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Research Article

Transcriptome Analysis Reveals Distinct Patterns Between the Invasive and Noninvasive Pituitary Neuroendocrine Tumors

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Abstract

Although most pituitary neuroendocrine tumors (PitNETs)/pituitary adenomas remain intrasellar, a significant proportion of tumors show parasellar invasive growth and 6% to 8% infiltrate the bone structures, thus affecting the prognosis. There is an unmet need to identify novel markers that can predict the parasellar growth of PitNETs. Furthermore, mechanisms that regulate bone invasiveness of PitNETs and factors related to tumor vascularization are largely unknown.

We used genome-wide mRNA analysis in a cohort of 77 patients with PitNETs of different types to explore the differences in gene expression patterns between invasive and noninvasive tumors with respect to the parasellar growth and regarding the rare phenomenon of bone invasiveness. Additionally, we studied the genes correlated to the contrast enhancement quotient, a novel radiological parameter of tumor vascularization.

Most of the genes differentially expressed related to the parasellar growth were genes involved in tumor invasiveness. Differentially expressed genes associated with bone invasiveness are involved in NF-κB pathway and antitumoral immune response. Lack of clear clustering regarding the parasellar and bone invasiveness may be explained by the influence of the cell lineage-related genes in this heterogeneous cohort of PitNETs.

Our transcriptomics analysis revealed differences in the molecular fingerprints between invasive, including bone invasive, and noninvasive PitNETs, although without clear clustering. The contrast enhancement quotient emerged as a radiological parameter of tumor vascularization, correlating with several angiogenesis-related genes. Several of the top genes related to the PitNET invasiveness and vascularization have potential prognostic and therapeutic application requiring further research.

Key Words: PitNET, transcriptomics, RNA-sequencing, pituitary adenoma, pathology, invasiveness

Abbreviations: bone-I-PitNET, bone invasive pituitary neuroendocrine tumor; bone-NI-PitNET, bone noninvasive pituitary neuroendocrine tumor; CS, cavernous sinus; I-PitNET, invasive pituitary neuroendocrine tumor; MRI, magnetic resonance imaging; PitNET, pituitary neuroendocrine tumor; NI-PitNET, noninvasive pituitary neuroendocrine tumors; PMOC, proopiomelanocortin; TUSC2, tumor suppressor candidate 2; VDAC1, voltage-dependent anion channel 1.

Pituitary neuroendocrine tumors (PitNETs), traditionally termed pituitary adenomas, constitute more than 15% of all surgically resected intracranial neoplasms [1], with a prevalence of more than 90 clinically diagnosed tumors per 100 000 [2]. The PitNETs are currently classified based on the pituitary cell lineages determined by immunohistochemical expression of adenohypophysial hormones and pituitary-specific transcription factors [3]. PitNETs can behave as nonfunctioning, ie, silent tumors, or functioning, manifesting clinically due to increased hormone levels in the blood. The designation pituitary adenoma implicates a benign clinical course, which characterizes most PitNETs. However, PitNETs may have a highly variable impact on health due to an expansion of intracranial tumor mass, hormonal hypersecretion, or adenohypophysial failure. Nonfunctioning PitNETs, in general, are larger and more invasive than the functioning ones [4].

Although the majority of tumors remain intrasellar, between approximately 20% and 40% of surgically resected tumors show local invasive growth [5], and up to 15% are designated as "giant adenomas" with a maximum diameter ≥40 mm [6]. A small subset of patients, probably 0.3% to 2% of the surgically treated patients [7, 8], have aggressive

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tumors, as defined by the European Society of Endocrinology guidelines for management of aggressive and malignant pituitary tumors [9]. The terms aggressive and invasive should not be used interchangeably, as aggressive PitNETs, in addition to signs of invasiveness, show unusually rapid tumor growth and/or fail to respond to standard surgical, radio-, and/or pharmacological therapy. Metastatic PitNETs or pituitary carcinomas constitute 0.1% to 0.6% [3, 10] of the surgically resected anterior pituitary neoplasms. They are defined solely based on metastatic spread within or outside the central nervous system [3].

Invasiveness of PitNETs has mainly been evaluated by assessing the parasellar growth and the tumor's relation to the anatomical structures in the cavernous sinus (CS) [11], as the CS ingrowth is a considerable limiting factor for completeness of surgery [12]. Biopsy of the medial wall of the CS with a histopathological examination is considered the gold standard for assessment of invasiveness, but biopsies cannot be routinely performed due to the high risk of complications [13]. Therefore, a widely accepted preoperative method for the assessment of tumor invasion is based on the radiological appearance of the tumor. The Knosp grading system, based on magnetic resonance imaging (MRI), is used to indirectly assess the parasellar tumor growth and the tumor's relation to the intrasinusoidal part of the internal carotid artery [11]. The Knosp grading system defines 5 grades of parasellar growth (0-4), with tumor grades 3 and 4 considered invasive. The intraoperative endoscopic technique allows for direct visualization with a panoramic view of the integrity of the medial wall of the CS and thus is highly accurate and superior to MRI in terms of assessment of PitNET invasiveness. Hence, the Knosp grading system has been reevaluated by intraoperative endoscopy, revealing a pronounced heterogeneity within grade 3 tumors and justifying the subdivision of Knosp grade 3 into 3A (invasion of the superior CS compartments) and 3B (invasion of the inferior CS compartments) [13]. Although this subdivision allows for a more reliable preoperative assessment of invasiveness, with grade 3B tumors showing a higher fraction of invasive tumors compared to 3A tumors (70.6% vs 26.5%, respectively) [13], there is still a need for improved precision for prognostic stratification of patients. So far, no agreement has been reached regarding what radiological criteria should be used for undoubtful proof of invasiveness [12].

Approximately 6% to 8% of all PitNETs infiltrate the bone structures [14], and only a few scientific reports address this phenomenon [14, 15]. Bone invasion is not incorporated into the preoperative radiological grading systems for tumor invasiveness. Hence, the prognostic significance of bone invasion is unclear. Molecular mechanisms underlying the bone invasiveness of PitNETs are also largely unknown.

Angiogenesis, the formation of new blood vessels, is crucial for tumor development and metastasis [16]. Unlike most solid tumors, PitNETs are less vascularized compared to normal pituitary tissue. Even invasive, aggressive, or malignant PitNETs do not demonstrate a significant increase in vascularity, with the exception of some rare secondary deposits of metastatic PitNETs [16]. The genetic background of low vascular density in PitNETs is unclear.

Transcriptome profiling based on massive parallel sequencing of mRNA is used to define specific gene expression signatures and has successfully been exploited by the Human Protein Atlas (www.proteinatlas.org) to map expression patterns in human cells and normal and tumor tissues [17].

Recent transcriptomics studies using genome-wide mRNA sequencing have demonstrated that the overall gene expression pattern in PitNETs is aligned with the current PitNET classification [18-23]. Studies with particular emphasis on identifying transcriptomic signatures underlying invasive PitNETs have been conducted in recent years, revealing genes involved in diverse signaling pathways [20, 24-27]. However, results are not always concordant, and the complex genetic and transcriptomic landscape of PitNETs showing different growth patterns and grades of invasiveness has not yet been fully characterized. There is, thus, an urgent and unmet need to identify novel markers that can predict invasive growth of PitNETs.

In the present study, we extended our well-characterized cohort of 51 PitNETs of different histological and secretory types with an additional 26 cases to obtain a higher proportion of invasive (Knosp grades 3 and 4) tumors. Genome-wide mRNA analysis was applied to investigate the differences in gene expression between invasive and noninvasive PitNETs. Comparisons were based on the traditionally accepted criterion of parasellar invasiveness and also on a criterion of bone invasiveness. We also aimed to explore the expression of genes that correlated with the contrast enhancement quotient, a parameter of tumor vascularization.

Material and Methods

Study Cohort and Tissue Samples

We extended our previously reported cohort of 51 well-characterized PitNETs [19] to include a comparable number of invasive (Knosp 3 and 4) and noninvasive (Knosp 0, 1, and 2) tumors. In brief, PitNET tissue samples were obtained from 77 adult patients who underwent transsphenoidal surgery at Uppsala University Hospital between 2014 and 2021. Fresh tumor tissue samples from all patients were frozen in liquid nitrogen, stored at −80 °C, and used for RNA extraction. The remaining tumor tissue was formalin fixed, paraffin embedded, and used for routine diagnostics. Tumors were classified clinically, based on the endocrine symptoms and the laboratory hormone tests. Histopathological classification was based on the immunohistochemical expression of anterior pituitary hormones and pituitary-specific transcription factors according to the current World Health Organization classification of pituitary tumors [3]: FSH (Agilent cat. no. M3504, RRID:AB_2079146), LH (Agilent cat. no. M3502, RRID:AB_2135325), TSH (Leica Biosystems cat. no. NCL-TSH-R2, RRID:AB_564033), ACTH (Agilent cat. no. M3501, RRID:AB_2166039), GH (Agilent cat. no. A0570, RRID:AB_2617170), prolactin (Agilent cat. no. A0569, RRID:AB_2893308), alpha-subunit of the glycoprotein hormones (Thermo Fisher Scientific cat. no. MA1-25038, RRID:AB_779817), pituitary-specific positive transcription factor 1 (Novus cat. no. NBP1-92273, RRID:AB_11030310), steroidogenic factor 1 (Abcam cat. no. ab217317, RRID:AB_2920891), and pituitary-restricted transcription factor (Atlas Antibodies cat. no. AMAb91409, RRID:AB_2716678). Distribution of the clinical and histological tumor types in the study cohort is presented in Table 1.

The Swedish Ethical Review Authority approved the study protocol (Dnr 2018/053).

Fresh frozen PitNETs tissue samples were partly obtained through the U-CAN project (www.u-can.uu.se) [28].

Table 1. Immunohistochemical and clinical features of the PitNETs

TF	n	Functioning status	n	IHC subtype			Clinical classification		Comment
SF1	29	NF	29	Gonadotroph			NF-PitNET		
PIT1	25	NF	3	Somatotroph		1	NF-PitNET		
				Somato-lactotroph		1			
				only Pit-1 positive		1			
		F	22	Somatotroph	3	14	Acromegaly		2 SG-GH tumors were mixed PitNET-gangliocytomas
					DG 2				
					SG 1				
				Somato-lactotroph	11				
					DG 2				
					SG 8				
					NG 1				
				Lactotroph		4	HyperPRL		
				Thyrotroph		1	HyperTSH		
				Plurihormonal GH + PRL + TSH		2	Acromegaly + HyperTSH	1	
							HyperPRL	1	
				Gonadotroph		1	HyperFSH		Paradoxical PIT1 expression and not SF1
TPIT	19	NF	13	Corticotroph			NF-PitNET		One Crooke cell tumor
		F	6				Cushing disease		
Double PitNET	1	NF	1	Gonadotroph + lactotroph			NF-PitNET		
Triple Pit-NET	1	F	1	Gonadotroph + GH + ACTH			Acromegaly		
Null cell	2	NF	2				NF-PitNET		Clustered with SF1 respectively TPIT tumors based on mRNA expression [19]
Total	77		77						

Abbreviations: DG, densely granulated; F, functioning; IHC, immunohistochemistry; NF, non-functioning; NF-PitNET: non-functioning pituitary neuroendocrine tumor; NG, no granulation; PIT1, pituitary transcription factor 1; PitNET, pituitary neuroenocrine tumor; PRL, prolactin; SF1, steroidogenic factor 1; SG, sparsely granulated; TF, transcription factor; TPIT, T-box family member 19 (TBX19).

Radiological Evaluation

Pituitary MRI, taken on the last examination before the first surgery, was reviewed for all the patients. The following variables were evaluated: tumor size and volume, parasellar and bone invasion, as well as the contrast enhancement quotient (CEQ). Tumor size, volume, and the degree of parasellar invasion were assessed using the same criteria as in our previous project [19]. Briefly, the tumor size was measured in 3 orthogonal directions (height, width, and depth) on T1 weighted images after contrast medium administration; tumor volume was calculated as follows: volume = (width × height × depth)/2. Modified Knosp classification [13] was used to evaluate the degree of parasellar invasiveness in grades 0 to 4. Tumors were considered bone-invasive if they showed MRI signs of infiltration into the clivus, posterior clinoid process, or sphenoid floor.

To compare the differentially expressed genes with respect to tumor invasiveness, 2 ways for grouping the tumors were applied: (1) Knosp grades 0 to 2 tumors considered as noninvasive (NI-PitNETs) vs Knosp grade 4 tumors considered as invasive (I-PitNETs), irrespective of their bone-invasiveness status; Knosp grade 3 cases were excluded to achieve a clear separation between the invasive and noninvasive tumors; and (2) bone invasive (bone-I-PitNETs) vs bone noninvasive (bone-NI-PitNETs) tumors, irrespective of the invasiveness into the cavernous sinus, in the entire cohort of 77 patients.

The contrast enhancement quotient was used to quantify the upload of contrast, which reflects the tumor vascularization and also the permeability across the vessel wall. As the MRI signal cannot be compared between patients and different sequence types, a ratio between the signal without contrast and the signal in the same area after contrast administration was calculated. This ratio, ie, contrast enhancement quotient, was considered a proxy for the degree of tumor vascularization. To explore genes with a significant correlation with the CEQ, a Spearman correlation analysis was performed.

Overview of the PitNET cohort, including all radiological parameters, is provided in Supplementary Table S1 [29].

Transcriptomics

The transcriptomic profiling was based on the Human Protein Atlas pipeline, and data analysis was performed using strategies previously described [17].

Hematoxylin–eosin stained cryosections confirmed that all specimens from the extended cohort contained representative tumor tissue. Total RNA from the 26 new cases was extracted according to the same protocol that was used for the primary cohort [19]. In short, 10 micrometers thick sections were cut from the fresh frozen specimens, and total RNA was extracted using a RNeasy Mini Kit (Quiagen, Hilden, Germany). Automated electrophoresis system Agilent 2100 Bioanalyzer system (Agilent Biotechnologies, Palo Alto, CA, USA) with the RNA 6000 Nano LabChip Kit was used to analyze the quality of the RNA samples. RNA sequencing was performed on the Illumina NovaSeq 6000 instrument.

Quality score and the percentage of reads cut-offs were set at 30% and 75%, respectively. The cut-off for the number of

reads was set at 10 million reads. All samples have passed these quality control limits. To obtain quantification scores for all human genes and transcripts, transcript expression levels were calculated as transcript per million (TPM) by mapping processed reads to the human reference genome GRCh37/hg19 ref and with gene models based on Ensembl (v92) using Kallisto (v.0.43.1). Next, the gene expression levels were calculated by summing up all the TPM values of all alternatively spliced protein coding transcripts of the corresponding gene for a total number of 19 670 protein-coding genes. The average TPM values are used to estimate the gene expression level. All TPM values were trimmed mean of M-values normalized between all the samples. The expression level cut-off is set at 1 TPM, and 13 348 genes expressed this cut-off or higher in all samples. The full TPM data matrix is shown in Supplementary Table S2 [29].

Immunohistochemistry

Additional immunohistochemistry was performed on the selected tumors belonging to the different categories (invasive vs noninvasive, bone invasive vs bone noninvasive, and low vs high CEQ) to explore the expression of some of the differentially expressed genes on the protein level (*JADE1,* Atlas Antibodies cat. no. HPA020016, RRID:AB_1855292*; PAPPA2,* Atlas Antibodies cat. no. HPA018412, RRID: AB_1854962; and *VDAC1,* Atlas Antibodies cat. no. HPA030780, RRID:AB_2673608). The immunohistochemistry and digitalization of the stained tissue slides were performed using a standardized protocol and workflow, as described previously [30, 31].

An overview of the antibodies used to classify PitNETs and validate transcriptomics results for the selected genes is provided in Supplementary Table S3 [29].

Statistical Analysis

Data analysis and visualization were performed using R (version 4.0.0). Pearson's Chi-squared test or Fisher's exact test was used for categorical variables of PitNETs patients dependent on the theoretical frequency. Wilcoxon rank-sum test was used for continuous variables. The DESeq2 R package was used for differential analysis based on the mRNA raw counts for gene expression analysis. Clustering in heatmaps and dendrograms based on Spearman correlation were created by first calculating a correlation matrix of Spearman's ρ between all the samples. Dendrograms showing the gene expression in heatmaps have been clustered using the Ward2 algorithm implementation of Ward's minimum variance method implemented as "Ward.D2" in the hclust function in the R package stats. A false discovery rate of less than 0.05 is considered significant (adjusted *P*-value is performed with the Benjamini–Hochberg method).

Results

Clinical, Histological, and Radiological Features of PitNETs with Respect to Invasiveness

Seventy-seven PitNETs of various histological and secretory types were analyzed (Table 1), showing the following distribution across Knosp grades: 48 cases (62%) were Knosp grades 0 to 2 tumors, 17 (22%) cases belonged to Knosp grade 3, and 12 (16%) were Knosp grade 4 tumors. To assess the differentially expressed genes (DEGs) between the invasive and the noninvasive tumors, Knosp grade 3 tumors were excluded, leaving 48 noninvasive PitNETs (Knosp grade 0–2 tumors) and 12 clearly invasive PitNETs (Knosp grade 4). CEQ could only be calculated for 70 patients. In the remaining 7 cases, there was either no measurable solid tumor component or no comparable sequences before and after contrast agent administration. All other parameters were assessed on the entire cohort of 77 cases, of which 17 (22%) were bone-I-PitNETs. Most cases were macroadenomas (84%). Maximal tumor diameter and volume were significantly larger in invasive (I-PitNETs) and bone-I-PitNETs compared to the noninvasive counterparts.

No significant differences were detected between I-PitNETs vs NI-PitNETs, and bone-I-PitNETs vs bone-NI-PitNETs regarding sex, age, endocrine status, and immunohistochemical tumor types. Similarly, there were no significant differences between I-PitNETs vs NI-PitNETs and bone-I-PitNETs vs bone-NI-PitNETs regarding the contrast enhancement quotient. Clinical, histological, and radiological features of the tumors with respect to invasiveness are presented in Table 2.

Molecular Features of PitNETs with Respect to the Invasiveness and CEQ

Transcriptome profiling

The proportion of viable tumor cells in cryosections stained with hematoxylin–eosin, which correlates with the amount of isolated RNA exceeded 80% for all cases, except 1 case with 75% and 1 case having 60% tumor cells.

Normalized mRNA levels determined for each of the 77 samples, calculated as TPM values, were analyzed; furthermore, 13 348 presumed protein-coding genes were expressed in all PitNETs when applying a cut-off value of 1 TPM. Global expression profiles of the 77 tumors were compared using hierarchical clustering. Overall, 3 main clusters were revealed, corresponding well to the expected categories of tumors based on the 3 main transcription factors (steroidogenic factor 1, pituitary-restricted transcription factor, and pituitary-specific positive transcription factor 1) with only a few intermingled samples (Fig. 1).

Gene expression patterns regarding the parasellar invasiveness of PitNETs

Differential expression analyses between I-PitNETs (Knosp grade 4) and NI-PitNETs (Knosp grade 0–2) were performed and revealed 21 significant differentially expressed genes, as shown in Supplementary Table S4 [29]. Most of these DEGs (*SERPINA1, LRRC8C, RAMP3, AVPR1A, TRIM71, SDK2, NPHP3-ACAD11, JADE1, FBXO11, MGA, C7,* and *ZYG11A*) were upregulated, and 9 genes (*FOXL2NB, CGA, GIPR, CHRNA5, CDH12, PLAC9, FOXP2, SFRP4,* and *KIAA1614*) were downregulated in I-PitNETs.

Most of the genes differentially expressed between the invasive and the noninvasive groups were genes with established roles in tumor biology, often related to invasiveness. Several of these genes, such as *SFRP4, SERPINA1, FOXP2, GIPR,* and *FBXO11,* are recognized as crucial in PitNETs pathogenesis. However, a few upregulated (*NPHP3-ACAD11, SDK2,* and *LRRC8C)* and downregulated *(FOXL2NB* and *KIAA1614)* genes in I-PitNETs are poorly characterized or have only suggested roles in tumor biology. The expression levels of 13 top differentially expressed genes based on the log fold are shown in Fig. 2.

Table 2. Clinical and radiological features of PitNETs with respect to invasiveness

Parasellar invasion (Knosp 0-2 vs 4)				
Variable	**n**	**NI-PitNETs, n = 48[a]**	**I-PitNETs, n = 12[a]**	***P*-value**
Sex (%)	60			1.000
Female		20 (42)	5 (42)	
Male		28 (58)	7 (58)	
Age[b]	60	52.5 (35.0, 64.0)	67.0 (43.3, 69.3)	.147
Size (mm)	60	24 (18.5, 30.8)	36 (28.0, 39.3)	**.002**
Volume (cm^3)	60	4.2 (1.8, 7.9)	12,9 (5.2, 18.7)	**.008**
CEQ	53	1.62 (1.41, 1.86)	1,44 (1.31, 1.78)	.195
NF vs F (%)				.602
NF		28 (58.3)	6 (50)	
F		20 (41.7)	6 (50)	
TFs[c] (%)				.608
SF1		16 (33.3)	4 (33.3)	
PIT1		17 (35.4)	6 (50)	
TPIT		15 (31.3)	2 (16.7)	
Bone invasion				
Variable	**n**	**Bone-NI-PitNETs, n= 60[a]**	**Bone-I-PitNETs, n = 17[a]**	***P*-value**
Sex (%)	77			.930
Female		24 (40)	7 (41)	
Male		36 (60)	10 (59)	
Age[b]	77	54 (35, 67)	67 (55, 70)	**.043**
Size (mm)	77	26 (20, 30)	34 (25, 38)	**.020**
Volume (cm^3)	77	4.7 (2.1, 9.2)	8.2 (5.5, 13.5)	**.011**
CEQ	70	1.62 (1.4, 1.8)	1.61 (1.4, 2.1)	.584
NF vs F (%)				.173
NF		35 (58.3)	13 (76.5)	
F		25 (41.7)	4 (23.5)	
TFs[c] (%)				.319
SF1		21 (35)	10 (58.8)	
PIT1		22 (36.7)	4 (23.5)	
TPIT		17 (28.3)	3 (17.6)	

Abbreviations: CEQ, contrast enhancement quotient; F, functioning PitNETs; I-PitNET, noninvasive pituitary neuroendocrine tumors; NF, nonfunctioning PitNETs; NI-PitNET, noninvasive pituitary neuroendocrine tumors; PIT1, pituitary-specific positive transcription factor 1; PitNET, pituitary neuroenocrine tumor; TF, transcription factor.
A double PitNET (ID50) grouped with SF1 corresponding to the dominant tumor component. A triple PitNET (ID51) grouped with PIT1 as the patient has acromegaly. Statistically significant values ($P < .05$) are written in bold.
[a]n (%); median (interquartile range).
[b]Age at first surgery.
[c]Two null-cell PitNETs (ID47 and 49) were classified as TPIT, respectively, SF1 based on mRNA expression [19].

Global expression profiles of 12 I-PitNETs and 48 NI-PitNETs were compared using hierarchical clustering. Results revealed that the 2 groups were not separated into distinct clusters based on the overall gene expression (Fig. 1).

Gene expression patterns in regard to bone invasiveness of PitNETs

To investigate the gene expression pattern in the bone-I-PitNETs, differential expression analyses were conducted between bone-I-PitNETs and bone-NI-PitNETs. The analyses revealed 7 DEGs (*TDRD12*, *PAPPA2*, *AKR1B10*, *OR7C1*, *GNLY*, *ZNF831*, and *POMC*) (Supplementary Table S5) [29], all except *TDRD12* with well-known functions in tumor biology. Interestingly, all these genes, except *TDRD12*, were downregulated in the bone-I-PitNETs in our cohort (Fig. 3). Several of the genes play a role in the NF-κB pathway and antitumoral immune response, ie, in processes related to bone destruction. Similar to the parasellar invasiveness, the comparison of tumors with bone invasiveness did not reveal clear clustering (Fig. 1).

Contrast enhancement quotient in PitNETs

As the CEQ on the MRI reflects tumor vascularization, we aimed to explore genes that correlate with this parameter. Our results revealed that the expression of 2127 genes varied

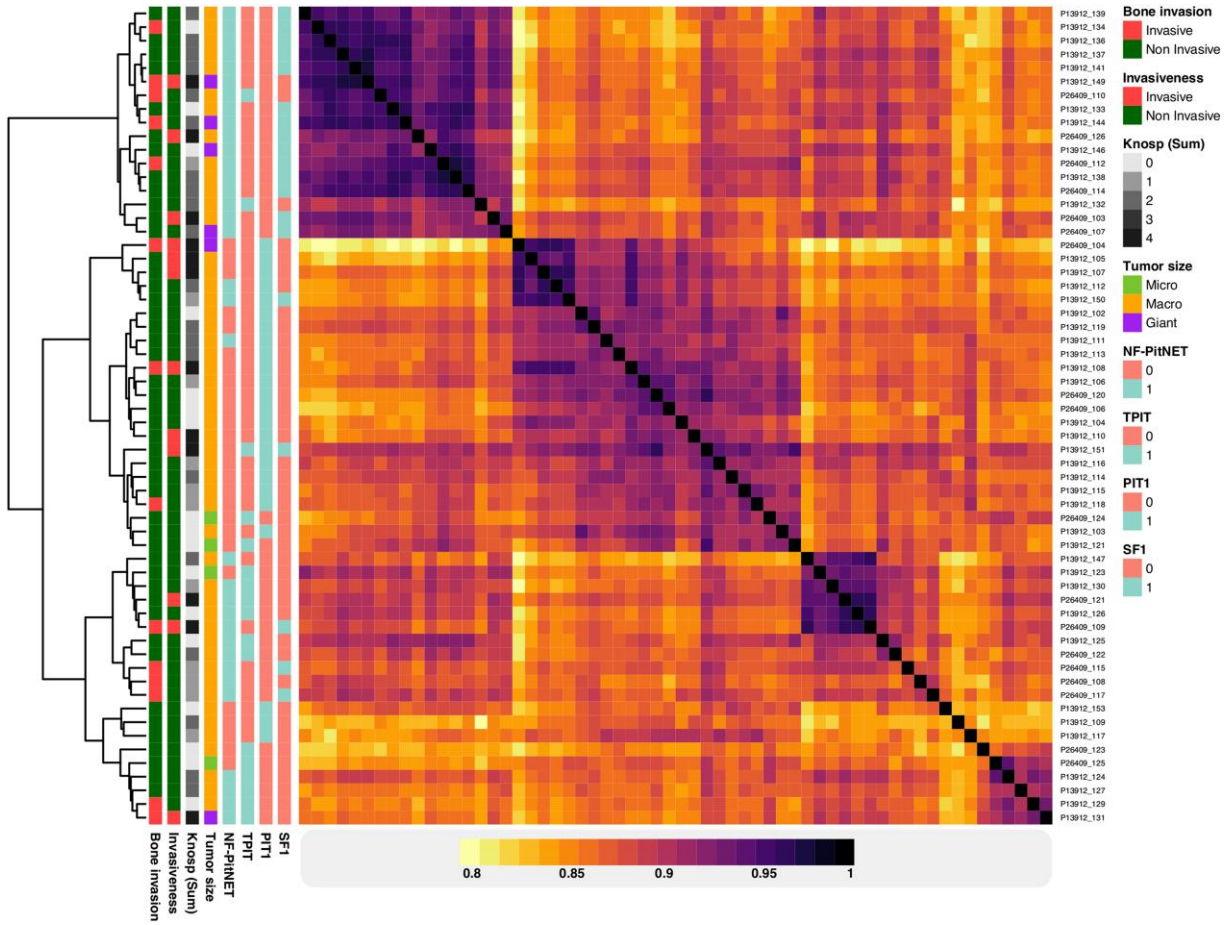

Figure 1. Overview of the expression profiles of the protein-coding genes in the human PitNETs. The heatmap shows the pairwise Spearman correlation between the global gene expression profiles for the 77 pituitary tumor tissues analyzed. Abbreviations: F-PitNET, functioning pituitary neuroendocrine tumor; NF-PitNET: nonfunctioning pituitary neuroendocrine tumor; PitNET, pituitary neuroenocrine tumor.

significantly with changes in the CEQ (adjusted *P*-value < .05) (Supplementary Table S6) [29].

Top 5 genes positively correlated with CEQ were *VDAC1*, *NPIPB11*, *CNIH3*, *TBKBP1*, and *TUSC*, whereas top 5 negatively correlated genes were *ENOX2*, *THAP1*, *ZNF398*, *TBC1D8B*, and *PEX12;* the majority of those genes have a well-established role in tumor biology. A few of the top genes that correlated with the CEQ are targetable genes involved in mitochondrial functioning and mTOR signaling; among them, *VDAC1* has a crucial role in angiogenesis.

Correlation patterns for the top 5 positively and negatively correlated genes are shown in Fig. 4, and an overview of the selected differentially expressed genes regarding the parasellar and bone invasiveness as well as the CEQ is provided in Table 3.

A more general overview of genes relevant for this study with details regarding expression levels in various subgroups of the PitNET cohort, normal pituitary gland, and other normal tissues as well as information regarding protein class and expression category is shown in Supplementary Table S7 [29]. The supplemental data has been extracted and summarized from the Human Protein Atlas (www.proteinatlas.org) [17].

Examples of differentially expressed genes at the protein level between tumors representing different categories regarding invasiveness (*JADE1)* and bone invasiveness (*PAPPA2),* as well as a gene correlated with CEQ (*VDAC1*) are illustrated in Fig. 5.

Furthermore, a thorough comparison of the DEGs and top correlated genes in our cohort to the cell lineage and/or functioning status related genes depicted in the recent studies [18-22, 27] is shown in Supplementary Table S8 [29].

Discussion

In the present study, we aimed to explore differences in gene expression patterns between the invasive and noninvasive PitNETs with respect to parasellar growth but also regarding the rare phenomenon of bone invasiveness in a well-characterized cohort of 77 tumors representing different subtypes of PitNETs. Our findings revealed differences in the molecular signatures between the invasive, including bone invasive, and the non invasive PitNETs that could facilitate prediction of the invasive behavior in PitNET. Genes that correlated with the contrast enhancement quotient, proxy for tumor vascularization, were disclosed. Several among the differentially expressed genes between the invasive and the noninvasive tumors and genes implicated in tumor vascularization are potential therapeutic targets.

Studies aiming to identify the genetic basis of invasiveness in PitNETs have been conducted in recent years [24-27], usually exploring exclusively nonfunctioning PitNETs, which are primarily of the gonadotroph type and less frequently of the

Figure 2. Gene expression patterns of the top differentially expressed genes related to invasiveness. (A) Barplots with samples colored according to their invasiveness status. Color intensity is correlated with the tumor size in mm. (B) Violin plots with samples colored according to their invasiveness status.

corticotroph type. Very few studies have addressed the important phenomenon of bone invasiveness in PitNETs [14, 15].

In our heterogeneous cohort composed of PitNETs of different types, we found that most of the DEGs between the invasive and the noninvasive tumors exemplify crucial genes involved in general mechanisms of tumor biology and related to invasiveness, a few with established roles in pituitary biology and PitNETs.

SFRP4 gene, a well-known tumor suppressor and WNT signaling antagonist involved in the process of epithelial-mesenchymal transition, was significantly downregulated in our cohort of I-PitNETs. Downregulation of the *SFRP4* gene has previously been described as essential in PitNETs tumorigenesis [49] and associated with acquisition of the invasive phenotype in nonfunctioning PitNETs [61], suggesting a potential role of *SFRP4* as a predictive biomarker of invasiveness and recurrence/progression in gonadotroph tumors.

SERPINA1, the gene involved in angiogenesis, tumor invasion, and metastasis [32], was upregulated in the I-PitNETs in our cohort. In line with our findings, *SERPINA1* is reported in the literature as being overexpressed in the invasive null cell PitNETs, which mainly belong to gonadotroph tumors [33].

Significant downregulation of the *SFRP4* and overexpression of *SERPINA1* in the I-PitNETs in our heterogeneous cohort comprising different PitNETs subtypes indicates that these genes influence tumor invasiveness, not only in the nonfunctioning PitNETs as previously suggested but also in the PitNETs in general.

FOXP2, downregulated in the I-PitNETs in our cohort, has been suggested as a novel transcription factor in normal pituitary, enriched in gonadotroph tumors [47], and also described to have an essential role in cancer initiation and progression [48]. However, as far as we know, the impact of *FOXP2* expression on the invasiveness of PitNETs has yet to be studied, and our study is the first to report an association between *FOXP2* downregulation and PitNET invasiveness.

An aberrant activation of *GIPR* has been described in several endocrine and neuroendocrine tumors, including a subset of *GNAS1wt* somatotroph tumors. The gene has been suggested as a potential diagnostic marker and a radiation therapy agent for neuroendocrine tumors [43]. In our study cohort, *GIPR* was downregulated in I-PitNETs, which is in line with the majority of reported *GIPR*-positive somatotrophs that are smaller noninvasive tumors with better responses to medical treatment (reviewed in [43]). According to the literature, other PitNETs subtypes, apart from *GNAS1wt* somatotroph tumors, do not exhibit aberrant *GIPR* activation. Our report of *GIPR* downregulation in

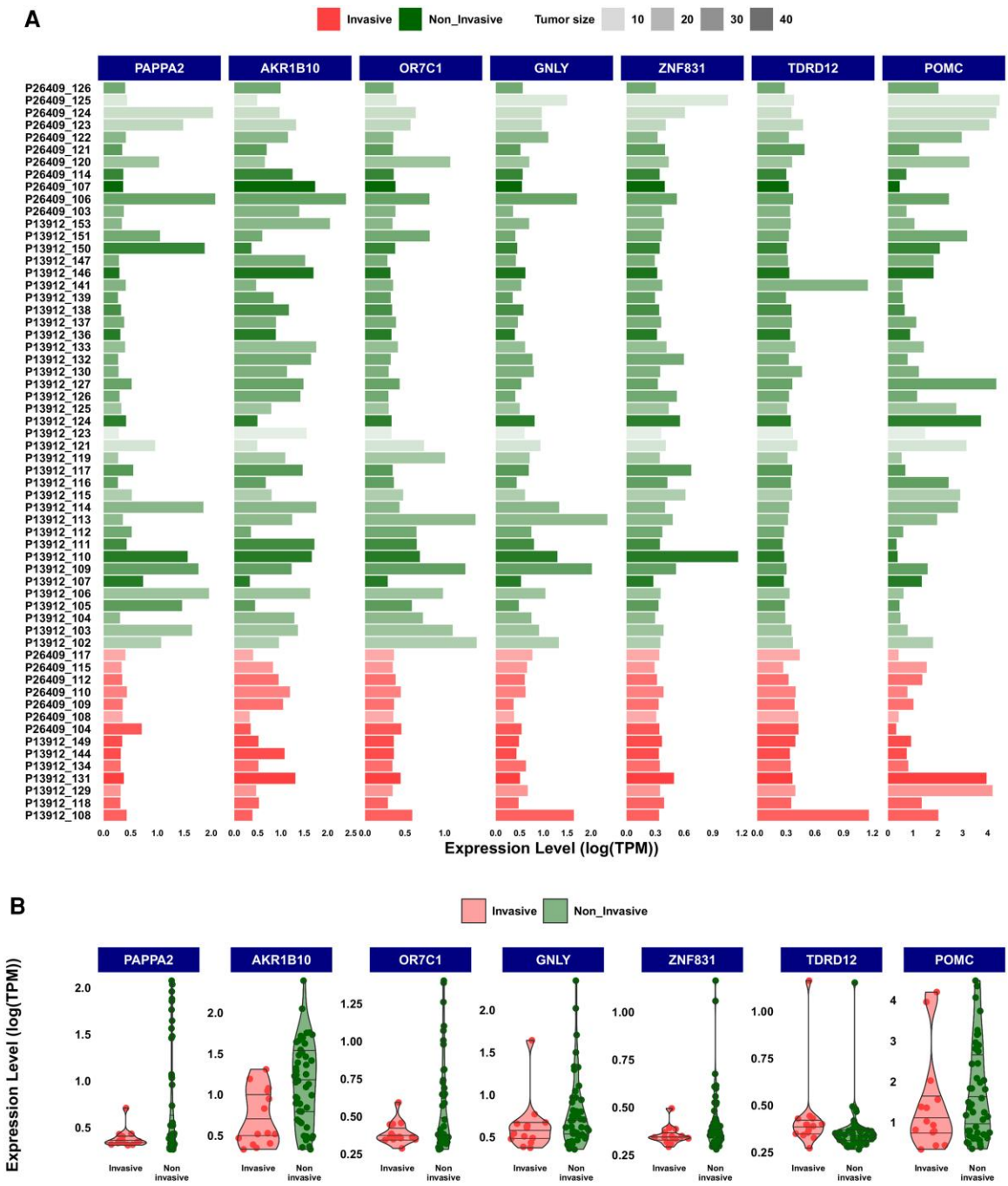

Figure 3. Gene expression patterns of the top differentially expressed genes related to bone invasion. (A) Barplots with samples colored according to their bone invasiveness status. Color intensity is correlated with the tumor size. (B) Violin plots with samples colored according to their invasiveness status.

I-PitNETs of different types suggests a role of *GIPR* in the invasiveness of PitNETs in general and its potential as a diagnostic and radiation therapeutic agent for invasive PitNETs.

Several well-known tumor-promoting genes, such as *RAMP3, FBXO11, ZYG11A,* and *AVPR1A*, suggested as therapeutic targets and with established function in tumor proliferation, migration, invasion, and metastasis [34-37], were overexpressed in the I-PitNETs. Further research is needed to decode the exact roles of these oncogenes in pathogenesis and invasiveness of PitNETs as well as its potential predictive, prognostic, or therapeutic application.

A few genes *(JADE1, TRIM71*, and *MGA)* described in the literature as tumor suppressors [38-40] were upregulated in the I-PitNETs. Moreover, a few genes reported as cancer promotors *(CHRNA5* and *CDH12)* [44, 45] were downregulated in the I-PitNETs. Seemingly contradictory findings for these genes reported across studies suggest context-dependent mechanisms of action and encourage further research.

Only a few studies addressed the phenomenon of bone invasiveness in a subset of PitNETs [14, 15]. Thus, mechanisms that regulate the invasion of PitNETs into the bone structures still need to be determined.

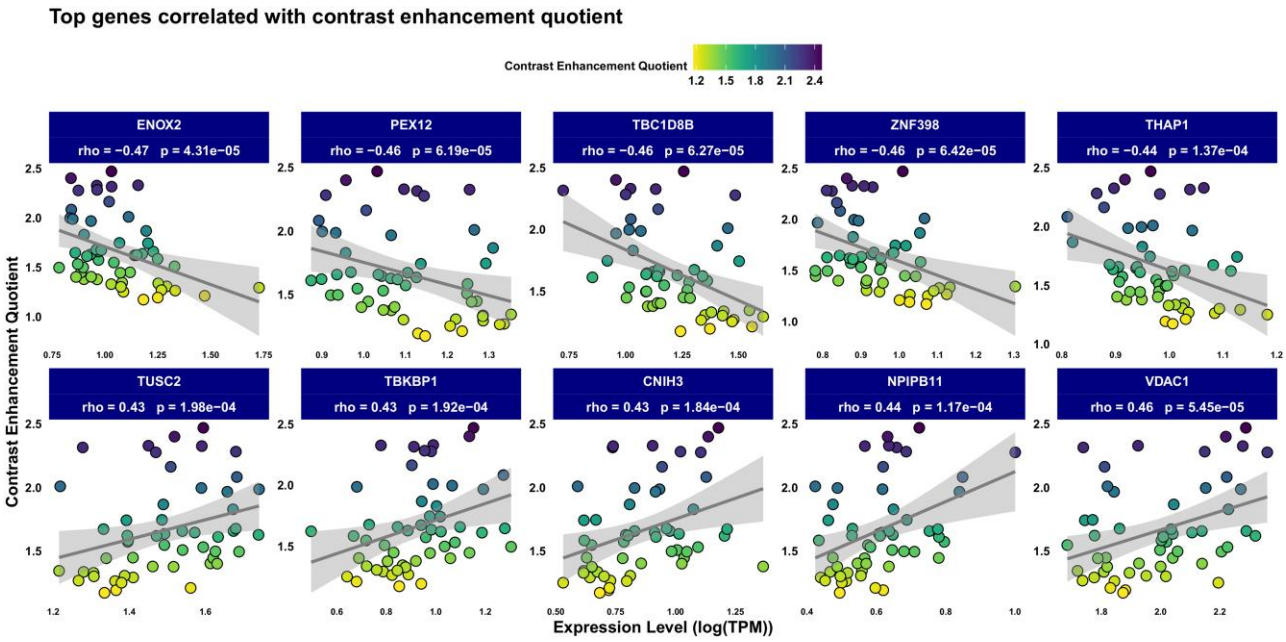

Figure 4. Top correlation patterns. Scatter plot showing top correlation trends between gene expression and contrast enhancement quotient.

It seems that tumor cells do not directly destroy bone; rather, they stimulate differentiation and maturation of osteoclasts, the process mediated by immune cells [14]. The NF-κB pathway is crucial in the generation of proinflammatory cytokines, which can lead to the stimulation of osteoclasts and the promotion of bone destruction [62]. Indeed, 4 out of the 7 DEGs related to bone invasiveness in our study were genes with the function in antitumoral immune response (*ZNF831, OR7C1*, *PAPPA2,* and *GNLY*) [51-54], while 1 gene, *AKR1B10,* was involved in the NF-Kb pathway [50]. All these 5 genes were downregulated in the bone-I-PitNETs, supporting the hypothesis that alterations in the immune mechanisms may play a role in bone invasiveness of PitNETs. Interestingly, the role of *PAPPA2* in silencing of PitNETs has been previously suggested, with higher expression of this gene in functioning corticotroph tumor compared to nonfunctioning ones [27]. No differences in the expression of *GNLY* on intratumoral immune cells have been reported between functioning and nonfunctioning corticotroph tumors [27].

POMC, encoding the ACTH precursor pro-opiomelanocortin, was downregulated in the bone-I-PitNETs. Proopiomelanocortin (POMC) is expected to be overexpressed in corticotroph tumors, which probably influenced the differential expression of this gene in our cohort, as corticotroph tumors were underrepresented among the bone-invasive tumors.

With the exception of *POMC*, *PAPPA2,* and *GNLY,* none of the genes differentially expressed in the bone-I-PitNETs vs bone-NI-PitNETs in our study was previously described in the context of pituitary tumors. This indicates a need for further research to decipher their function in bone invasiveness of PitNETs and their potential use as biomarkers.

The PitNETs in our cohort failed to cluster in compact groups with respect to parasellar and bone invasiveness. One possible explanation for the lack of clustering could be the impact of the variety of histological and secretory types represented in the cohort, with the strong influence of the cell lineage-related genes. A recent transcriptomic study exploring invasiveness in a more compact cohort of nonfunctioning PitNETs, applying the same criterion for invasiveness (Knosp grade 0–2 vs 4), showed tight clustering into invasive respectively noninvasive tumors [25]. However, Bao et al also demonstrated transcriptome heterogeneity between corticotroph and gonadotroph tumors, pointing out the potential impact of tumor subtypes on the expression of invasiveness-related genes [25].

The CEQ quantifies the upload of the contrast medium on MRI and, as such, may be considered as a parameter of tumor vascularization. In our study, the CEQ correlated with several genes having critical roles in angiogenesis.

Among the top 5 positively correlated genes, *VDAC1* and *TUSC2* both have well-known roles in mitochondrial function, calcium regulation, and mTOR signaling [55, 56]; CNIH3 is reported as a protooncogene with a potential diagnostic and therapeutic role [57], and TBKBP1 has an established role in tumor growth promotion and tumor-mediated immunosuppression [58]. The mitochondrial voltage-dependent anion channel 1 (VDAC1) protein is directly involved in angiogenesis with a crucial role in the proliferation of endothelial cells [55]. Overexpression of tumor suppressor candidate 2 (TUSC2) downregulates mTOR signaling and decreases PDL-1 expression allowing for more effective use of PD-1 blockers [56]. Both genes are promising therapeutic targets as VDAC1 can be targeted by the antifungal drug itraconazole [55], and the TUSC2-based drug in combination with an anti-PD-1 therapy is currently being tested in a clinical trial for patients with non–small-cell lung carcinoma [56] (https://clinicaltrials.gov/ct2/show/NCT05062980).

Regarding the genes negatively correlated with CEQ, *ENOX2 (tNOX)* overexpression seems to be associated with a poor prognosis of cancers. Its lower expression in benign PitNETs is not surprising as *ENOX2* downregulation is detected in slow-proliferating tumors [59].

Several among the top 5 genes positively and negatively correlated with CEQ (*NPIPB11*, *PEX12*, *ZNF398*, and *THAP1*) were genes sparsely or not at all described in tumor biology.

Table 3. Overview of the selected genes differentially expressed between PitNETs in regard to invasiveness and contrast enhancement quotient

	Gene	Function
Upregulated in I-PitNETs	SERPINA1	Involved in angiogenesis, tumor invasion, and metastasis [32] Overexpressed in invasive null cell PitNETs [33]
	RAMP3 FBXO11 ZYG11A AVPR1A	Function in tumor proliferation, migration, invasion, and metastasis [34-37] Suggested as therapeutic targets
	JADE1 TRIM71 MGA	Tumor suppressors [38-40]
	C7	Related to the maintenance of stemness [41]
Downregulated in I-PitNETs	CGA	Tumor suppressors or oncogenes, depending on the cancer type and signaling pathway [42]
	GIPR	Aberrant activation in several endocrine and neuroendocrine tumors, including subset of GNAS1wt somatotroph tumors [43]
	CHRNA5 CDH12	Cancer promotors [44, 45]
	PLAC9	Tumor suppressor [46]
	FOXP2	Novel transcription factor in normal pituitary, enriched in gonadotroph tumors [47] Essential role in cancer initiation and progression [48]
	SFRP4	Tumor suppressor gene and WNT signaling antagonist Essential in PitNETs tumorigenesis [49]
Upregulated in bone-I-PitNETs	TDRD12	Probable ATP-binding RNA helicase required during spermatogenesis [17]
Downregulated in bone-I-PitNETs	AKR1B10	Involved in the NF-κb pathway [50]
	ZNF831 OR7C1 PAPPA2 GNLY	Function in antitumoral immune response [51-54]
	POMC	Encoding the ACTH precursor pro-opiomelanocortin
Top genes positively correlated with CEQ	VDAC1 TUSC	Roles in mitochondrial function, calcium regulation, and mTOR signaling; promising therapeutic targets [55, 56]
	CNIH3	Protooncogene with a potential diagnostic and therapeutic role [57]
	TBKBP1	Role in tumor growth promotion and tumor-mediated immunosuppression [58]
Top genes negatively correlated with CEQ	ENOX2	Overexpression associated with a poor prognosis of cancers [59]
	TBC1D8B	GTPase-activating protein Promotor of apoptosis [60]

Abbreviations: bone-I-PitNETs, bone invasive pituitary neuroenocrine tumor; CEQ, contrast enhancement quotient; I-PitNETs, invasive pituitary neuroenocrine tumor (parasellar invasiveness); PitNET, pituitary neuroenocrine tumor.

To affirm that the genes identified in our cohort are the indicators of invasion rather than cell lineage-related, we performed a thorough comparison of the DEGs and top correlated genes in our cohort to the cell lineage and/or functioning status related genes depicted in the recent studies [18-22, 27], as shown in Supplementary Table S8 [29]. Not surprisingly, the comparisons revealed some overlapped genes. However, besides POMC which was expectedly overexpressed in corticotroph tumors no other clear link to the cell-lineage related genes was found.

Our study demonstrated that several genes with a known role in tumor growth and progression are dysregulated in invasive PitNETs. Some of the genes were previously described in association with pituitary tumors (*SFRP4*, *GIPR*, *SERPINA1*, and *FOXP2*), whereas others do not have any known role in pituitary tumor biology (*RAMP3*, *FBXO11*, and *ZYG11A*). Invasion of PitNETs into bone seems to be regulated primarily by genes involved in immune modulation and anti-tumoral immune response (*ZNF831*, *OR7C1*, *PAPPA2*, and *GNLY*). As expected, some of the differentially expressed genes between the invasive, including bone-invasive, and the non-invasive PitNETs are involved in the process of epithelial-mesenchymal transition (*SFRP4* and *AKR1B10*), previously shown to be activated in larger and more invasive PitNETs [63-65]. Genes involved in angiogenesis correlate with contrast enhancement quotient, making this novel radiological measurement a potential radiological parameter of tumor vascularization. Further research is warranted to explore the potential utility of this novel parameter in the radiological diagnostics and prognosis of pituitary tumors. Importantly, we identified several potential therapeutic targets among the genes seemingly involved in the mechanisms of invasiveness, bone invasiveness, and tumor vascularization (*GIPR*, *RAMP3*, *FBXO11*, *ZYG11A*, *OR7C1*, *VDAC1*, and *TUSC2*).

There are a few limitations of the present study. The sample and feature coverage related to the chosen PTM cut-off value should be considered for the generalizability of the findings. Additionally, excluding noncoding RNAs and other functional elements might also be limiting. Indeed, excluding these elements may impact the comprehensive understanding of the regulatory network involved in PitNETs that are not included in the present work. Furthermore, given the limiting size of the retrieved DEG signatures, comprehensive functional analyses were limited. Indeed, when applied to gene signatures with a limited number of genes, enrichment functional analyses face challenges related to statistical power, increased susceptibility to multiple testing issues, and potential overfitting in biological representations. Instead, we opted for a more curated analysis approach to overcome, to some extent, these limitations. Our aim was to prioritize the interpretability and relevance of findings.

The clearly invasive PitNETs (Knosp 4) and bone-I-PitNETs were represented with fewer cases compared to the noninvasive and bone-NI-PitNETs. Therefore, studies including a larger number of clearly invasive tumors are warranted.

Preoperative computed tomography imaging is a more accurate method than MRI to assess cortical bone destruction and can thus aid in the evaluation of possible bone invasion. However, computed tomography is not a routine workup for sellar tumors.

Posttranscriptional modifications such as alternative RNA splicing impact protein burden in a cell and can influence invasive features of PitNETs. However, we have not addressed these aspects in our study.

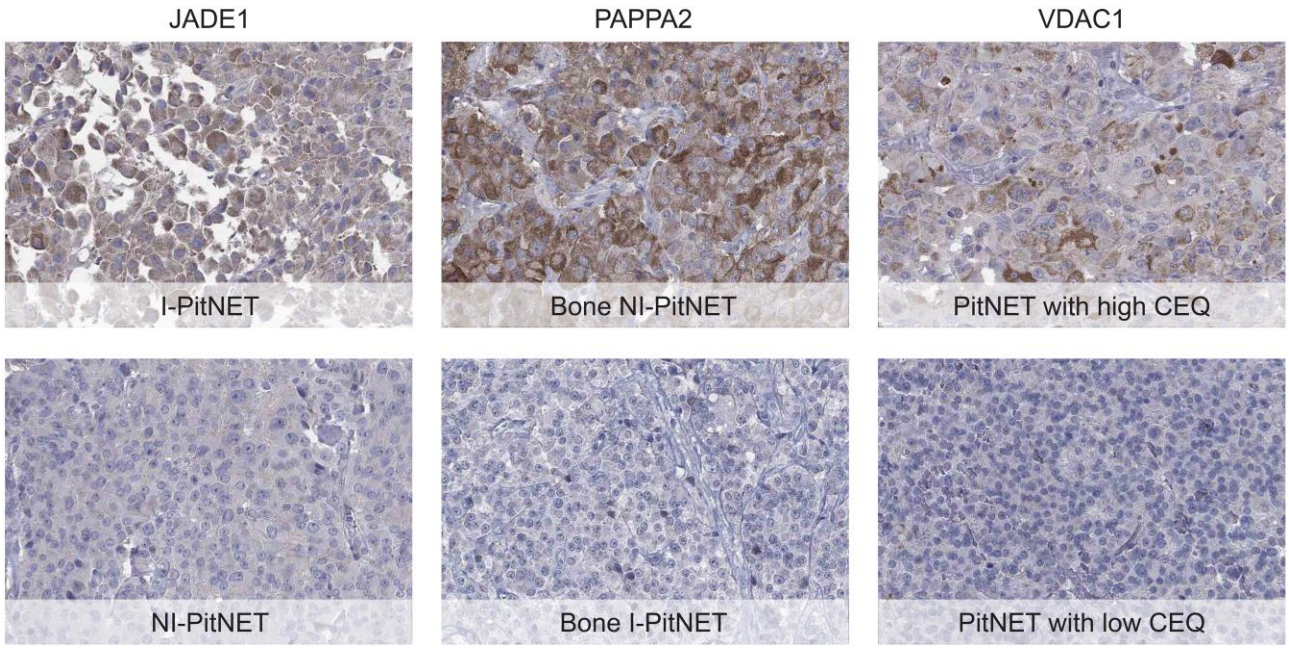

Figure 5. Immunohistochemical analysis of the selected gene products demonstrates positive labeling for the upregulated genes (upper row) and lack of staining for downregulated genes (lower row) in relation to invasiveness, bone invasiveness, and contrast enhancement quotient. All microphotographs taken with magnification 200x.

We used a contrast enhancement quotient between the enhanced and nonenhanced images to minimize the influence of differences in the MRI sequence parameter settings. However, a residual effect of technical differences, eg, using spin echo or gradient echo sequences, cannot be excluded.

In conclusion, our transcriptomics data, based on a well-characterized cohort of PitNETs of different histological types and growth patterns, reveal both known and novel genes impacting the parasellar and bone invasiveness of PitNETs, although without clear clustering. Several of the genes differentially expressed between invasive, including bone-invasive, and noninvasive PitNETs represent potential prognostic markers and therapeutic targets. The contrast enhancement quotient emerges as a radiological parameter of tumor vascularization, correlating with several angiogenesis-related genes. The interplay of many factors needs to be considered and further research conducted to elucidate the exact roles of the reported genes in the biology of pituitary neuroendocrine tumors and their potential prognostic and therapeutic benefits.

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Author Contributions

J.J. contributed to the study design, collected the tissue specimens, interpreted histological and immunohistochemical results, drafted the first version of the manuscript, and had a substantial role in writing the manuscript. A.T. analyzed and interpreted bioinformatics data and substantially contributed to the manuscript writing. N.H. and Å.S. were responsible for RNA extraction and sequencing. C.L. contributed with immunohitochemical analyses. M.U. provided infrastructure and basic work that allowed for this study and revised the manuscript. O.G. collected the tissue specimens. E.T. and B.E.E. contributed with collection and analysis of clinical data. J.W. performed radiological analysis, interpreted radiological data, and contributed to the manuscript writing. F.P. and O.C.B. designed the study, revised the manuscript, and had overall responsibility for accuracy and integrity of any part of the work. All the authors read and approved the final version of the manuscript.

Disclosures

The authors have nothing to disclose.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References.

Ethics Approval and Consent to Participate

The Swedish Ethical Review Authority approved the study protocol, Dnr 2018/053.

Consent for Publication

The manuscript does not contain any individual person´s data in any form.

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