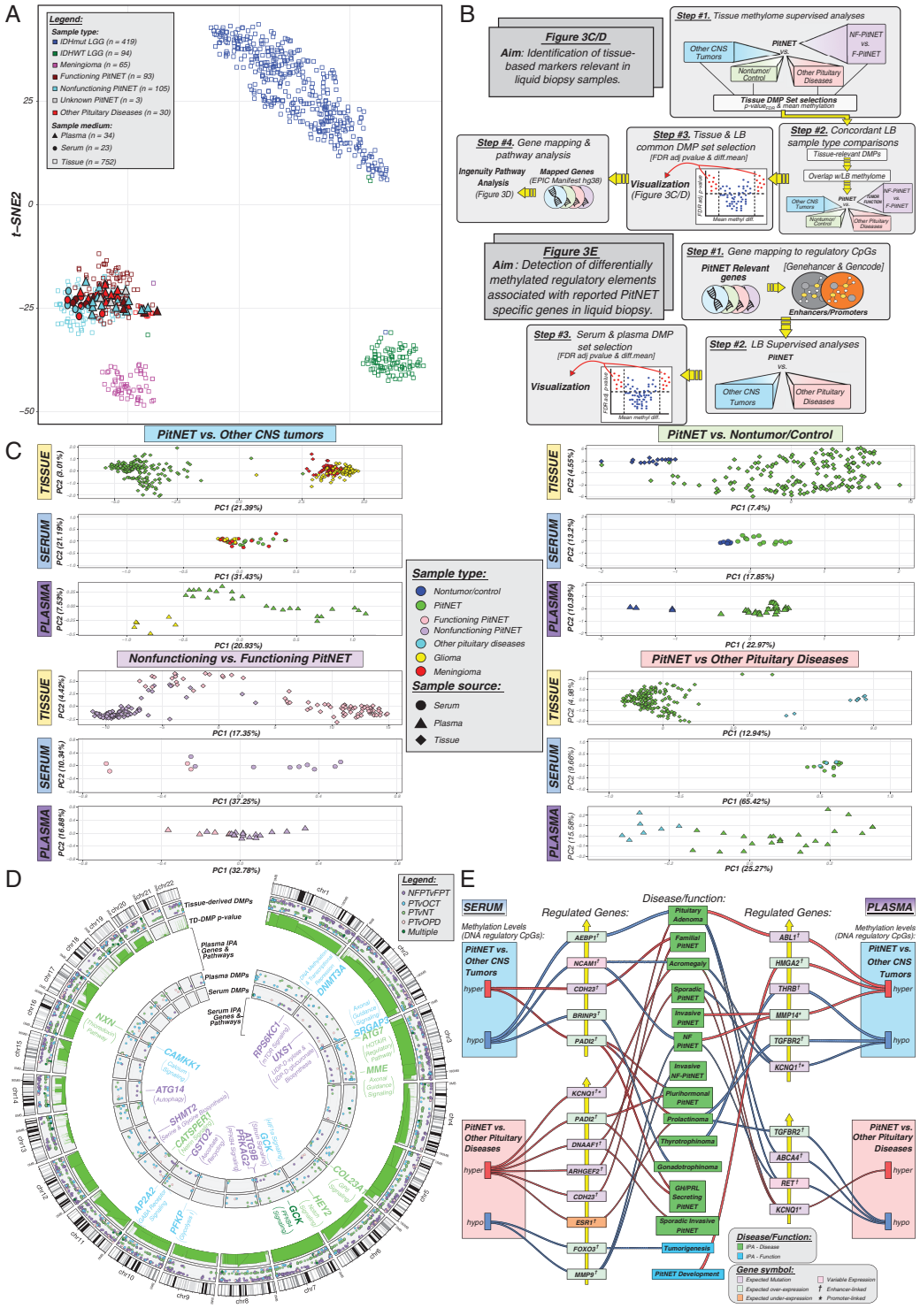

Fig. 3. Relationship between tissue and liquid biopsy methylome. (A) t-Distributed Stochastic Neighbor Embedding (t-SNE) using the 250 most variably methylated and PitNET-specific CpG sites across multiple cohorts; (B) Schematic detailing the analysis aims, comparison groups, and sources associated with the following: (C) Principal component analyses of tissue-derived DMPs which retained significance in concordant liquid biopsy comparisons; (D) A circos plot (ShinyCirco) depicting molecular and biological features associated with the aforementioned DMPs: supervised group assignment; chromosomal location, target genes, pathways output from the ingenuity pathway analysis (IPA), and significances ($-\log_{10}$ *P*-value; *y*-axis: difference in mean methylation [diff.mean]); (E) A river plot depicting disease-related genes, mapped to regulatory DMPs (enhancer/promoter) derived from the liquid biopsy comparisons of PitNET vs non-PitNET or PitNET vs other pituitary diseases. Abbreviations: DMPs, differentially methylated probes; PitNETs, pituitary neuroendocrine tumors.

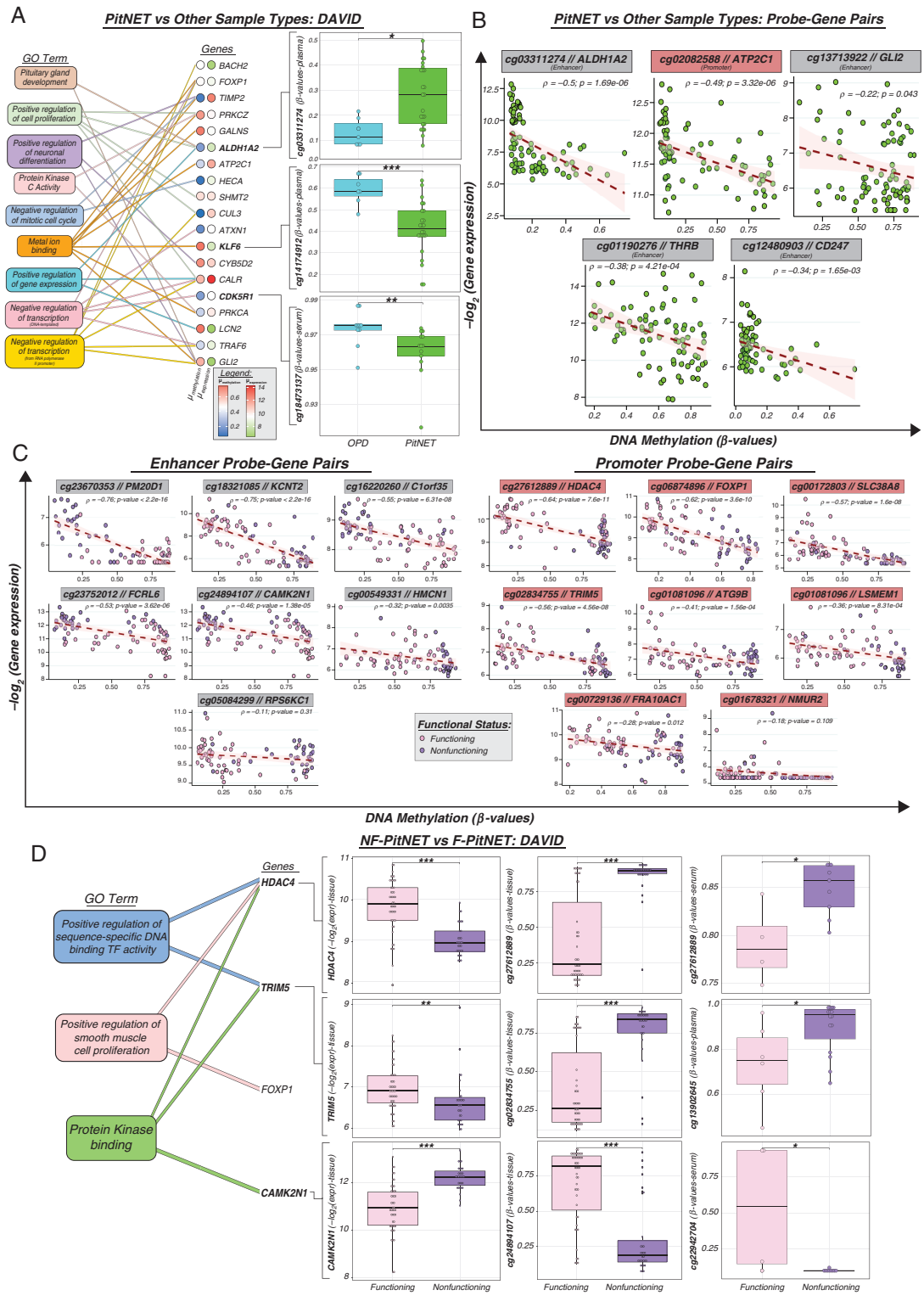

Fig. 4 In silico validation of probe and putative target gene pairs and exploration of gene ontologies. (A) Negatively correlated methylation and expression levels of PitNET relevant probe-gene pairs, with associated gene ontologies (DAVID); (B) PitNET tissue-methylation and -expression levels[18]of negatively correlated and potentially PitNET relevant probe-gene pairs derived from multiple supervised analyses in liquid biopsy specimens; (C) PitNET tissue methylation and gene expression levels of negatively correlated probe-gene pairs annotated in regulatory regions of the genes (ELMER); (D) Negatively correlated methylation and expression levels of nonfunctioning PitNET-specific probe-gene pairs showing differential methylation and expression levels between nonfunctioning and functioning PitNETs across liquid biopsy and tissue specimens (box plots), and associated DAVID results. Abbreviation: PitNETs, pituitary neuroendocrine tumors.

with publicly available PitNET tissue methylation and expression datasets.[18] We identified significant negative correlations between the methylation of CpG located in gene regulatory regions and the expression levels of their putative target genes suggesting that these genes are epigenetically regulated by these DMPs (Figure 4B; Supplementary File S5). This hypothesis was corroborated by the finding that genes mapped to DMPs derived from the comparison between functioning and nonfunctioning in serum/plasma were differentially expressed in PitNET tissue. Overall, these target genes were involved in pathways related to pituitary development, function, tumorigenesis, and behavior, as well as innate immune responses, cellular signaling and cell cycle (proliferation and apoptosis), etc. and some were potential prognostic markers (TXNRD1) in other tumors or reported as therapeutic targets in prolactinomas (ERBB2) (Figures 3D and 4A and D; Supplementary Figure S4A and D; Supplementary Files S2–S5).[14,17,39–41] These results suggest that serum and plasma are resourceful for the identification of biologically and clinically relevant methylation markers and lay the groundwork for future functional and clinical studies.[42,43]

Most reported LB studies have used plasma instead of serum as a source of cfDNA to perform omics analysis. Although certain molecular results could be impacted by the use of serum profiling, for example, detection of somatic mutations due to possible dilution or contamination with genomic DNA derived from blood and other cells during the coagulation process,[10,12,44] it does not seem to interfere with the detection of cell-specific methylation markers as shown in this study. However, no formal head-to-head comparisons of molecular results between both sources have been previously reported. Here, we performed a head-to-head comparison between methylome analyses results derived from serum and plasma cfDNA and observed that these 2 sources of DNA are different in several aspects, such as cell composition estimations and genome-wide and supervised mean methylation levels; however, they both provided unique or common PitNET-specific markers that were able to differentiate PitNETs from other tumors, suggesting that the use of either blood element is useful, and possibly complementary, as a noninvasive methylation-based diagnostic tool (Supplementary Figure S2C; Supplementary File S5).[45,46]

Although grounded on robust bioinformatic analysis, our results are limited by the relatively small cohort of PitNETs, specifically NF-PitNETs and OPD, the inclusion of controls with known epigenetic abnormalities in blood samples (eg, epilepsy); unavailability of paired methylation and expression across OPD and validation with functional studies.

Altogether, our results provide evidence that PitNETs release DNA methylation markers in the serum/plasma and that blood-based LB constitutes a reliable approach for the noninvasive detection of clinically relevant epigenetic signatures specific to PitNETs. Specifically, using serum or plasma specimens, it is feasible to generate methylation-based prediction models that successfully distinguish PitNET and NF-PitNET from OPD. These results lay the groundwork for the potential application of these models to complement imaging to differentiate challenging cases of sellar diseases that mimic PitNETs and ultimately optimize diagnostic and therapeutic management using noninvasive techniques.

Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

Keywords

liquid biopsy | methylation | PitNET | plasma | serum

Funding

This work was supported by the Department of Neurosurgery, HBTC and HFHS; A.V.C. and K.P.A. are supported by the Henry Ford Hospital (A30935, A30957; GME Research Grants 202199); A.E.S. is supported by the Peter D. Cristal Chair of Neurosurgical Oncology and the Kimble Family Foundation.

Acknowledgments

The authors are grateful to the HFHS patients and UH patients who consented to the usage of PitNETs for research purposes. We thank Nancy Takacs, Nicolette Lineberry, and Heather Mengel for their administrative support; Simona L. Cazacu and Bartow Thomas for the collection, handling, and maintenance of the tumor bank at the Hermelin Brain Tumor Center (HBTC); Andrea Transou for tumor pathology processing; Laura A. Hasselbach for DNA extraction; Daniel Weisenberger and team at USC Epigenome Center for assistance with DNA methylation profiling; CWRU/UH team: Penelope Miron, Kristy Miskimen, Kristin Waite, Gino Cioffi, Tuesday Gibson for plasma sample selection, cfDNA extraction and shipping and clinicopathological curation; Susan MacPhee for proofreading the manuscript.

Conflict of interest statement. The authors declare to have no conflict of interest.

Authorship statement. Overall concept and coordination of the study: A.V.C., J.R., H.N., G.A.H., and K.P.A.; retrieval of publicly available molecular and clinical data: G.A.H., K.P.A., M.W., and A.V.C.; bioinformatic and statistical analyses: G.A.H., M.W., T.S.S., T.M.M., M.S.M., H.N., A.V.C., and input from L.M.P.; pathology review (HFHS): A.M., and J.S.B.; serum and tissue collection, tumor bank storing, and maintenance: K.N., A.C.C., and L.S.; molecular data generation: T.M.M. and A.C.C.; procurement of biospecimens/clinical data (CWRU/UH): J.S.B., A.E.S., W.R.S., and J.C.; the manuscript was written by A.V.C., G.A.H., H.N., and M.W.; intellectual contribution: K.P.A., J.S., J.C., T.M.M., S.K., T.W., J.S.B., M.R., T.M., A.B., and S.K. All authors contributed to the revision of the manuscript.

References

1. Asa SL, Casar-Borota O, Chanson P, et al. From pituitary adenoma to pituitary neuroendocrine tumor (PitNET): an International Pituitary Pathology Club proposal. *Endocr Relat Cancer.* 2017;24(4):C5–C8.
2. Ostrom QT, Cioffi G, Gittleman H, et al. CBTRUS Statistical Report: primary brain and other central nervous system tumors diagnosed in the United States in 2012-2016. *Neuro Oncol.* 2019;21(Suppl 5):v1–v100.
3. Molitch ME. Diagnosis and treatment of pituitary adenomas: a review. *JAMA.* 2017;317(5):516–524.
4. Mete O, Lopes MB. Overview of the 2017 WHO classification of pituitary tumors. *Endocr Pathol.* 2017;28(3):228–243.
5. Wang J, Liu Z, Du J, et al. The clinicopathological features of pituicytoma and the differential diagnosis of sellar glioma. *Neuropathology.* 2016;36(5):432–440.
6. Freda PU, Post KD. Differential diagnosis of sellar masses. *Endocrinol Metab Clin North Am.* 1999;28(1):81–117, vi.
7. Al-Dahmani K, Mohammad S, Imran F, et al. Sellar masses: an epidemiological study. *Can J Neurol Sci.* 2016;43(2):291–297.
8. Bagley SJ, Nabavizadeh SA, Mays JJ, et al. Clinical utility of plasma cell-free DNA in adult patients with newly diagnosed glioblastoma: a pilot prospective study. *Clin Cancer Res.* 2020;26(2):397–407.
9. Sabedot TS, Malta TM, Snyder J, et al. A serum-based DNA methylation assay provides accurate detection of glioma. *Neuro Oncol.* 2021;23(9):1494–1508.
10. Shen SY, Singhania R, Fehringer G, et al. Sensitive tumour detection and classification using plasma cell-free DNA methylomes. *Nature.* 2018;563(7732):579–583.
11. Nassiri F, Mamatjan Y, Suppiah S, et al. DNA methylation profiling to predict recurrence risk in meningioma: development and validation of a nomogram to optimize clinical management. *Neuro Oncol.* 2019;21(7):901–910.
12. Megnis K, Peculis R, Rovite V, et al. Evaluation of the possibility to detect circulating tumor DNA from pituitary adenoma. *Front Endocrinol (Lausanne).* 2019;10:615.
13. Ling C, Pease M, Shi L, et al. A pilot genome-scale profiling of DNA methylation in sporadic pituitary macroadenomas: association with tumor invasion and histopathological subtype. *PLoS One.* 2014;9(4):e96178.
14. Kober P, Boresowicz J, Rusetska N, et al. DNA methylation profiling in nonfunctioning pituitary adenomas. *Mol Cell Endocrinol.* 2018;473:194–204.
15. Salomon MP, Wang X, Marzese DM, et al. The epigenomic landscape of pituitary adenomas reveals specific alterations and differentiates among acromegaly, Cushing's disease and endocrine-inactive subtypes. *Clin Cancer Res.* 2018;24(17):4126–4136.
16. Capper D, Jones DTW, Sill M, et al. DNA methylation-based classification of central nervous system tumours. *Nature.* 2018;555(7697):469–474.
17. Gu Y, Zhou X, Hu F, et al. Differential DNA methylome profiling of nonfunctioning pituitary adenomas suggesting tumour invasion is correlated with cell adhesion. *J Neurooncol.* 2016;129(1):23–31.
18. Neou M, Villa C, Armignacco R, et al. Pangenomic classification of pituitary neuroendocrine tumors. *Cancer Cell.* 2020;37(1):123–134.e5.
19. Moran S, Martínez-Cardús A, Sayols S, et al. Epigenetic profiling to classify cancer of unknown primary: a multicentre, retrospective analysis. *Lancet Oncol.* 2016;17(10):1386–1395.
20. Hoadley KA, Yau C, Hinoue T, et al. Cell-of-origin patterns dominate the molecular classification of 10,000 tumors from 33 types of cancer. *Cell.* 2018;173(2):291–304.e6.
21. Hao X, Luo H, Krawczyk M, et al. DNA methylation markers for diagnosis and prognosis of common cancers. *Proc Natl Acad Sci USA.* 2017;114(28):7414–7419.
22. Mosella MS, Sabedot TS, Silva TC, et al. DNA methylation-based signatures classify sporadic pituitary tumors according to clinicopathological features. *Neuro Oncol.* 2021;23(8):1292–1303.
23. Nassiri F, Chakravarthy A, Feng S, et al. Detection and discrimination of intracranial tumors using plasma cell-free DNA methylomes. *Nat Med.* 2020;26(7):1044–1047.
24. Ceccarelli M, Barthel FP, Malta TM, et al. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. *Cell.* 2016;164(3):550–563.
25. Sahm F, Schrimpf D, Stichel D, et al. DNA methylation-based classification and grading system for meningioma: a multicentre, retrospective analysis. *Lancet Oncol.* 2017;18(5):682–694.
26. Moss J, Magenheim J, Neiman D, et al. Comprehensive human cell-type methylation atlas reveals origins of circulating cell-free DNA in health and disease. *Nat Commun.* 2018;9(1):5068.
27. Colaprico A, Silva TC, Olsen C, et al. *TCGAbiolinks*: an R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Res.* 2016;44(8):e71.
28. Triche TJ, Weisenberger DJ, Van Den Berg D, Laird PW, Siegmund KD. Low-level processing of Illumina Infinium DNA methylation beadarrays. *Nucleic Acids Res.* 2013;41(7):e90.
29. Zhou W, Laird PW, Shen H. Comprehensive characterization, annotation and innovative use of Infinium DNA methylation BeadChip probes. *Nucleic Acids Res.* 2017;45(4):e22.
30. Krämer A, Green J, Pollard J, Tugendreich S. Causal analysis approaches in ingenuity pathway analysis. *Bioinformatics.* 2014;30(4):523–530.
31. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009;4(1):44–57.
32. Silva TC, Coetzee SG, Gull N, et al. ELMER v.2: an R/Bioconductor package to reconstruct gene regulatory networks from DNA methylation and transcriptome profiles. *Bioinformatics.* 2019;35(11):1974–1977.
33. Chakravarthy A, Furness A, Joshi K, et al. Pan-cancer deconvolution of tumour composition using DNA methylation. *Nat Commun.* 2018;9(1):3220.
34. Chicco D, Tötsch N, Jurman G. The Matthews correlation coefficient (MCC) is more reliable than balanced accuracy, bookmaker informedness, and markedness in two-class confusion matrix evaluation. *BioData Min.* 2021;14(1):13.
35. Mitchell AJ. Sensitivity × PPV is a recognized test called the clinical utility index (CUI+). *Eur J Epidemiol.* 2011;26(3):251–252, author reply 252.
36. Constâncio V, Nunes SP, Moreira-Barbosa C, et al. Early detection of the major male cancer types in blood-based liquid biopsies using a DNA methylation panel. *Clin Epigenetics.* 2019;11(1):175.
37. Kang S, Li Q, Chen Q, et al. CancerLocator: non-invasive cancer diagnosis and tissue-of-origin prediction using methylation profiles of cell-free DNA. *Genome Biol.* 2017;18(1):53.
38. Melmed S. Pituitary-tumor endocrinopathies. *N Engl J Med.* 2020;382(10):937–950.
39. Sun Z, Xue H, Wei Y, et al. Mucin O-glycosylating enzyme GALNT2 facilitates the malignant character of glioma by activating the EGFR/PI3K/Akt/mTOR axis. *Clin Sci.* 2019;133(10):1167–1184.
40. Pacini V, Petit F, Querat B, et al. Identification of a pituitary ERα-activated enhancer triggering the expression of Nr5a1, the earliest gonadotrope lineage-specific transcription factor. *Epigenetics Chromatin.* 2019;12(1):48.
41. Sapochnik M, Nieto LE, Fuertes M, Arzt E. Molecular mechanisms underlying pituitary pathogenesis. *Biochem Genet.* 2016;54(2):107–119.
42. Cooper O, Mamelak A, Bannykh S, et al. Prolactinoma ErbB receptor expression and targeted therapy for aggressive tumors. *Endocrine.* 2014;46(2):318–327.

43. Fu B, Meng W, Zeng X, et al. TXNRD1 is an unfavorable prognostic factor for patients with hepatocellular carcinoma. *Biomed Res Int.* 2017;2017:4698167.

44. Johansson G, Andersson D, Filges S, et al. Considerations and quality controls when analyzing cell-free tumor DNA. *Biomol Detect Quantif.* 2019;17:100078.

45. Cristall K, Bidard F-C, Pierga J-Y, et al. A DNA methylation-based liquid biopsy for triple-negative breast cancer. *npj Precis Oncol.* 2021;5(1):53.

46. Carson JJK, Di Lena MA, Berman DM, Siemens DR, Mueller CR. Development and initial clinical correlation of a DNA methylation-based blood test for prostate cancer. *Prostate.* 2020;80(12):1038–1042.