PITX2 Expression in Non-functional Pituitary Neuroendocrine Tumor with Cavernous Sinus Invasion

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Abstract

Although most pituitary neuroendocrine tumors (PitNETs) show benign behavior, a significant number of PitNETs exhibit an aggressive course including cavernous sinus (CS) invasion. To date, the cause of CS invasion has not been fully elucidated. In this study, we analyzed the relationship between CS invasion in PitNETs and the expression of PITX2 and SNAIL1, which are the transcription factors associated with the morphogenesis of pituitary gland. Sixty cases with non-functional PitNETs were classified into four types: type 1a, none of CS invasion and suprasellar expansion; type 1b, suprasellar expansion without CS invasion; type 2a, CS invasion without suprasellar expansion; and type 2b, CS invasion with suprasellar expansion. We analyzed the expression of PITX2 and SNAIL1 employing quantitative real-time polymerase chain reaction (qPCR) and immunohistochemistry. Other parameters such as mitotic count, Ki-67 index, and p53 expression were also analyzed, which were previously reported as potential tumor proliferative markers in PitNETs. PITX2 expression was significantly higher in PitNETs with CS invasion than PitNETs without CS invasion ($P = 0.019$). Expression of SNAIL1 was significantly elevated in PitNETs with suprasellar expansion compared with PitNETs without suprasellar expansion ($P = 0.02$). There was no apparent relationship between CS invasion and mitotic count, Ki-67 index, and p53 expression (mitotic count, $P = 0.11$; Ki-67 index, $P = 0.61$; p53, $P = 0.66$). High PITX2 expression was observed in non-functional PitNETs with CS invasion, suggesting that PITX2 may be involved in CS invasion of PitNETs.

Keywords Pituitary adenoma · PITX2 · SNAIL1 · Cavernous sinus · Invasion

Introduction

Atypical morphologic features such as cavernous sinus (CS) invasion are often demonstrated as one of the aggressive behaviors of pituitary neuroendocrine tumors (PitNETs) (formerly known as pituitary adenoma) [1–3]. According to the World Health Organization (WHO) classification in 2014, atypical pituitary adenoma is a distinct adenoma subtype, with Ki-67 index > 3%, elevated mitotic activity, and high p53 immunoreactivity [3]. Later in 2017, WHO classification was revised and the term of histopathological atypical pituitary adenoma was no longer recommended. Clinically aggressive parameters, such as radiographic tumor invasion, were highly recommended for consideration as high-risk PitNETs as well having histopathological proliferative potential by Ki-67 index, p53 staining, and mitotic count [4]. The cause of CS invasion in PitNETs has not been fully elucidated. Some PitNETs easily invade into the CS despite their small tumor size, leading to the difficulty in surgical excision. In contrast, some PitNETs became enlarged without CS invasion.

In the present study, we focused on PITX2 and SNAIL1, which are the transcription factors associated with the morphogenesis of pituitary gland in the early stage of development [5]. In some specific genes involving in development and patterning, PITX2 and SNAIL1 are particularly essential factors for normal morphogenesis, which engage in cross-talk with one another. It has been described that the left-specific PITX2 axis mediates left identity in vertebrates, and this PITX2 axis is repressed by the epithelial-mesenchymal

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transition (EMT) inducer SNAIL1, on the right-hand side [6, 7]. PITX2, a member of the bicoid/paired-like homeobox gene family, plays a role at various stages of pituitary organogenesis and highly expressed in the fetal pituitary gland during pituitary primordium formation, cell specification, and proliferation. PITX2 gene-deleted mice have shown arrest of proliferation events of the anterior pituitary gland [6–9]. EMT is a highly conserved physiological process by which immotile and polarized epithelial cells become migratory mesenchymal cells by losing their adherent phenotype and tight junctions. SNAIL1 increases stability and availability for the EMT process, which is involved in pituitary gland regulation during the embryonic phase [10, 11].

We evaluated the expression of PITX2 and SNAIL1 in non-functional PitNETs, and the expression of PITX2 was associated with CS invasion. To date, there are no reports focusing on the expression of both PITX2 and SNAIL1 in relation to the aggressive behavior of PitNETs.

Materials and Methods

Immunohistochemical Analysis

We have obtained the necessary approvals from the Institutional Review Board at our institutes (reference number: 20050002) for the work presented here. We also obtained written informed consent from all 60 consecutive patients. Tumor tissues from each of the 60 patients with newly diagnosed non-functional PitNETs, which was determined based upon hormonal status, were formalin-fixed, paraffin-embedded, and 4 μm sections were prepared for histopathological analyses. All patients did not receive no radio-chemotherapy before the operation. Of these, 36 exhibited CS invasion. Mitotic activity was assessed using hematoxylin and eosin (H&E) staining. Classical histological subtypes (diffuse, sinusoidal, and papillary patterns) were evaluated based on H&E findings [12]. Immunohistochemistry was performed by standard procedures [13]. After the tissue sections were deparaffinized and rehydrated, antigen retrieval was performed using microwave irradiation in citrate buffer (pH 6.0). The sections were blocked for 60 min in 2.5% horse serum (ImmPRESSTM Detection Systems, Vectorlabs, CA, USA). Then, the sections were incubated at 4 °C overnight with anti-Ki-67 monoclonal antibody (1:200, M7249, DAKO, Agilent Technologies, CA, USA), anti-p53 monoclonal antibody (1:100, DO-7, DAKO, Agilent Technologies) [13], anti-PITX2 polyclonal antibody (1:250, ab32832, Abcam, Cambridge, UK), and anti-SNAIL1 polyclonal antibody (1:250, ab53519, Abcam), and then incubated with anti-mouse, rabbit or goat Ig secondary antibody (ImmPRESSTM Detection Systems, Vectorlabs) for 60 min at room temperature. Visualization of the products was done using peroxidase-diaminobenzidine reaction.

For the assessment of Ki-67 index, manual counting of 1000 tumor cells was routinely done at high-power field (× 40). The expression of PITX2 was assessed as the following: ++, diffuse nuclear staining in tumor cells; +, faint staining only in a subset of tumor cells; −, negative staining. The expression of SNAIL1 was assessed as the following: ++, diffuse cytoplasmic staining in tumor cells; +, faint staining only in a subset of tumor cells; −, negative staining. All expressions were assessed by consensus of three authors (RT, YM, and KO) who were blinded to the clinical information.

Immunofluorescent Analysis

Immunofluorescence staining for PITX2 and SNAIL1 expressions was performed to evaluate the nuclear or cytoplasmic staining patterns. Tissue sections were incubated overnight at 4 °C in the presence of antibodies against PITX2 (1:250, ab32832, rabbit polyclonal IgG; Abcam) and SNAIL1 (1:250, ab53519, goat polyclonal IgG; Abcam). Corresponding secondary antibodies, Alexa Fluor 568 donkey anti-goat IgG, or Alexa Fluor 488 goat anti-rabbit IgG were used (1:200). Last, the sections were mounted with a medium containing 4′,6′-diamidino-2-phenylindole (DAPI) for nucleus staining. Cell images were captured by fluorescence microscopy.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

For quantitative real-time polymerase chain reaction (qPCR), RNA was isolated from 10-μm sections of formalin-fixed, paraffin-embedded tissue in 60 samples using a NucleoSpin totalRNA FFPE XS kit (Macherey-Nagel, Düren, Germany). cDNA was subsequently obtained using the SuperScript III First Strand Synthesis System with random hexamers (Invitrogen, Carlsbad, CA, USA). Triplicate qPCR assays were performed on an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) using 10 μl SYBR Green reagent (Applied Biosystems), 2 μl synthesized cDNA, 2 μl of the same primers that were used in the standard PCR, and 6 μl of molecular grade H_2O in a final volume of 20 μl. The conditions for thermal cycling were 10 min at 95°C, initially, followed by 55 cycles of 95 °C for 15 s and 60 °C for 60 s. The comparative CT method was used to analyze the qPCR data. Briefly ΔCT (the difference in cycle threshold), was determined as the difference between human GAPDH and the tested gene. Data were then normalized to GAPDH cDNA. The fold change was calculated as 2-ΔΔCT [14]. The primer sequences are listed as the following: PITX2 forward (fwd), CAGCCTGAGACTGAAAGCA; PITX2 reverse (rev), GCCCACGACCTTCTAGCAT; SNAIL1 fwd, AATCGGAAGCCTAACTACAGCG; SNAIL1 rev, GTCCCAGATGAGCATTGGCA; GAPDH fwd, TGAACGGGAAGCTCACTGG; GAPDH rev, TCCACCACCCTGTTGCTGTA.

Type 1a Type 1b Type 2a Type 2b
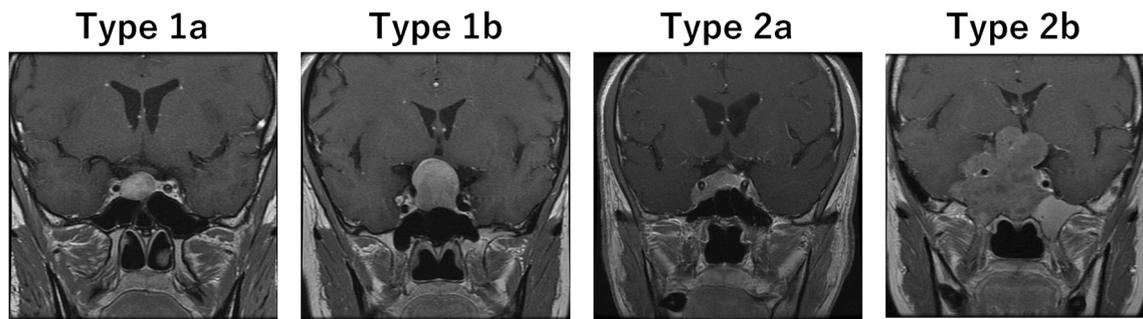
Fig. 1 Pituitary neuroendocrine tumor (PitNET) classification according to cavernous sinus (CS) invasion and suprasellar expansion. Type 1a: none of CS invasion and suprasellar expansion; type 1b: suprasellar expansion without CS invasion; type 2a: CS invasion without suprasellar expansion; and type 2b: CS invasion with suprasellar expansion

Radiographical Analysis

The Knosp grading scale was created to predict the invasion of the medial wall of the CS based on the relationship of the PitNETs to the cavernous internal carotid artery [15]. Although this grading is a well-organized system for CS invasion, it does not reflect the suprasellar expansion. Therefore, in the present study, we classified PitNETs into four types: type 1a, none of CS invasion and suprasellar expansion; type 1b, suprasellar expansion without CS invasion; type 2a, CS invasion without suprasellar expansion; and type 2b, CS invasion with suprasellar expansion (Fig. 1).

Statistical Analysis

The chi-squared test was used to evaluate the classical histological subtype, immunohistopathological expressions of p53, PITX2, and SNAIL1. Student's t test was used to compare the recurrent rate, Ki-67 index, mitotic count, and relative expressions of PITX2 and SNAIL1 analyzed by qPCR between patients with and without CS invasion. Analyses were performed with IBM SPSS statistics (IBM Corp., Armonk, NY, USA). A P value of < 0.05 was considered statistically significant.

Table 1 Patient characteristics and results

	CS invasion	Without CS invasion	P value
Number	36	24	–
Age (years old)	61.5 (31~85)	51.4 (26~80)	0.02
Sex	Male: 16, female:20	Male: 11, female: 13	0.79
Post-operative recurrent rate (%)	5 (14%)	2 (8%)	0.81
Knosp classification	Grade2: 5	Grade1: 24	–
	Grade3: 5	–	–
	Grade4: 26	–	–
Type in the present study ( )	Type 2a:8	Type 1a: 9	–
	Type 2b:28	Type 1b:15	–
Ki-67 index	<1%: 34	<1%:22	0.61
	2%: 1, 15%:1	3%:1, 18%:1	
Mitotic count	0/10HPF:28	0/10HPF: 22	0.11
	1/10HPF:5	1/10HPF: 2	
	2/10HPF:2		
	3/10HPF:1		
p53 IHC	+: 1	+: 1	0.66
	−: 35	−: 23	
Histological subtype	Diffuse: 24	Diffuse: 16	0.78
	Sinusoidal: 8	Sinusoidal: 6	
	Papillary: 4	Papillary 2	

CS, cavernous sinus; IHC, immunohistochemistry

Type 1a, none of CS invasion and suprasellar expansion

Type 1b, suprasellar expansion without CS invasion

Type 2a, CS invasion without suprasellar expansion

Type 2b, CS invasion with suprasellar expansion

Results

Patient Characteristics

Patient characteristics of 60 patients with non-functional PitNETs are summarized in Table 1. Patients with CS invasion included Knosp classification grade 2 ($n = 5$), 3 ($n = 5$), and 4 ($n = 26$) [15]. Patients without CS invasion included only Knosp classification grade 0. Infrasellar of sphenoid sinus invasion was not observed in all patients. In our study, PitNETs were classified into four different settings according to the CS invasion and suprasellar expansion: types 1a ($n = 9$), 1b ($n = 15$), 2a ($n = 8$), and 2b ($n = 28$). The average patient age of those with PitNETs exhibiting CS invasion was higher than those without CS invasion. There was no significant difference in sex or the post-operative recurrent rate between the patients with and without CS invasion.

Histopathological Analysis

No significant differences were observed in classical histological subtype, Ki-67 and p53 expression or mitotic count between PitNETs with and without CS invasion (Table 1 and Fig. 2). In immunohistopathological analysis, the expression of PITX2 exhibited nuclear staining pattern. The expression of SNAIL1 mainly exhibited cytoplasmic staining pattern (Fig. 3a and b). In seven out of nine type1a adenomas, negative or faint staining of PITX2
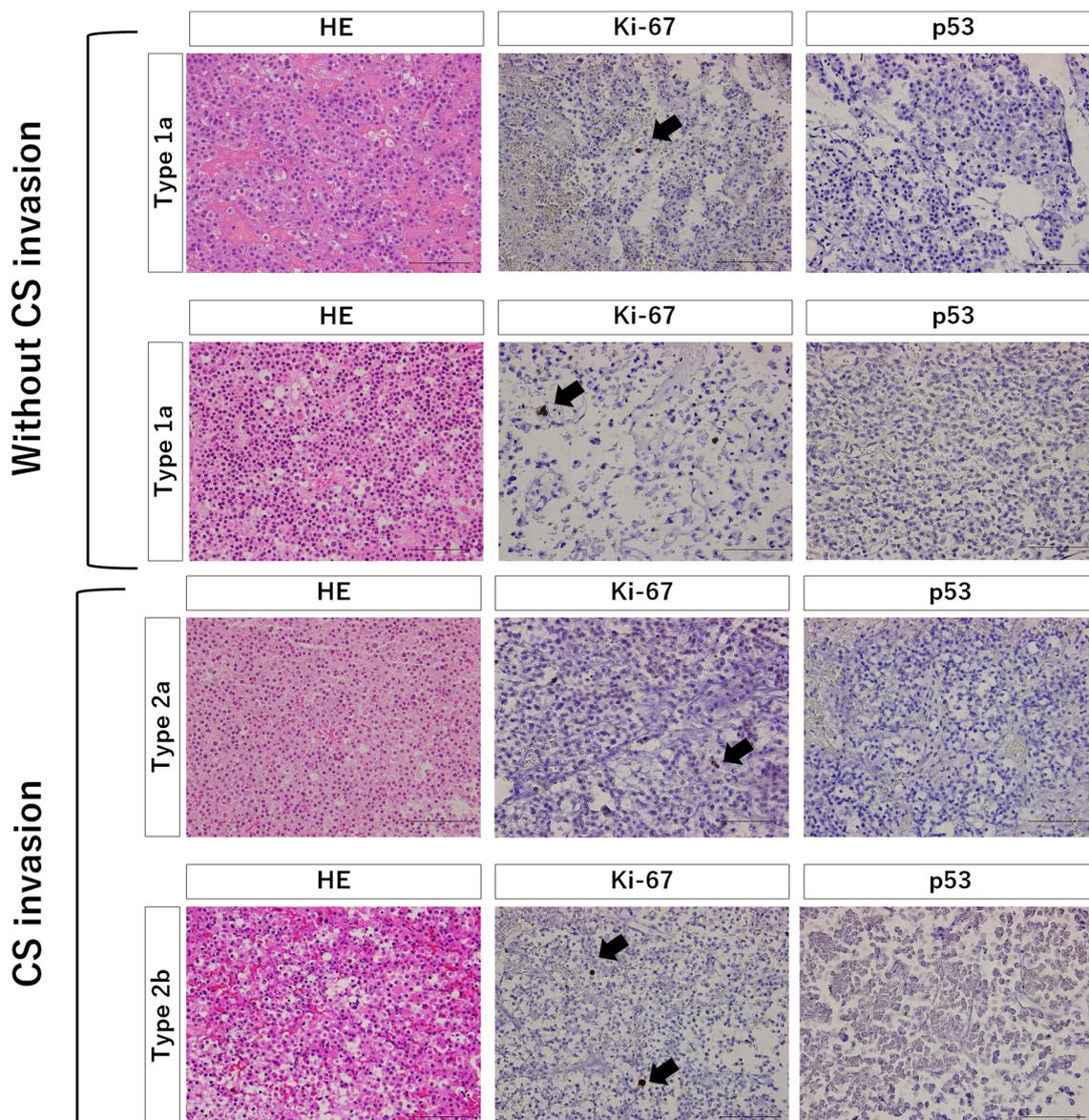
Fig. 2 Immunostaining of Ki-67 and p53 in four types of pituitary neuroendocrine tumors (PitNET s). No significant differences were observed in Ki-67 and p53 expression or mitotic count between PitNETs with and without CS invasion. Black arrow: tumor cell with positive Ki-67 expression. Original magnification, × 40; magnification bar, 100 μm

Fig. 3 Immunostaining pattern of PITX2 and SNAIL1. The expression of PITX2 exhibited nuclear staining pattern, and SNAIL1 mainly exhibited cytoplasmic staining pattern in immunohistochemical (**a**) and immunofluorescent analysis (**b**) (original magnification, × 100; magnification bar, 10 μm (**a**) and × 40; magnification bar, 100 μm (**b**))
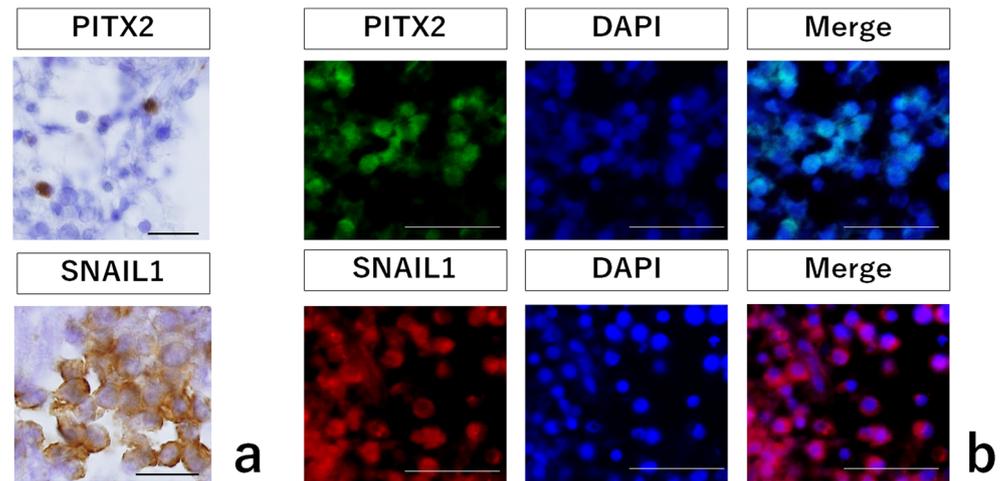
was observed, and five cases demonstrated faint or negative staining of SNAIL1 (PITX2, ++: 2, + or −: 7; SNAIL1,++: 4, + or −:5) (Fig. 4a). In 14 out of 15 type 1b adenomas, negative or faint staining of PITX2 and diffuse positive staining of SNAIL1 (PITX2,++: 1, + or −: 14; SNAIL1,++: 14, + or −:1) was observed (Fig. 4b). All eight type 2a adenomas showed diffuse positive staining of PITX2, and four cases showed negative or faint staining of SNAIL1 (PITX2, ++: 8, + or −: 0; SNAIL1,++:4, + or −:4) (Fig. 4c). In the 28 type 2b adenomas, 26 cases showed diffuse positive staining of PITX2 and 27 cases diffuse positive staining of SNAIL1 (PITX2,++: 26, + or −: 2; SNAIL1,++: 27, + or −:1) (Fig. 4d). The diffuse positive ratio of PITX2 in cases with CS invasion (type 2a and 2b) was significantly higher than that in cases without CS invasion (type 1a and 1b) ($P < 0.01$). The diffuse positive ratio of SNAIL1 in cases with suprasellar expansion (type1b + 2b) was significantly higher than that in cases without suprasellar expansion (type1a + 2a) ($P < 0.01$).

qPCR Analysis

qPCR analysis revealed PITX2 was significantly higher in cases with CS invasion (type 2a and 2b) compared with cases without CS invasion (type 1a and 1b, Fig. 5a). SNAIL1 was significantly higher in cases with suprasellar expansion (type 1b and 2b) than cases without suprasellar expansion (type 1a and 2a, Fig. 5b). In the cases without CS invasion, SNAIL1 was significantly higher in cases with suprasellar expansion (type 1b) than those without suprasellar expansion (type 1a, Fig. 5c). In the cases with CS invasion, SNAIL1 tended to be higher in cases with suprasellar expansion (type 2b) compared with those without suprasellar expansion (type 2a, Fig. 5d).

Discussion

It is difficult to completely resect PitNETs with CS invasion. Thus, critical factors for CS invasion should be established to predict high-risk PitNETs. Rutkowski et al. [16] analyzed the characteristics of patients, tumor features, and outcomes of treatment in PitNETs. PitNETs with atypical characteristics (Ki-67 index ≥ 3%, mitotic index, and extensive p53 staining) were more likely to be seen in younger patients at a larger size, more often hormonal hypersecretion, and associated with early recurrence. However, these factors did not reflect the invasive characteristics into the CS. In the present study, Ki-67 index, mitotic count, and p53 expression, which have been previously reported as the markers of aggressive PitNETs, did not demonstrate a significant difference between PitNETs with and without CS invasion. These three parameters may not be used as biomarkers for the clinically aggressive PitNETs. Although DNA methylation and deletions affecting 11q13, 13q12–14, and 10q26 have also been previously reported as aggressive factors, these factors could not reflect CS invasion [17, 18]. Even though activating mutations of *ras* protooncogenes are identified in various types of human cancers, such mutations were uncommon in PitNETs [19, 20].

We focused on the transcription factors of the pituitary gland. PITX2 is necessary for pituitary organogenesis, specification (especially gonadotrope) and expansion [21–25]. In addition, PITX2 expression was shown to be related to pituitary stem cells (PSCs) [26]. PSCs were detected in the adult pituitary gland, and were shown to play a role in pituitary gland homeostasis throughout life as these cells are able to maintain multipotent capacity [26–30]. PITX2 is involved in the upregulation of *Sox2*, leading to PSC proliferation [30–32]. Increasing stemness was shown to correlate with the abilities of cell invasion in the central nervous system tumors such as glioblastomas [33]. PSCs have also been reported to play a role in pituitary tumorigenesis [34]. Moreno
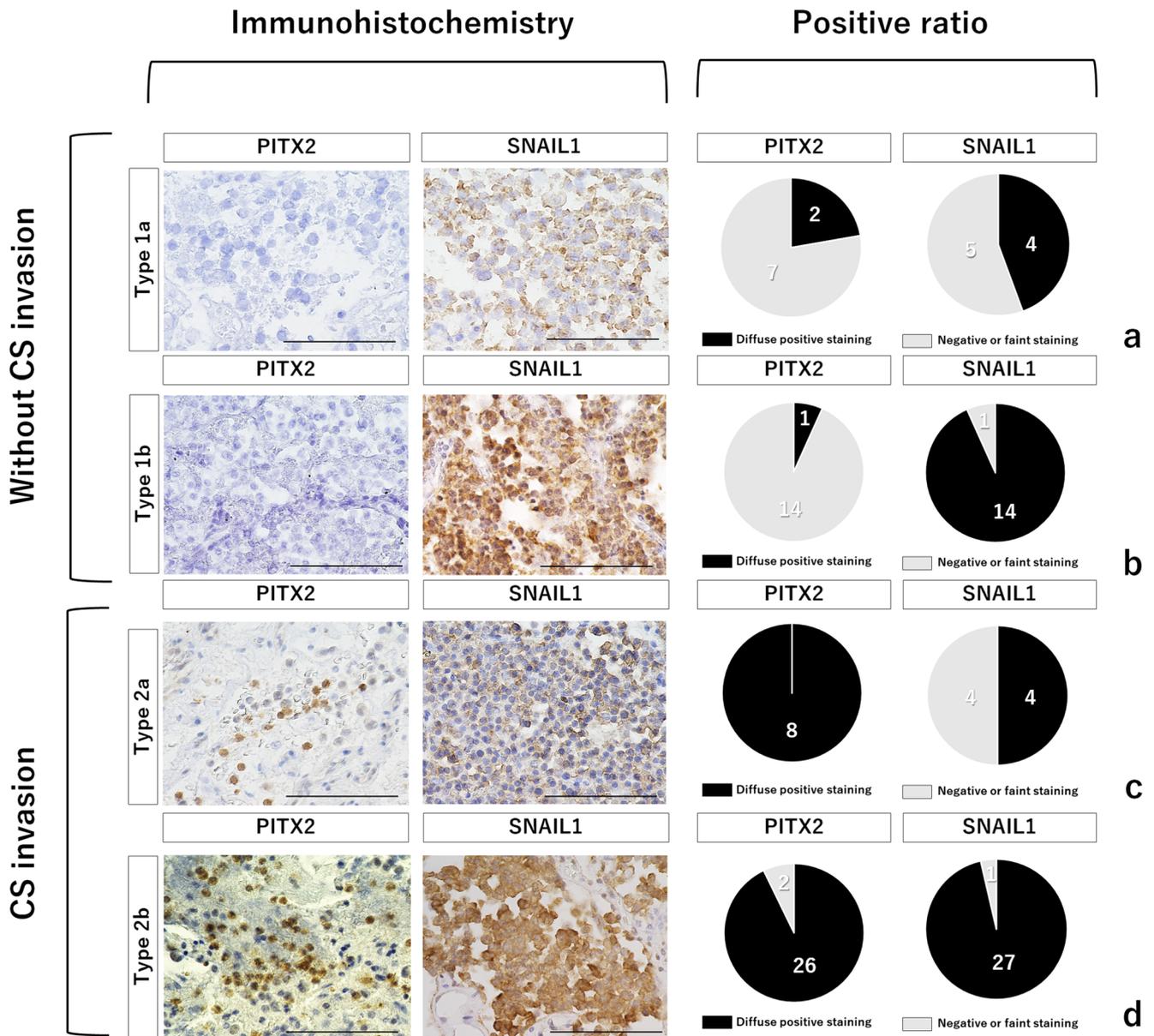
Fig. 4 Immunostaining of PITX2 and SNAIL1 in the four types of pituitary neuroendocrine tumor (PitNETs). The expression and positive ratio of PITX2 and SNAIL1 in type 1a (a), type 1b (b), type 2a (c), and Type 2b (d) (original magnification, × 40; magnification bar, 100 μm)

et al. [35] examined relative expression of PITX2 mRNA by RT-qPCR in nonfunctional PitNETs compared with normal pituitaries, which showed that PITX2 mRNAs was significantly upregulated in non-functional PitNETs. The Wnt/Dvl/β-catenin pathway can rapidly induce PITX2, which is important in the progression of pituitary malignancy. Acunzo et al. [36] demonstrated that inactivation of PITX2 transcription factor could induce apoptosis of gonadotroph tumor cells, suggesting that PITX2 overexpression might play an antiapoptotic role during non-functional PitNETs tumorigenesis. Recently, Cui et al. [37] also demonstrated that invasive PitNETs showed significantly elevated levels of PITX2, and lower levels of miR-21 compared with non-invasive tumors.

MiR-21 was found to target the 3′-UTR of PITX2 gene to block its expression. In the present study, PitNETs with high PITX2 expressions showed invasive behavior particularly into CS (not suprasellar expansion).

SNAIL1, by repressing E-cadherin [38, 39], plays an essential role in the modulation of EMT, by which the adherent phenotype of epithelial cells is altered to a migratory, mesenchymal phenotype. The morphological changes of the PSCs into the loosely distributed cells from the tightly packed columnar-type cells, is also mediated by EMT. SNAIL1 expression also accelerates invasion activity by upregulating matrix metalloproteinase expression, resulting in poor prognosis [40–43]. Jia et al.
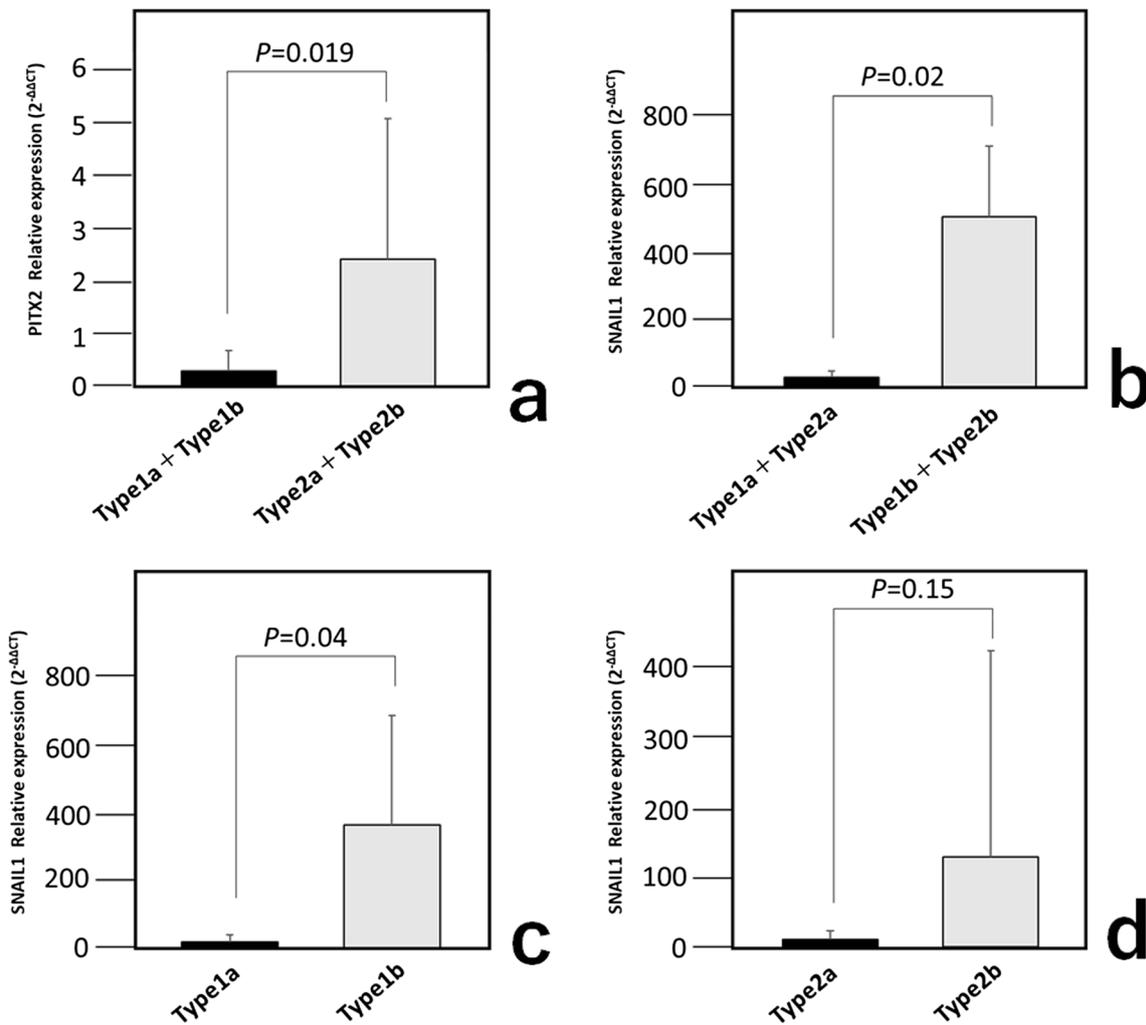
Fig. 5 Gene expression of PITX2 and SNAIL1 by quantitative real-time polymerase chain reaction (qPCR). PITX2 expression (**a**) and SNAIL1 expression (**b**, **c**, and **d**)

[44] demonstrated that EMT induced by SNAIL1 was significant indicators of bone destruction, tumor progression, appearance of cystic lesions, and endocrine functions in PitNETs [44, 45]. In the present study, the expression of SNAIL1 was significantly associated with suprasellar expansion. However, this was not related to CS invasion, suggesting that invasion the dural leaflets of the medial wall in the CS and passive expansion of the diaphragm sellae might be caused by different mechanisms.

PITX2 and SNAIL1 were evaluated from some specific genes involving in development and patterning, because they were highly engaged factors with one another for morphogenesis [6, 7]. Further analysis of other morphogenetic factors such as transforming growth factor signaling and beta-catenin signaling associated with PITX2 and SNAIL1 would be warranted to confirm the results of this study. In addition, non-functional PitNETs are morphologically heterogeneous.

The new classification by WHO in 2017 was based on the immunohistochemistry, in particular, the hormone immunohistochemistry. Furthermore, it is also encouraged to use the pituitary transcription factors. Gonadotroph adenomas are the most common subtype of non-functional adenomas [4, 46]. Immunolabeling in 10% of the cases is absent (null-cell adenoma). Remarkably, immunolabeling might be positive for TSH, GH, ACTH, or PRL, even if there is no secretion, which are known as silent adenomas [4, 46]. The sparsely granulated somatotroph tumor, Crooke's cell tumor, silent corticotroph tumor, lactotroph tumor in men and Pit-1 positive plurihormonal tumor (also known as poorly differentiated Pit-1 lineage tumor and previously referred to as silent subtype III pituitary adenoma) were defined as high-risk PitNETs in the 2017 WHO classification [4, 46]. The relationship between PITX2 and SNAIL1 expressions, and these hormonal and/or transcriptional characteristics should be examined for future research.

Conclusions

High PITX2 expression was associated with CS invasion in PitNETs. PITX2 may be involved in aggressive PitNETs.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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