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Expression of Insulinoma-Associated Protein 1 (INSM1) and Orthopedia Homeobox (OTP) in Tumors with Neuroendocrine Differentiation at Rare Sites

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Abstract
Insulinoma-associated protein 1 (INSM1) and orthopedia homeobox (OTP) are transcription factors that play a critical role in neuroendocrine (NE) and neuroepithelial cell development. INSM1 has been identified in multiple tumors of NE or neuroepithelial origin, whereas OTP expression has been mainly studied in NE tumors of pulmonary origin. Expression of OTP appears to correlate with poorer prognosis in pulmonary carcinoids; however, its expression patterns in other NE/neuroepithelial tumors need further investigation. Here, we assessed the diagnostic utility of INSM1 and OTP in tumors with NE differentiation at relatively uncommon sites including prostate, breast, and tumors of gynecologic origin. Thirty-two formalin-fixed, paraffin-embedded cases were used to construct a tissue microarray. Immunohistochemistry for INSM1 and OTP was performed and scored semi-quantitatively. INSM1 was diffusely expressed in 60% of gynecologic tumors, 71.4% of mammary carcinoma, and 25% of prostate adenocarcinoma with NE differentiation. Diffuse expression of OTP was detected in 50% of prostate adenocarcinoma with NE differentiation and 100% neuroendocrine carcinoma of the ovary. Immunostain for achaete-scute homolog 1, chromogranin, synaptophysin, and CD56 supported the NE and/or neuroepithelial differentiation of the tumors. In summary, INSM1 is expressed in most of the tumors with NE and neuroepithelial differentiation in this study, confirming the diagnostic utility of INSM1 as a novel and sensitive marker of NE/neuroepithelial differentiation. The expression of OTP in some NE tumors outside of lung expands the spectrum of tumors that may express this biomarker and should be considered when working up a NE tumor of unknown primary site.

Keywords Insulinoma-associated protein 1 (INSM1) · Orthopedia homeobox (OTP) · Ovarian carcinoma with neuroendocrine differentiation · Breast carcinoma with neuroendocrine differentiation · Prostate carcinoma with neuroendocrine differentiation

Introduction

Insulinoma-associated protein 1 (INSM1) and orthopedia homeobox (OTP) are transcription factors that play critical roles in neuroendocrine (NE) and neuroepithelial cell development [1–5]. In adult tissues, INSM1 has been identified in multiple tumors of NE or neuroepithelial origin; however, its expression in tumors of prostate, breast, and gynecologic malignancy with NE differentiation has not been studied extensively [6–8]. OTP expression has been studied mainly in NE tumors of pulmonary origin and appears to correlate with poorer prognosis in pulmonary carcinoids [7–10]; however, its expression patterns in other NE/neuroepithelial tumors are still under investigation.

Unlike most other solid cancers, NE tumors can arise within a wide variety of tissues and organ systems. Some NE tumors are functional due to their biologic nature whereby production and secretion of neuropeptides produce clinical symptoms/syndromes. Despite the diversity of presentations of these neoplasms, the fundamental biological mechanisms driving the progression of NE tumors are somewhat similar, and NE tumors very often share some histologic features, genetic pathways, and pathologic biomarkers.

Very few NE tumors of the prostate arise "de novo," i.e., diagnosed without a prior diagnosis of prostatic

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adenocarcinoma [11]. Immunohistochemical studies are typically positive for NE markers, and negative for androgen receptor (AR) and prostate specific antigen (PSA). NE tumors include small-cell carcinoma of the prostate (< 0.1% of all diagnosed PCs), large cell NE carcinoma (only a few cases worldwide), and low-grade carcinoid, comparable morphologically to carcinoid tumors in other locations [11, 12]. These tumors can be pure or a component of a prostatic adenocarcinoma or with an unusual "hybrid" tumor morphology showing both NE and conventional epithelial phenotypes. Transdifferentiation from an epithelial-like phenotype to an NE-like phenotype has been well established in a substantial number of heavily treated castrate resistant prostate cancer cases that exhibit features of de novo small-cell carcinomas, admixed with or without an adenocarcinoma component. The exact prevalence is difficult to establish as metastatic castrate resistant prostate cancer patients are not routinely biopsied. It is estimated that at least 25% of patients with advanced disease may eventually progress [11, 12].

Primary NE carcinoma of the breast is rare, often underdiagnosed, and is typically a disease of postmenopausal women. The tumors are subclassified into well-differentiated and poorly differentiated NE tumors and invasive breast carcinoma with NE features [13]. While well-differentiated NE tumors resemble carcinoids of other sites and poorly differentiated NE tumors resemble small cell carcinoma of the lung, invasive breast carcinomas with NE features are most commonly associated with hypercellular variant of mucinous carcinoma and solid papillary carcinoma. Confirmation of NE differentiation is established by immunohistochemistry. The consensus on prognosis is debated but most reported studies suggest a poor outcome and resistance to chemotherapy [14–18].

In the gynecologic tract, NE carcinoma can arise in any component, including the vulva, vagina, cervix, endometrium, and ovary. These tumors in the gynecologic tract are quite uncommon, constituting only 2% of all gynecologic cancers and can pose diagnostic challenges. A panel of NE differentiation markers is usually necessary to establish the diagnosis [19, 20]. Among the gynecologic malignancies, most NE cancers of the cervix are small cell carcinomas with a morphologic appearance similar to the pulmonary small cell carcinoma. These cases are strongly associated with HPV18 and less often with HPV16 [21]. Large cell NE carcinomas are poorly differentiated, and confirmation of NE differentiation by immunohistochemistry is necessary for diagnosis [19].

Ovarian neuroendocrine carcinomas comprise of small cell carcinoma, pulmonary type, and large cell neuroendocrine carcinoma [22, 23]. It is important to keep in mind that small cell carcinoma of the ovary, hypercalcemic type, used to be grouped within the NETs of the ovary; however, it is now considered a distinct entity of uncertain origin since it lacks NE differentiation, and nearly 100% of these cases have deleterious germline or somatic mutations of SMARCA4, a member of the SWI/SNF chromatin remodeling complex [24]. Small cell carcinoma of the ovary, pulmonary type, is rare and occurs in women 28–85 years of age (mean age of 59); and at laparotomy, the tumors are often bilateral, and majority of cases have extraovarian spread [22]. The even rarer non-small cell NE carcinoma or large cell NE carcinoma of the ovary occur in women 22–63 years (mean age of 46.7) are typically admixed with other histologic subtypes (epithelial and germ cell) and are generally associated with poor patient outcomes [23].

Small cell carcinoma in the endometrium is also quite uncommon and can be admixed with other components of an endometrial cancer. Confirmation of diagnosis requires at least one positive neuroendocrine marker. Abnormal bleeding is the primary presenting symptom, although patients may also present with metastatic disease [19, 20]. Primary small cell carcinoma of the vagina is also a rare entity, and patients typically present with post-menopausal bleeding. These lesions have a propensity for early widespread metastasis, and 85% die within 1 year of diagnosis. In the vulva, the primary differential diagnosis is Merkel cell carcinoma. Both tumor types demonstrate NE differentiation, and therefore, diagnostic accuracy of the pathologic interpretation is essential [19, 20].

Among the ovarian germ cell tumors, immature teratoma is the only ovarian germ cell neoplasm that is histologically graded based upon the proportion immature neural elements. It can be found either in pure form or as a component of a mixed germ cell tumor [25]. Histological grade is an important prognostic factor that predicts extra ovarian spread and overall survival in these patients, and therefore, identification of the immature neural elements is crucial and can be aided by immunohistochemistry in difficult cases.

While chromogranin, synaptophysin, and CD56 have been in clinical use for diagnosis of NE lesions for many years, there are limitations in their sensitivity and specificity including, notably, reduced expression in poorly differentiated tumors. On the other hand, INSM1 and OTP are novel neuroendocrine markers that emerge to be more sensitive markers of NE differentiation [6, 26]. Here, we assessed the diagnostic utility of these two markers in uncommon tumors with NE differentiation including tumors of prostate, breast, and of gynecologic origin.

Materials and Methods

Case Selection

Duplicate cores from 32 formalin-fixed, paraffin-embedded cases were used to construct a tissue microarray (TMA). Cases included malignant tumors with neuroendocrine differentiation from the gynecologic organs (n=16), breast (n=8),

Table 1 Antibody source and dilution

Antibody	Source	Dilution
INSM1	Santa Cruz #SC-271408	1:2500
OTP	Novus Biological #NBP1–85861	1:500
ASCL1	ThermoFisher Scientific #PA5–36556	1:200
CHGA (LK2H10)	Ventana Medical Systems, Inc. #760–2519	Predilute
SYP (SP11)	Ventana Medical Systems, Inc. #790–4407	Predilute
CD56 (MRQ-42)	Cell Marque, #156R-98	Predilute

OTP, orthopedia homeobox; *INSM1*, insulinoma-associated protein 1; *ASCL1*, achaete-scute homolog 1; *CHGA*, chromogranin A; *SYP*, synaptophysin

and prostate gland (n=6). Cases were retrieved and histories were reviewed with appropriate institutional review board (IRB) approval.

Immunohistochemistry

Immunohistochemical staining was performed on a Ventana Discovery XT BioMarker platform (Ventana Medical Systems, Tucson, AZ). Deparaffinization and heat-induced epitope retrieval were performed by "cell conditioning" with CC1 buffer (Ventana #950-500), an EDTA-based buffer. The antibodies used are summarized in Table 1. INSM1 and OTP antibodies were diluted using Da Vinci Green diluent (#PD900H Biocare Medical, Pacheco, CA). INSM1 was incubated for 1 h at ambient temperature; OTP was incubated for 28 min at 37 C. The secondary antibodies used were Discovery UltraMap anti-Mouse HRP (Ventana #760-4313) incubated for 16 min at ambient temperature for INSM1 and Discovery UltraMap anti-Rabbit HRP (Ventana #760-4315) for 8 min @37 °C for OTP. The chromogens Discovery ChromoMap DAB (diaminobenzidine, Ventana #760-159) was utilized for antigen localization for INSM1 and OTP. Counterstaining was performed using Harris Hematoxylin (#SH26-500D Fisher Scientific, Hampton, NH) diluted 1:5 and stained for 45 s.

INSM1 and OTP expression were scored, and results were categorized into four different groups based on intensity (0–3) and distribution (rare 1–5%, focal 5–25%, diffuse > 25%). Positive cases were defined as having a scoring index > 1 and negative cases displayed scoring indices ≤ 1 [6, 11]. IHC for achaete-scute homolog 1 (ASCL1), chromogranin (CHGA), synaptophysin (SYP), and CD56 were used as positive controls for NE differentiation in these tumors.

Results

Nuclear expression of INSM1 and OTP expression was interpretable in 26 of 32 cases (26/32) after excluding lost tissues during immunostaining and cases with recurrent disease. Neuroendocrine lineage was confirmed by additional immunohistochemical stains including ASCL1, CHGA, SYP, and CD56, with true NE differentiation being positive for at least one or more of these markers (Table 2).

INSM1 was diffusely expressed in 60% of gynecologic tumors (9/15), 71.4% (5/7) of invasive mammary carcinoma, and 25% (1/4) of prostate adenocarcinoma with NE differentiation (Table 2). While 9 of 15 cases of gynecologic tumors were positive for INSM1, including tumors in ovary (7 of 8, including 5 of 6 cases of immature teratoma, Fig. 1) and in the

Table 2 Expression of INSM1 and OTP in malignant neoplasms of prostate, breast, and gynecologic origin with NE differentiation

Site	*N*	INSM1 (%)	OTP (%)	ASCL1 (%)	CHGA (%)	SYP (%)	CD56 (%)
Prostate	4	25.0	50.0	100.0	0.0	50.0	50.0
Vagina	1	0.0	0.0	100.0	0.0	0.0	0.0
Cervix	2	100.0	0.0	50.0	50.0	100.0	100.0
Endometrium	4	0.0	0.0	50.0	50.0	25.0	50.0
Ovary, neuroendocrine carcinoma	2	100.0	100.0	0.0	50.0	100.0	100.0
Ovary, immature teratoma	6	83.3	0.0	83.3	16.7	100.0	100.0
Breast	7	71.4	0.0	85.7	71.4	85.7	71.4
All cases	26	57.7	15.4	73.1	38.5	73.1	73.1

OTP, orthopedia homeobox; *INSM1*, insulinoma-associated protein 1; *ASCL1*, achaete-scute homolog 1; *CHGA*, chromogranin A; *SYP*, synaptophysin

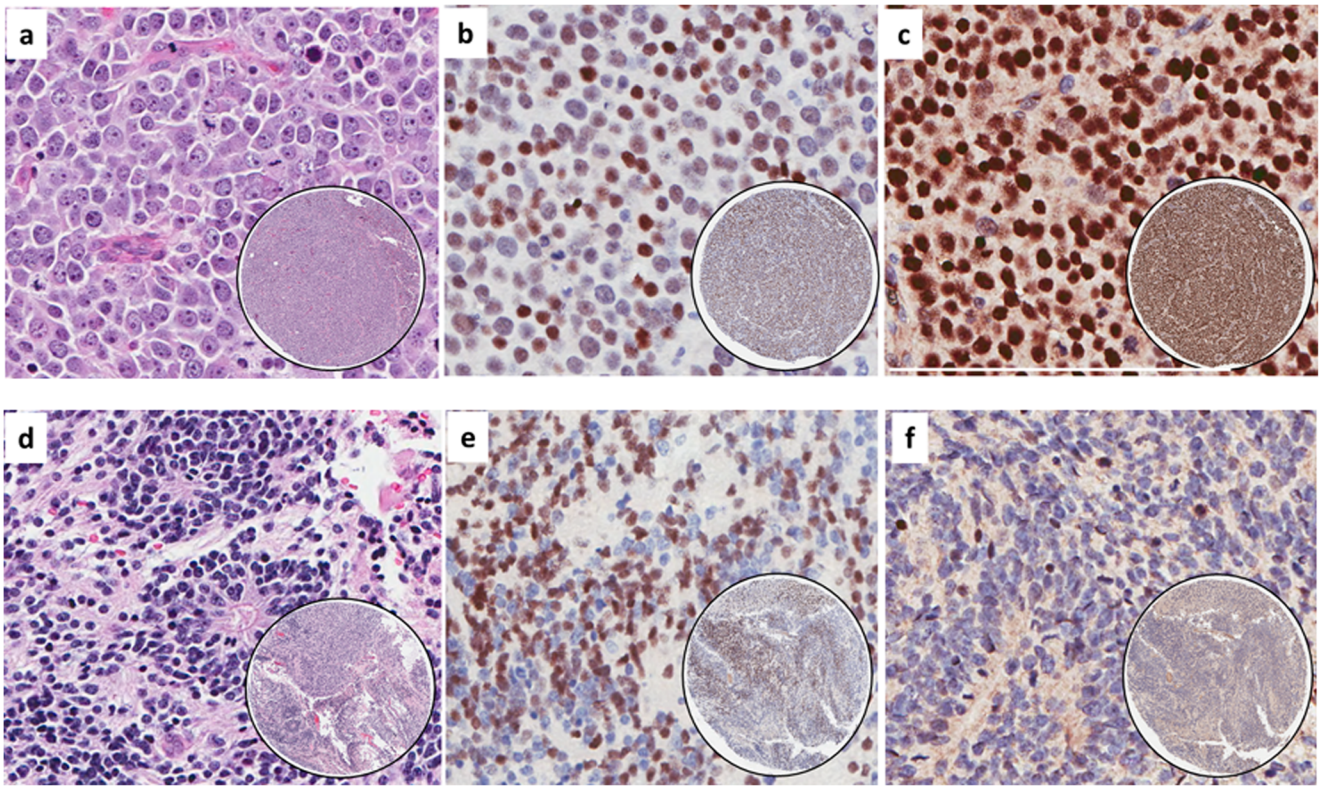

Fig. 1 Ovarian carcinoma with neuroendocrine differentiation (**a**–**c**) and immature teratoma (**d**–**f**) express INSM1 (**b** and **e**, respectively). OTP is expressed in ovarian carcinoma with neuroendocrine differentiation (**c**), but not in immature teratoma (**f**). Scale bar 200 μM

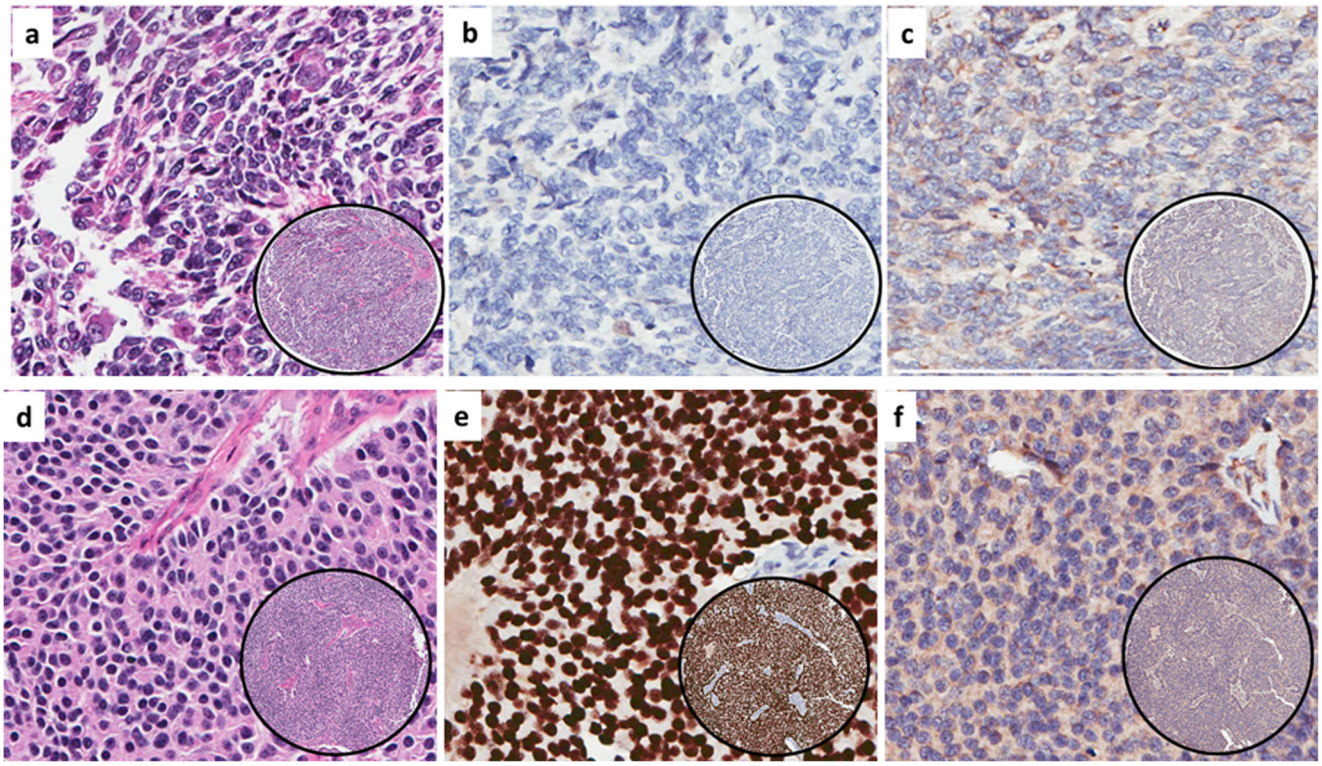

Fig. 2 Endometrial (**a**–**c**), and cervical (**d**–**f**) carcinoma with neuroendocrine differentiation. INSM1 (**b**) and OTP (**c**) are both negative in endometrial adenocarcinoma (**a**–**c**). Diffuse expression of INSM1 (**e**) is observed in cervical carcinoma (**d**–**f**) while OTP is negative (**f**). Scale bar 200 μM

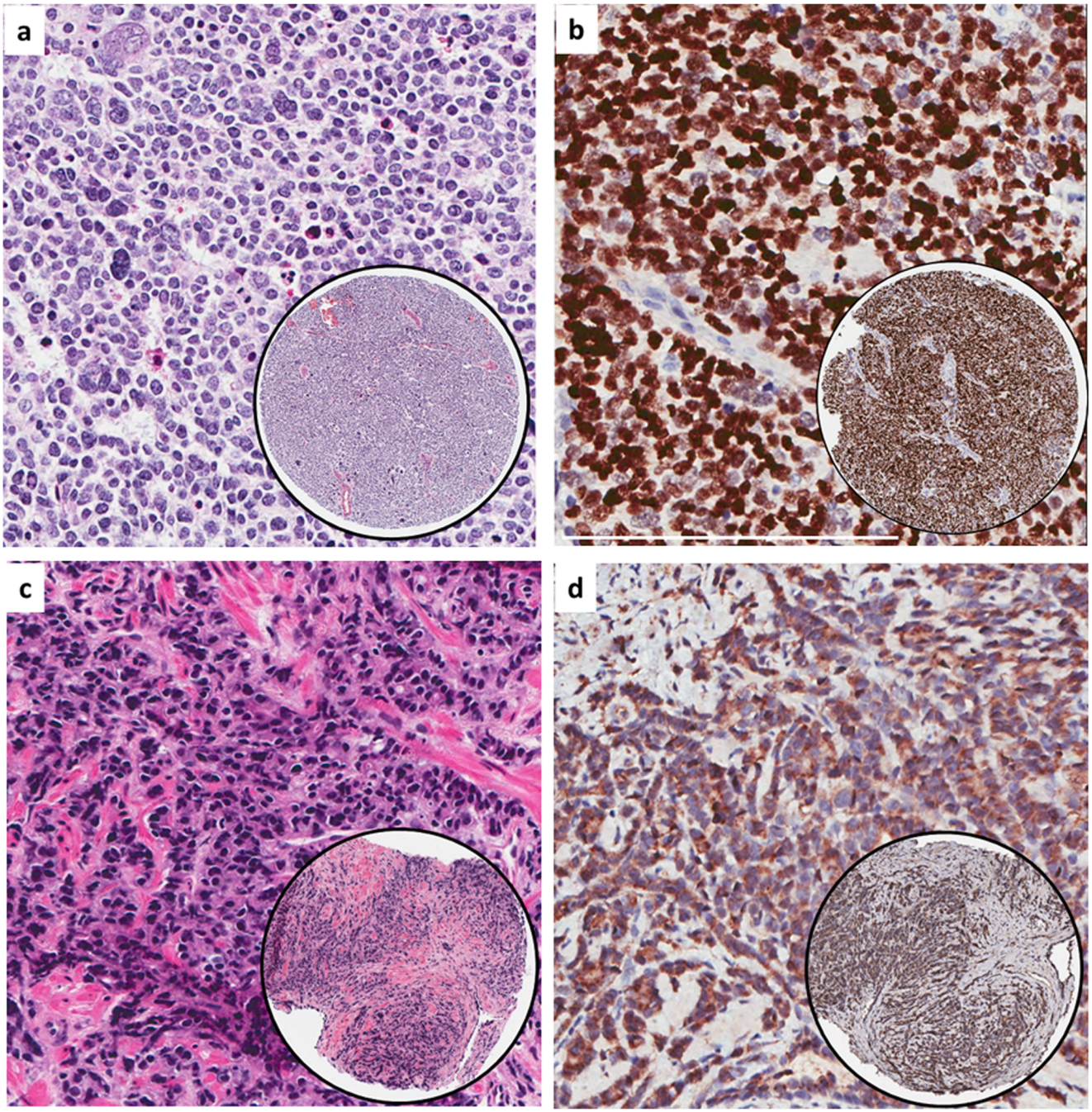

Fig. 3 Prostatic carcinoma with neuroendocrine differentiation. Diffuse expression of INSM1 (**b**) and corresponding H&E in (**a**) and OTP (**d**), corresponding H&E in (**c**). Scale bar 200 μM

cervix (2 of 2, Fig. 2d–e), INSM1 expression was not detected in cases of adenocarcinoma with NE differentiation arising in the endometrium (0/4, Fig. 2a–b) and vagina (0/1, not shown). Overall, INSM1 performed better compared to CHGA alone (57.1 vs 38.1%) but was less often positive when compared to ASCL1, SYP, or CD56 (57.1 vs 73.1%, Table 2).

In contrast to INSM1, diffuse nuclear expression of OTP was detected in only 15.4% cases, including 50% (2/4) of prostate adenocarcinoma with NE differentiation (Fig. 3c–d) and 100% in ovary (2/2, Fig. 1a, c). OTP expression was not detected in the remainder of the cases of gynecologic malignancy (Figs. 1d, f and 2a, c, d, f), and in invasive mammary carcinoma with NE differentiation (Fig. 4a, c). While the neuroendocrine carcinoma in the ovary (2/2) demonstrated diffuse and strong nuclear staining pattern, the cases of prostate carcinoma (2/4) demonstrated nuclear expression with a perinuclear accentuation.

Discussion

As the therapeutic options for cancer are evolving toward personalized, genome-based prognostication and treatment, understanding the fundamental molecular biology of a neoplasm is essential. This is particularly challenging for neuroendocrine neoplasms because traditional clinical classification and subclassification on NE tumors derives from the diverse sites of origin and clinical symptoms of NE tumors. Even in the era of progressive personalized medicine, surgery remains the mainstay of treatment in these patients. However, additional treatment with chemotherapy or targeted therapies may be needed for locally advanced, recurrent or metastatic disease.

We sought to investigate the utility of INSM1 and OTP as diagnostic markers in rare malignant tumors with NE differentiation. Most gynecologic tumors with histologic evidence of NE differentiation were positive for INSM1, whether originating in the cervix or the ovary (including immature teratomas, Figs. 1 and 2). INSM1 expression was not detected in cases of adenocarcinoma with NE differentiation arising in the endometrium (Fig. 2) and vagina (not shown). It is possible that in cases where NE differentiation is focal, a small section of tumor captured on a tissue microarray may not be the optimal strategy for assessment of INSM1 expression. In contrast, INSM1 was diffusely expressed in 71.4% of invasive mammary carcinoma (Fig. 4) and 25% of prostate adenocarcinoma with NE differentiation (Fig. 3). Our data suggest that INSM1 can be used as a part of a panel of NE markers to establish NE differentiation.

In contrast to INSM1, diffuse nuclear expression of OTP was detected in only 50% (2/4) of prostate adenocarcinoma with NE differentiation (Fig. 3a, b) and 100% (2/2) neuroendocrine carcinoma of the ovary (Fig. 1a, c). OTP expression was not detected in the remainder of the cases of gynecologic malignancy and in invasive mammary carcinoma with NE differentiation (overall 15.4%, Table 2), indicating that OTP is not a very sensitive broad spectrum NE marker.

Because of the relatively small number of cases analyzed from different groups, additional studies with more cases should be done to support these novel findings. Nevertheless, our findings suggest that INSM1 is a useful biomarker to recognize NE differentiation in tumors from the breast, prostate, or gynecologic organs. INSM1 has been used and shown to be a highly specific biomarker for NEN from the lung and from other sites [6, 26]. More recently, additional studies have found INSM1 to be a useful marker in small cell carcinoma of the prostate; however, according to this study, while INSM1 was positive in 92.3% of prostatic small cell carcinoma, 3.4% of benign prostatic tissue and 4.0% of prostate adenocarcinomas were INSM1 positive [27]. A study by Kuji et al. found that 95% of cervical small carcinoma express INSM1, which is in keeping with our findings [21]. Our current study extends these earlier findings to include invasive breast carcinoma with neuroendocrine features and provides evidence that majority of these cases express INSM1 (71.4%, Table 2, Fig. 4).

Primary neuroendocrine/neuroepithelial tumors in the ovary are rare. They may arise from the ovarian stroma and surface epithelium, but they may also arise from teratomas [28]. Identification of the immature neural elements is crucial for prognosis and management, and these elements have many histologic mimics; thus, an immunohistochemical marker to aid in this distinction would be very helpful. However, chromogranin is at best, a modestly sensitive marker for neuroendocrine/neuroepithelial differentiation in immature teratomas [29]. The current study showed superior sensitivity of INSM1 immunohistochemistry for the detection of immature neuroepithelial component in these tumors, with 83.3% of cases being positive. In contrast, only 16.7% of the immature teratomas were positive for chromogranin A. These findings indicate that INSM1 can be used as a sensitive neuroendocrine/neuroepithelial marker for immature teratomas and further support our original findings reported previously [6]. Of note, our study also found expression of ASCL1 in most of the cases neuroendocrine differentiation. These

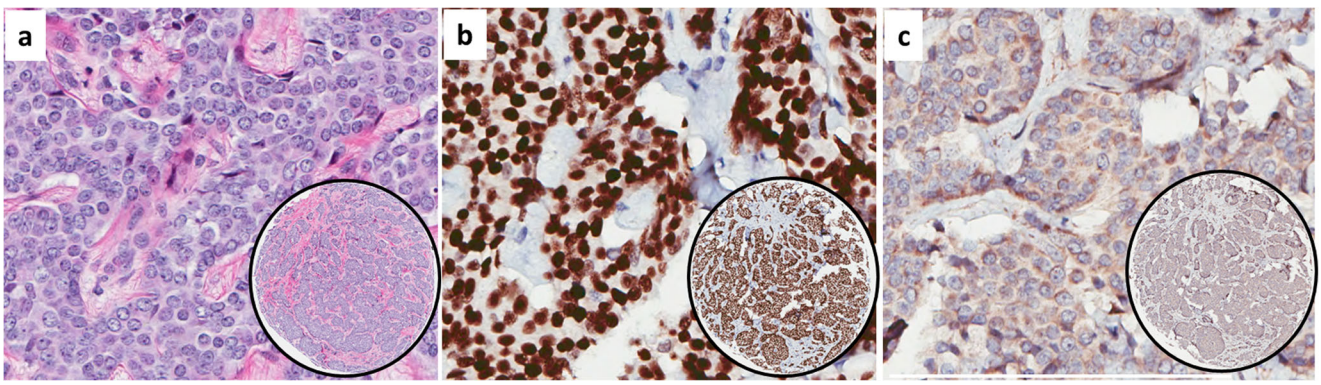

Fig. 4 Breast carcinoma with neuroendocrine differentiation. Diffuse expression of INSM1 is observed (**b**) and corresponding H&E in (**a**), while OTP (**c**) is negative. Scale bar 200 μM

findings agree with earlier studies on ASCL1 expression in neuroendocrine carcinoma [30] and on recent studies indicating cross talk of ASCL1 and INMS1 in the sonic hedgehog signaling pathway [31].

The finding of OTP expression in lung carcinoids suggests that this biomarker may be useful in the diagnosis of low-grade NE tumor. Our observation of OTP expression in non-pulmonary carcinomas with NE differentiation such as in the prostate or ovary, indicates that OTP is more widely distributed than previously recognized.

From the perspective of currently available treatments for NE tumors, which is primarily surgery, the classification and subclassification of NE tumors by site is important. Some of the differences between different classifications are certainly biological and have varied clinical implications and behavior. While a well-differentiated NEN typically confers a good prognosis, outcome is dramatically dismal with entities such as small cell carcinoma, or carcinoma with high-grade neuroendocrine differentiation [12, 18, 20]. The focus solely on subdividing NE tumors likely based on their underlying biological and clinical similarities may impair the development of novel, targeted treatment strategies. Despite obvious morphologic similarities, the existing histology-based grading of gastrointestinal and pulmonary well differentiated NE tumors is vastly different, and the application of different criteria to histologically similar lesions results in different treatment approaches. This poses a technical challenge as the histologic grading is especially problematic and subject to considerable interobserver variation. Moreover, even with existing grading schemes, prediction of clinical behavior of NE tumors can be difficult. Therefore, it would be ideal to categorize these tumors not only based on their origin and clinical and biological behavior but also to take into account their underlying molecular signature to allow for novel-targeted therapy. By studying common histologic and molecular features across a broad spectrum of NE tumors, it may help to improve existing histologic grading schemes in this tumor type. A number of different immunohistochemical markers may even find improved diagnostic or prognostic utility when evaluated in such a manner.

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