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Molecular Classification of Extrapulmonary Neuroendocrine Carcinomas With Emphasis on POU2F3-positive Tuft Cell Carcinoma

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Abstract: Extrapulmonary neuroendocrine carcinomas (EP-NECs) are associated with a poor clinical outcome, and limited information is available on the biology and treatment of EP-NECs. We studied EP-NECs by applying the recent novel findings from studies of pulmonary neuroendocrine carcinomas, including POU2F3, the master regulator of tuft cell variant of small cell lung carcinomas. A cohort of 190 patients with surgically resected EP-NECs or poorly differentiated carcinomas (PDCs) were established. Immunohistochemistry (IHC) for POU2F3 along with ASCL1, NEUROD1, YAP1, and conventional neuroendocrine markers was performed on tissue microarrays. Selected cases with or without POU2F3 expression were subjected to targeted gene expression profiling using nCounter PanCancer Pathway panel. POU2F3-positive tuft cell carcinomas were present in 12.6% of EP-NEC/PDCs, with variable proportions according to organ systems. POU2F3 expression was negatively correlated with the expression levels of ASCL1, NEUROD1, and conventional neuroendocrine markers ($P < 0.001$), enabling IHC-based molecular classification into ASCL1-dominant, NEUROD1-dominant, POU2F3-dominant, YAP1-dominant, and not otherwise specified subtypes. Compared wih POU2F3-negative cases, POU2F3-positive tuft cell carcinomas showed markedly higher expression levels of *PLCG2* and *BCL2*, which was also validated in the entire cohort by IHC. In addition to POU2F3, YAP1-positive tumors were a distinct subtype among EP-NEC/PDCs, characterized by unique T-cell inflamed microenvironment. We found rare extrapulmonary POU2F3-positive tumors arising from previously unappreciated cells of origin. Our data show novel molecular pathologic features of EP-NEC/PDCs including potential therapeutic vulnerabilities, thereby emphasizing the need for focusing on unique features of EP-NEC/PDCs.

Key Words: neuroendocrine carcinoma, poorly differentiated carcinoma, POU2F3, PLCG2, BCL2

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Neuroendocrine carcinoma (NEC) is a rare malignancy with poor prognosis.[1] Subtypes of NEC include small cell NEC and large cell NEC. Although most NECs arise from the lower respiratory tract, extrapulmonary neuroendocrine carcinomas (EP-NECs) account for about 2% to 9% of all NECs.[2–5] EP-NECs can originate from any organ system, including gastrointestinal tract, hepatopancreatobiliary (HBP) tract, genitourinary (GU) system, gynecologic tract (GYN) and head and neck (HN), or unknown primary site.[6–10]

EP-NECs resemble their pulmonary counterparts and are diagnosed on the basis of morphologic features and immunohistochemistry (IHC) for neuroendocrine (NE) markers. Due to the rarity of EP-NECs, molecular pathologic data on these cancers are extremely limited, and no specific therapeutic strategies have been developed for EP-NECs. Currently, platinum-based chemotherapy is empirically used for the treatment of EP-NECs[7,11] based on the similarity between small cell lung carcinoma

(SCLC) and EP-NECs. It is crucial to understand the biology of EP-NECs to develop novel treatment strategies.

Recent studies on SCLC have reported that a subset show low expression of NE-related genes including *ASCL1* and *NEUROD1*.[12] Huang et al identified certain SCLC cell lines that strongly expressed and showed powerful dependency on POU2F3,[13] which is a master transcription factor (TF) involved in the differentiation of tuft cells. The so-called tuft cell-like variant of SCLC retains the characteristic morphology of conventional SCLC but lacks the expression of conventional NE markers on IHC.

Tuft cells are specialized epithelial chemosensory cells found in various organ systems, including tongue taste buds, intestinal epithelium, skin, pancreas, and bronchial epithelium.[14–16] The wide distribution of tuft cells suggests that tuft cell carcinomas may arise from organs other than the lungs. In line with this, occasional subsets of poorly differentiated carcinomas (PDCs) in extrapulmonary sites show classic NE morphology but negative stains for NE markers,[3,17,18] indicating a potential role of POU2F3 in this subset.

In the present study, we identified POU2F3-positive tuft cell carcinomas among patients with EP-NEC/PDCs and compared the clinicopathologic features and gene expression profiles between POU2F3-positive and POU2F3-negative EP-NEC/PDCs.

MATERIALS AND METHODS

Study Population

We retrospectively reviewed the pathology database of Seoul National University Hospital (SNUH) for patients histopathologically diagnosed with EP-NEC or PDC during a period of 20 years (from 2000 to 2019). Formalin-fixed, paraffin-embedded (FFPE) tissue from an excisional biopsy or surgical resection samples were collected. Hematoxylin and eosin (H&E)-stained slides were reviewed. We included PDC cases with NE features on H&E-stainings, regardless of classic NE marker expression. Cases with overt squamous differentiation on H&E or IHC examination, and NUT carcinoma, a specific entity with poorly differentiated morphology, were excluded. We selected representative 2 mm cores from the FFPE blocks and constructed tissue microarrays.

Clinical data were collected from medical records, including sex, age, primary organ involved, clinical stage, pathologic diagnosis, and overall survival (OS). OS was defined as the intervals between initial diagnosis and date of death. This study was approved by the institutional review board (IRB) of SNUH (IRB number: H-1905-046-1031), and the written patient consent process was waived.

Immunohistochemistry

Using tissue microarray blocks, IHC was performed for POU2F3, ASCL1, NEUROD1, YAP1, CD56, synaptophysin, chromogranin A, INSM1, TTF-1, p16, p63, p53, NUT, BCL2, PLCG2, c-MYC, CD3, CD20, CD8, PD-1, and PD-L1. Supplementary Table S1 (Supplemental Digital Content 1, http://links.lww.com/PAS/B432)

presents detailed information regarding the antibodies used for IHC.

A histoscore (H-score) was calculated for each case based on the expression level of markers, except for TTF-1, p16, p63, NUT, CD3, CD20, CD8, PD-1, and PD-L1, by multiplying staining intensity with percent of positive cells. We used H-score 50 as the cutoff value defining low group (H-score <50) and high group (H-score ≥ 50) for categorization of individual marker expression.[19] Combined NE score, defined as the average H-score of CD56, synaptophysin, chromogranin A, and INSM1[19] was used to categorize cases into low (NE score <150) and high (NE score ≥ 150) NE score groups.

We assessed the expression level of PD-L1 using Combined Proportion Score.[20] CD3, CD20, CD8, and PD-1 immunostains were digitally scanned using Aperio AT2 (Leica Biosystems). Positive cells in representative tumor areas were quantified using Nuclear V9 algorithm of ImageScope (Aperio Technologies), and the number of positive cells per analyzed area was used for further analyses.

Gene Expression Profiling

A total of 12 cases were selected for gene expression profiling (GEP), of which 6 were POU2F3-positive, and 6 were POU2F3-negative. The whole FFPE block of each case were used to prepare five 10 μm sections. Areas rich in tumor cells were identified by H&E staining and macrodissected. The total RNA from each sample was extracted and quantified using DS 11 spectrophotometer (DeNovix Inc.). In all, 1200 ng total RNA was used for hybridization and sample preparation using nCounter Prep Station (Nanostring Technology Inc.). GEP was based on the nCounter PanCancer Pathway panel encompassing 770 genes and was digitally assessed using nCounter Digital Analyzer (Nanostring Technology Inc.).

Raw expression data were normalized against the expression levels of reference genes, and the differentially expressed genes (DEGs) between POU2F3-positive and POU2F3-negative groups were analyzed using nSolver Analysis Software version 4.0 (NanoString Technologies Inc.). DEGs were defined as genes with a $\log_2 FC > \pm 2.0$ and adjusted *P* value <0.05. Gene set enrichment analysis[21] was performed using gene set permutation mode, and gene sets from MSigDB (http://software.broadinstitute.org/gsea/msigdb),[22] Reactome,[23] and BioCarta.[24] Gene sets with false discovery rate *q*-values of ≤ 0.25 were considered significantly enriched.

Statistical Analysis

We used the χ^2, and linear-by-linear, and Fisher exact tests to compare categorical variables, and the Mann-Whitney, Kruskal-Wallis, and Tukey multiple comparison tests to compare continuous variables, as appropriate. The Pearson correlation test was performed, and Kaplan-Meier survival analyses were performed using the log-rank test. Statistical significance was defined as *P* value <0.05. The analyses were performed using SPSS software (version 25; IBM Corp.) and the R statistical package (version 3.6.0; R Foundation for Statistical Computing; http://www.r-project.org).

RESULTS

Clinicopathologic Characteristics

In all, 190 EP-NEC/PDC patients were included in the study (Table 1). The study patients had a median age of 61 years (18–92), and 50.5% were males. EP-NEC/PDCs originated from diverse primary sites, including GYN (33.2%), HBP (27.4%), GU tract (17.9%), HN (11.1%), and skin (5.3%). A small number of cases originated from thymus, breasts, and adrenal glands, whereas 1 patient had cancer of unknown primary.

Initial histopathologic diagnoses were reviewed from the pathology reports, where major diagnostic classes included mixed adenocarcinoma and NEC (MANEC; 23.7%), small cell NEC (17.9%), undifferentiated carcinoma (12.6%), large cell NEC (7.4%), and PDC (6.8%). Some organ-specific diagnoses were noted; 8 of 10 skin cancers were Merkel cell carcinomas, and 1 patient had mesonephric carcinoma arising from the uterus. TTF-1 and p16 positivity were observed in 16.7% (31/186) and 59.5% (113/190) of cases, respectively; 61.3% (111/180) cases showed null or overexpression of p53 (Supplementary Table S2, Supplemental Digital Content 2, http://links.lww.com/PAS/B433; Supplementary Fig. S1, Supplemental Digital Content 3, http://links.lww.com/PAS/B434).

In addition, 38.9% (74/190) of EP-NEC/PDC patients presented with distant metastases. Various treatment modalities were administered, including curative or palliative operation (85.9%), and were followed by adjuvant chemotherapy, radiation therapy, or both in some patients. Among the 190 patients, death was documented in 141 (74.2%), and the median survival was 30.0 months (range, 0.5 to 217.3 months).

POU2F3-positive Tuft Cell Carcinomas Originated From Various Organ Systems

POU2F3 expression was found in 12.6% (24/190) of EP-NEC/PDCs. The proportions of POU2F3-positive tuft cell carcinomas varied widely according to organ system (Table 1, Fig. 1A); 20.6% (7/34) of EP-NEC/PDCs originating from GU tract were POU2F3-positive, followed by 19.0% (4/21) from HN, 14.3% (9/63) from GYN, and 7.7% (4/52) from HBP. More specifically, POU2F3-positive tuft cell carcinomas in the GU tract originated from the urinary bladder (4/7), renal pelvis and ureter (2/7), and prostate (1/7). HN origin tuft cell carcinomas also included rarer sites such as paranasal sinus, larynx, and parotid glands. In the present study, none of the cancers originating from the skin, thymus, adrenal glands, breasts, or unknown primary were POU2F3-positive. POU2F3-positive tuft cell carcinoma more frequently originated from the GU tract and HN (11/55) compared with the other sites combined (13/135), with marginal statistical significance of difference ($P = 0.051$; χ^2). There were no significant differences in terms of patient age, sex, initial metastasis, and expression of TTF-1, p63, p16, and p53 according to POU2F3 expression.

Taken together, these results confirm that POU2F3-positive tuft cell carcinomas can arise from organs other than the pulmonary system, and that certain organs are somehow more likely to harbor POU2F3-positive tumors.

TABLE 1. Clinicopathological Characteristics of Study Population

	POU2F3 expression, n (%)			
	Positive	**Negative**	**Total**	***P***
Age, median (range)	60 (37–88)	61 (18–92)	61 (18–92)	0.976
Sex				0.956
Male	12 (12.5)	84 (87.5)	96 (50.5)	
Female	12 (12.8)	82 (87.2)	94 (49.5)	
Primary organ				0.051*
GU	7 (20.6)	27 (79.4)	34 (17.9)	
HN	4 (19.0)	17 (81.0)	21 (11.1)	
GYN	9 (14.3)	54 (85.7)	63 (33.2)	
HBP	4 (7.7)	48 (92.3)	52 (27.4)	
Skin	0 (0.0)	10 (100.0)	10 (5.3)	
Breast	0 (0.0)	2 (100.0)	2 (1.1)	
Thymus	0 (0.0)	5 (100.0)	5 (2.6)	
Adrenal	0 (0.0)	2 (100.0)	2 (1.1)	
MUO	0 (0.0)	1 (100.0)	1 (0.5)	
Pathologic diagnosis				0.086
SCNEC	8 (23.5)	26 (76.5)	34 (17.9)	
LCNEC	3 (21.4)	11 (78.6)	14 (7.4)	
NEC, type unspecified	1 (8.3)	11 (91.7)	12 (6.3)	
MANEC	4 (8.9)	41 (91.1)	45 (23.7)	
ADC	1 (12.5)	7 (87.5)	8 (4.2)	
PDC with NE features	2 (33.3)	4 (66.7)	6 (3.2)	
UCC with NE features	2 (28.6)	5 (71.4)	7 (3.7)	
SQCC with NE features	1 (11.1)	8 (88.9)	9 (4.7)	
Basaloid/PD SQCC	0 (0.0)	3 (100.0)	3 (1.6)	
PDC	1 (7.7)	12 (92.3)	13 (6.8)	
UDC	1 (4.2)	23 (95.8)	24 (12.6)	
Carcinosarcoma	0 (0.0)	6 (100.0)	6 (3.2)	
Meckel	0 (0.0)	8 (100.0)	8 (4.2)	
Mesonephric carcinoma	0 (0.0)	1 (100.0)	1 (0.5)	
Distant metastasis				0.293
Absent	17 (70.8)	99 (59.6)	116 (61.1)	
Present	7 (29.2)	67 (40.4)	74 (38.9)	
Treatment				0.122
OP/RT/CT	8 (14.3)	48 (85.7)	56 (29.5)	
OP/RT	3 (20.0)	12 (80.0)	15 (7.9)	
OP/CT	9 (13.4)	58 (86.6)	67 (35.3)	
RT/CT	1 (16.7)	5 (83.3)	6 (3.2)	
OP	3 (12.0)	22 (88.0)	25 (13.2)	
CT	0 (0.0)	10 (100.0)	10 (5.3)	
RT	0 (0.0)	3 (100.0)	3 (1.6)	
BSC	0 (0.0)	5 (100.0)	5 (2.6)	
NA	0 (0.0)	3 (100.0)	3 (1.6)	
Death				0.925
Dead	18 (75.0)	123 (74.1)	141 (74.2)	
Alive	6 (25.0)	43 (25.9)	49 (25.8)	
Total	24 (12.6)	166 (87.4)	190 (100.0)	

*Compared between GU/HN group versus others, by χ^2 test.

ADC indicates adenocarcinoma; BSC, best supportive care; CT, chemotherapy; LCNEC, large cell neuroendocrine carcinoma; MANEC, mixed adenocarcinoma and neuroendocrine carcinoma; MUO, metastasis of unknown origin; NA, not applicable; OP, operation; PD, poorly differentiated; RT, radiation therapy; SCNEC, small cell neuroendocrine carcinoma; SQCC, squamous cell carcinoma; UCC, urothelial carcinoma; UDC, undifferentiated carcinoma.

POU2F3 Expression Is Associated With Low NE Score

Because POU2F3-positive SCLC had low expression levels of NE markers, we compared POU2F3 expression and NE score in EP-NEC/PDCs. We also compared other TFs introduced in the molecular classification of SCLC: ASCL1, NEUROD1, and YAP1. Expression levels of
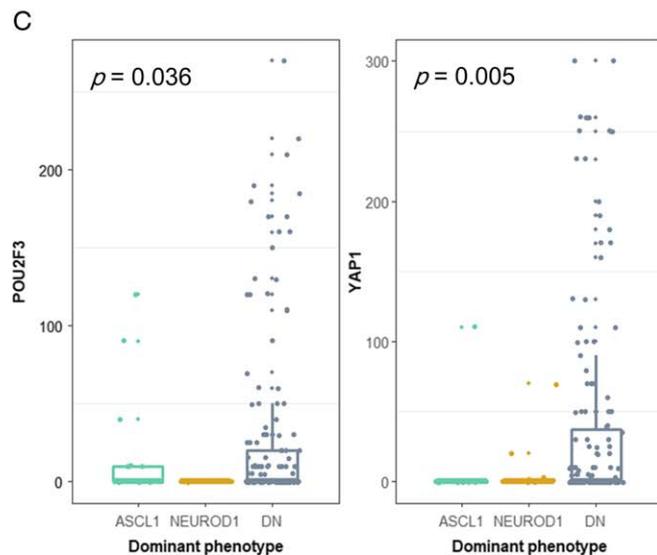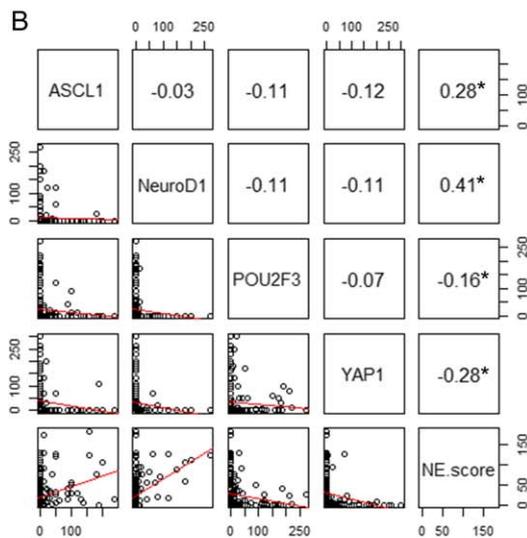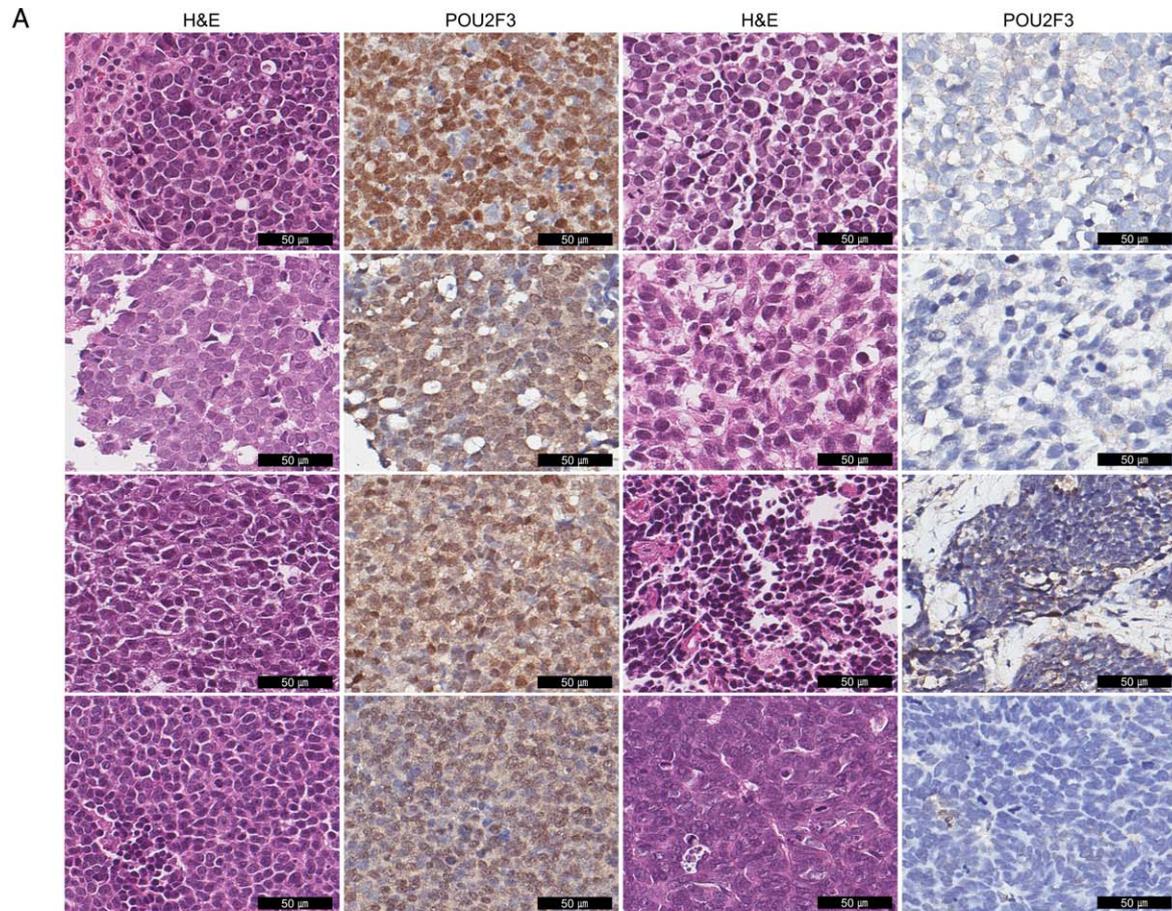
FIGURE 1. Expression of POU2F3 and its association with various TFs. A, POU2F3 expression levels varied in EP-NECs and PDCs with NE features. Representative cases from each organ system are shown: GU tract (first row), HN (second row), nasal cavity (second row, right), GYN (third row, right and left), and biliary tract (fourth row) cancers. B, The expression levels of ASCL1 and NEUROD1 were positively correlated with combined NE score (Pearson $R = 0.28$ and 0.41), while expression levels of POU2F3 and YAP1 were inversely correlated with combined NE score (Pearson $R = -0.16$ and -0.28). Asterisks indicate $P < 0.05$. C, Tumors with low ASCL1 and NEUROD1 expression levels were classified as DNs. POU2F3-positive and YAP1-positive cases had increased proportions in the DN group ($P = 0.036$ and 0.005).

POU2F3 and YAP1 were negatively correlated with NE score (Pearson $R = -0.16$ and -0.28; $P < 0.05$ for both), while ASCL1 and NEUROD1 expression levels were positively correlated with NE score (Pearson $R = 0.28$ and 0.41, respectively; $P < 0.05$ for both) (Fig. 1B).

Using the H-scores for ASCL1 and NEUROD1, we classified EP-NEC/PDCs into 3 groups: ASCL1-dominant (ASCL1 H-score $\geq$ 50), NEUROD1-dominant (NEUROD1 H-score $\geq$ 50), and double negative (DN; both ASCL1 and NEUROD1 H-scores <50). We found POU2F3 expression was significantly increased in DN group ($P = 0.036$ by Kruskal-Wallis test; Fig. 1C), and higher YAP1 H-scores were associated with the DN phenotype of EP-NEC/PDCs ($P = 0.005$ by Kruskal-Wallis test; Fig. 1C). These results confirm the previous findings from SCLCs that POU2F3 is expressed in variant SCLC tumors that have low NE marker expression.[13]

The unique expression pattern of YAP1 suggests the existence of a YAP1-positive subtype among EP-NEC/PDCs, although the existence of YAP1-positive tumors among SCLCs still remain controversial.[19,25,26] Moreover, in line with a previous report,[27] c-MYC expression by IHC showed a positive association with NEUROD1 expression (Pearson $R = 0.16$; $P = 0.034$), and the H-scores of c-MYC in NEUROD1-dominant were significantly higher than ASCL1-dominant or DN group ($P = 0.008$ by Kruskal-Wallis test; Supplementary Fig. S2, Supplemental Digital Content 4, http://links.lww.com/PAS/B435).

Immunohistochemically Defined Molecular Subtypes of EP-NEC/PDC

Based on the aforementioned findings, we applied the molecular classification strategy for SCLCs to our EP-NEC/PDC cohort.[19,28] The DN subtype were further divided into 3 categories: POU2F3-dominant (POU2F3 H-score $\geq$ 50), YAP1-dominant (YAP1 H-score $\geq$ 50), and not otherwise specified (NOS). The proportions of each molecular subtype were as follows: ASCL1-dominant, 10.5%; NEUROD1-dominant, 5.8%; POU2F3-dominant, 11.6%; YAP1-dominant, 14.7%; NOS, 57.4% (Fig. 2A). Figure 2B and Supplementary Figure S3 (Supplemental Digital Content 5, http://links.lww.com/PAS/B436) presents representative cases along with expression of TFs and conventional NE markers.

The distribution of molecular subtypes varied according to tumor site (Fig. 2C). GU cancers had a higher proportion of ASCL1-dominant subtype (23.5%) compared with other sites, while none of the cases originating from the HN region were ASCL1-dominant. Among HN cancers, the proportion of YAP1-dominant subtype (33.3%) was only second to that of NOS subtype (42.9%). The 10 EP-NEC/PDCs arising from the skin were not classified into any TF-dominant subgroups.

There were no differences in terms of OS between to the molecular subtypes ($P = 0.830$; Fig. 2D). We performed survival analyses using the expression levels of each TF, where no significant survival differences were observed (Supplementary Fig. S4, Supplemental Digital Content 6, http://links.lww.com/PAS/B437), unlike a previous report on SCLC.[28] Of note, higher expression of c-MYC was associated with shorter OS in the total population and ASCL1-dominant subtype ($P = 0.001$ and 0.019, respectively; Supplementary Fig. S4, Supplemental Digital Content 6, http://links.lww.com/PAS/B437).

Key Oncogenic Pathways in POU2F3-positive Tuft Cell Carcinomas

POU2F3 SCLC showed distinct biological features, including dependence on the IGF1R pathway.[13] To assess whether certain biomarkers of oncogenic pathways are enriched in POU2F3-positive EP-NEC/PDCs, we performed GEP in 12 selected cases (6 POU2F3-positive and 6 POU2F3-negative EP-NEC/PDCs). Supplementary Table S3 (Supplemental Digital Content 7, http://links.lww.com/PAS/B438) presents detailed characteristics of 12 patients who underwent GEP.

DEG analysis revealed that *PIK3R5*, *MYB*, *PLCG2*, *BCL2*, and *PAX5* were significantly upregulated in POU2F3-positive tuft cell carcinomas (Fig. 3A; Supplementary Table S4, Supplemental Digital Content 8, http://links.lww.com/PAS/B439). In addition, gene set enrichment analysis revealed significant enrichment of cellular pathways involved in the immune response, phosphatidylinositol metabolism, complement signaling, and STAT signaling (Fig. 3B).

PLCG2 and BCL2 Are Highly Expressed in POU2F3-positive Tuft Cell Carcinomas

We focused on the high expression levels of *PLCG2* and *BCL2* in POU2F3-positive tuft cell carcinomas and performed immunostainings for each marker in the entire study population. We confirmed the tumor cell expression of PLCG2 and BCL2 (Fig. 3C). The H-scores of PLCG2 and BCL2 were significantly higher in POU2F3-positive cases compared with POU2F3-negative cases ($P < 0.001$ and 0.003, respectively; Fig. 3D). The expression levels of PLCG2 and BCL2 were compared among the 5 molecular subtypes of EP-NEC/PDCs, and BCL2 expression was significantly higher in POU2F3-dominant subtype than NOS subtype ($P = 0.049$ by Tukey multiple comparison test; Fig. 3E). The PLCG2 H-score was significantly higher in POU2F3-dominant subtype than YAP1-dominant subtype; however, no significant differences were observed compared with other TF-dominant subtypes, possibly owing to the small numbers of cases in each class.

PLCG2 Expression as a Prognostic Factor in POU2F3-positive Tuft Cell Carcinomas

Based on a previous report suggesting worse prognosis of SCLCs with PLCG2-high phenotype,[26] we sought to explore the clinical implications of PLCG2 expression in EP-NEC/PDCs. Considering the low positivity rate of PLCG2 in EP-NEC/PDCs compared with other NE-related TFs, we used a cutoff H-score of 10 for distinguishing between PLCG2-high and PLCG2-low cases. There was no significant differences in the OS between PLCG2-high and PLCG2-low cases, in the entire study population ($P = 0.191$; Fig. 4). Interestingly, among the TF-based molecular subgroups, high PLCG2 expression
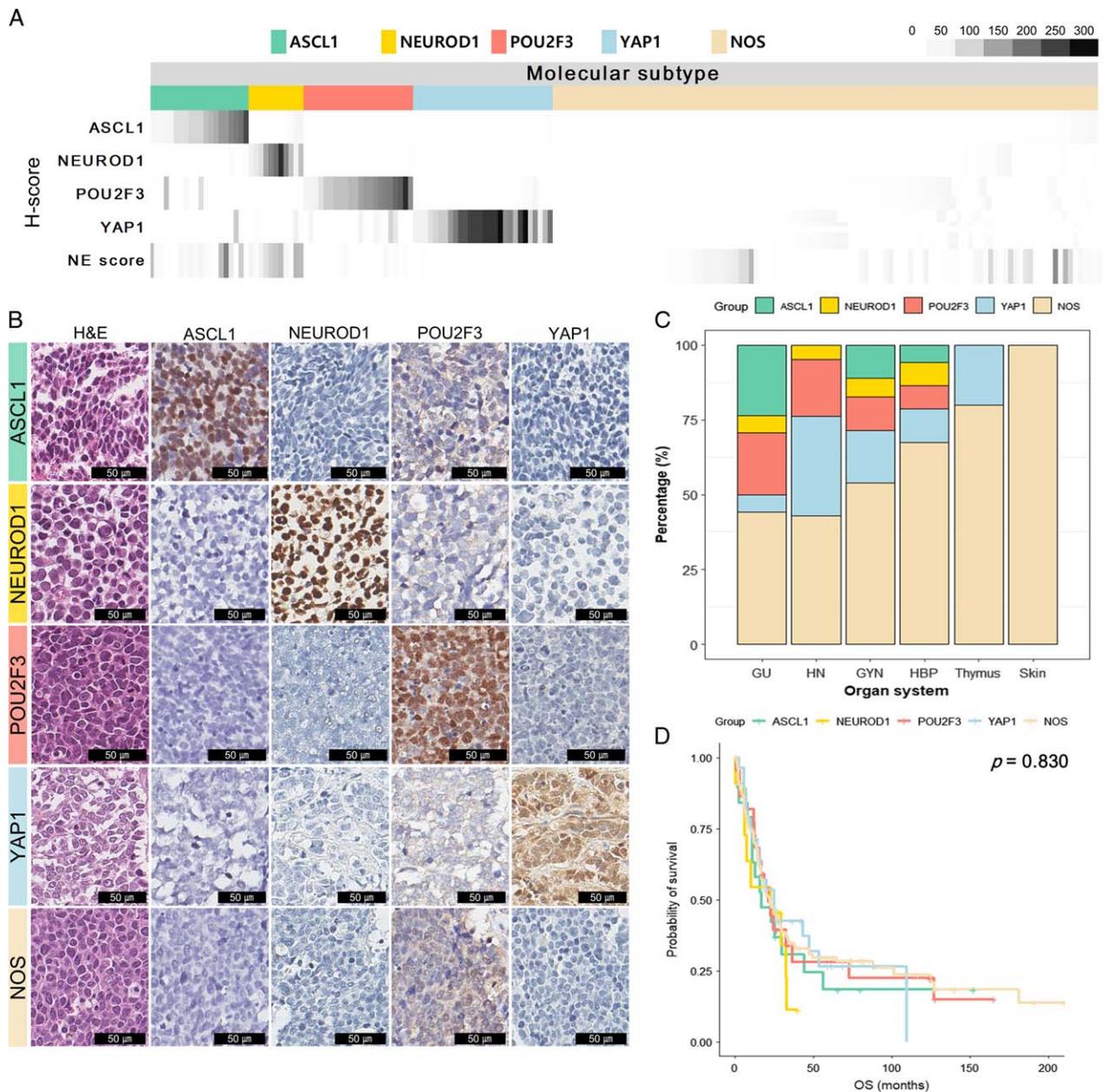
FIGURE 2. IHC-based molecular classification of EP-NECs and PDCs with NE features. A and B, Five classes were defined based on the expression of 4 TFs. C, The proportions of each subtype varied according to the primary organ system. D, No significant differences were observed in OS according to the subtypes.

was significantly associated with reduced OS only in the POU2F3-dominant subgroup ($P = 0.008$; Fig. 4). Taken together, these data indicate that PLCG2 expression is significantly increased in POU2F3-positive tuft cell carcinomas and suggests a poor prognosis.

Profiles of Tumor-infiltrating Lymphocytes in EP-NEC/PDC

We then focused on the *PAX5* upregulation in POU2F3-positive tuft cell carcinomas. Because PAX5 is involved in immune responses and B-cell activation,[29,30]

we hypothesized that the immune microenvironment would differ according to POU2F3 expression. To test this hypothesis, we performed IHC for CD3, CD20, PAX5, CD8, PD-1, and PD-L1 in the EP-NEC/PDC cohort, and found no differences in the CD20-positive B-cell distribution according to POU2F3 expression level (Fig. 5A).

Regarding T cells, CD3-positive tumor-infiltrating lymphocytes (TILs) were more densely present in POU2F3-negative cancers compared with POU2F3-positive cancers ($P = 0.019$; Fig. 5A). Conversely, a unique immune microenvironment was noted in YAP1-positive EP-NEC/PDCs.
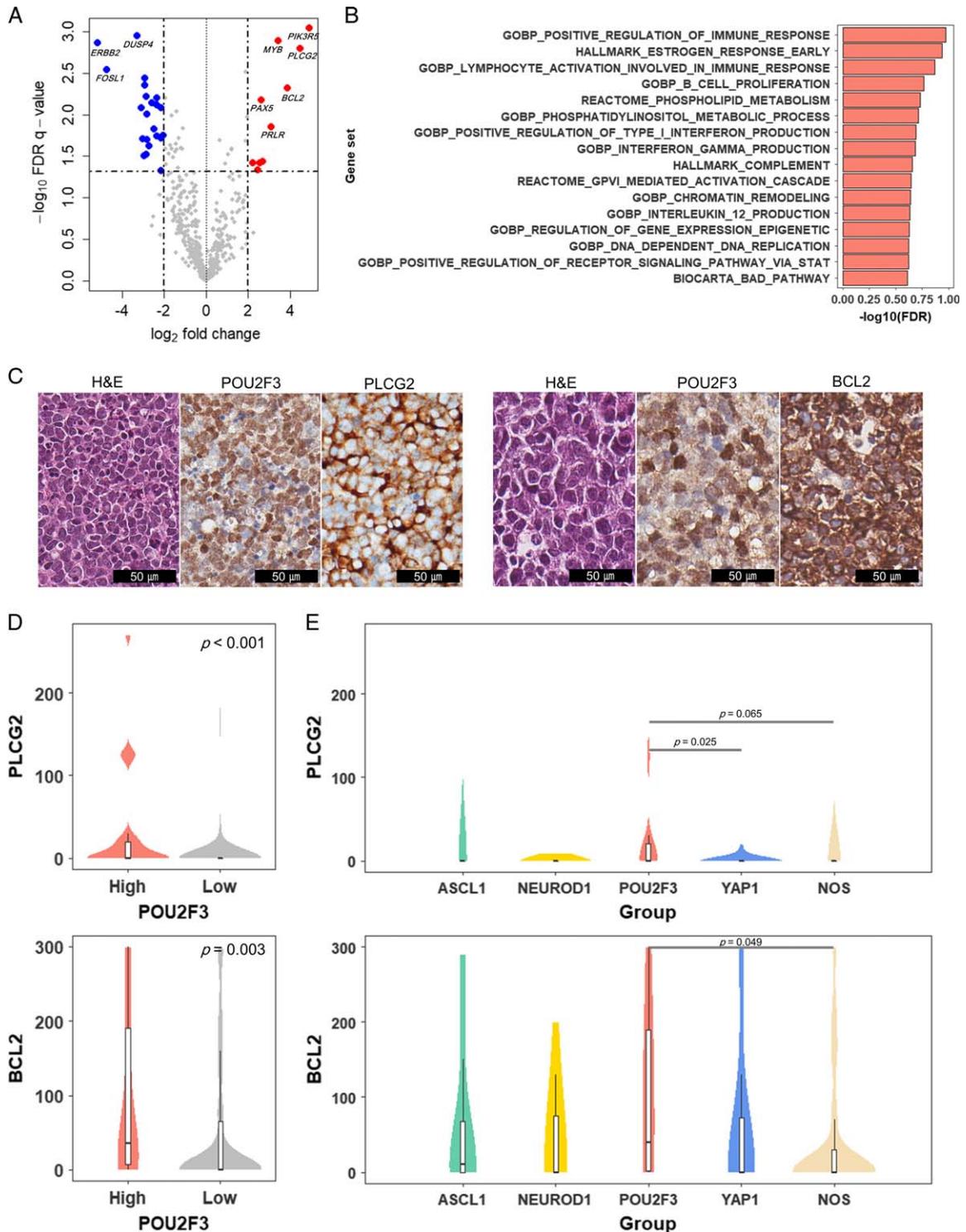
FIGURE 3. Gene expression profiles of POU2F3-positive tuft cell carcinomas and high expression levels of PLCG2 and BCL2. A, DEGs according to POU2F3 expression are depicted. Notable upregulation of *PIK3R5*, *PLCG2*, *MYB*, *BCL2*, and *PAX5* was observed. B, Gene sets representing immune response were enriched in POU2F3-positive tuft cell carcinomas. C and D, Significant over-expression of PLCG2 and BCL2 among POU2F3-positive tuft cell carcinoma cells was validated by IHC. E, Among the 5 subtypes, slight increases in PLCG2 and BCL2 expression levels were noted in the POU2F3-dominant subtype. FDR indicates false discovery rate.
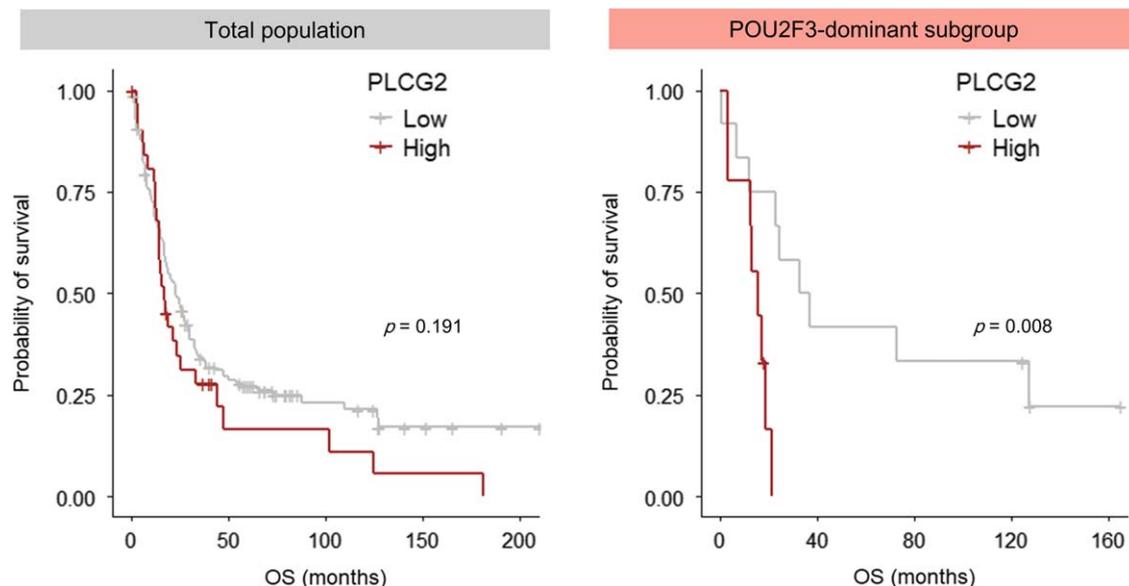
FIGURE 4. Survival analyses according to PLCG2 expression. PLCG2 expression was significantly associated with poor prognosis in the POU2F3-dominant subgroup ($P = 0.008$).

The patients had T-cell inflamed microenvironment, evidenced by significantly increased infiltration of CD3-positive and CD8-positive T cells ($P = 0.006$ and 0.029; Fig. 5B, Supplementary Fig. S5, Supplemental Digital Content 9, http://links.lww.com/PAS/B440). The TIL profiles were compared among 5 IHC-defined subtypes, which again showed the higher CD3-positive and CD8-positive T cells in YAP1-dominant subtype compared with the POU2F3-dominant subtype ($P = 0.012$ and 0.069, respectively; Fig. 5C). There was nonsignificant PD-L1 expression in EP-NEC/PDCs, and only 14 of 190 cases (7.4%) had PD-L1 Combined Proportion Score of > 1, without any predilection toward any particular TF expression or subtype.

DISCUSSION

In this study, we established a large cohort of EP-NEC/PDCs and identified the presence of POU2F3-positive tuft cell carcinomas arising from extrapulmonary sites. In addition, we investigated the clinicopathologic features as well as oncogenic pathways and microenvironmental factors of these carcinomas.

To date, POU2F3-positive tuft cell carcinomas have only been reported in about 15% of SCLCs,[12,31] 70% of thymic squamous cell carcinomas,[31] and 66.7% of 30 cases with castration-resistant prostate cancer and NE prostate cancer.[32] Our study was the first to assess the expression of POU2F3 in a wide range of clinical samples with EP-NEC/PDCs arising from various organ systems. Tuft cells have been found in the urethra,[33] nasal cavity,[34] biliary-pancreatic tract,[35,36] and salivary glands,[37] which is in line with our finding of tuft cell carcinomas in GU, HN, and HBP systems. Interestingly, tuft cells have not been well-described in female genital organs; our discovery of

POU2F3-positive tuft cell carcinomas of uterine origin suggest the possible existence of chemosensory receptor tuft cells, a previously unappreciated cell-of-origin.

The minor but notable enrichment of POU2F3 positivity among GU and HN cancers requires further investigation. In particular, the majority of POU2F3-positive tuft cell carcinomas in our cohort of GU cancers originated from the urinary tract, except for a single case arising from the prostate. While NE differentiation is well-known in prostate cancers,[38] it has been less investigated in urothelial carcinomas. Although the data on POU2F3 expression in extrapulmonary tumors are limited, recent studies on thymic tumors have reported significant POU2F3 expression.[31,39] However, none of the thymic origin tumors among EP-NEC/PDCs expressed POU2F3. In our patients, thymic epithelial tumors were selected on the basis of atypical histologic characteristics, including basaloid or NE features; therefore, thymomas or thymic squamous cell carcinomas showing typical histopathologic characteristics were not included, which may explain the discrepancy in POU2F3 level in thymic tumors.

Similar to previous studies on SCLC,[12,13,19,40,41] we found that POU2F3 expression was inversely associated with the expression of conventional NE markers. Moreover, POU2F3-dominant tumors were enriched in the DN group, and demonstrated low ASCL1 and NEUROD1 expression levels. These findings suggest that POU2F3-positive carcinomas make up a distinct subset of EP-NEC/PDCs, and more importantly, gives a valuable lesson in therapeutic perspective. A recent study on the transcriptional mechanism of the tuft cell lineage identified a critical transcriptional complex composed of POU2F3, OCA-T1, and OCA-T2; these interactions may become an important target for pharmacological blockade in tuft cell-like SCLC.[42] Therefore, assessment of POU2F3 expression should be encouraged when diagnosing unusual
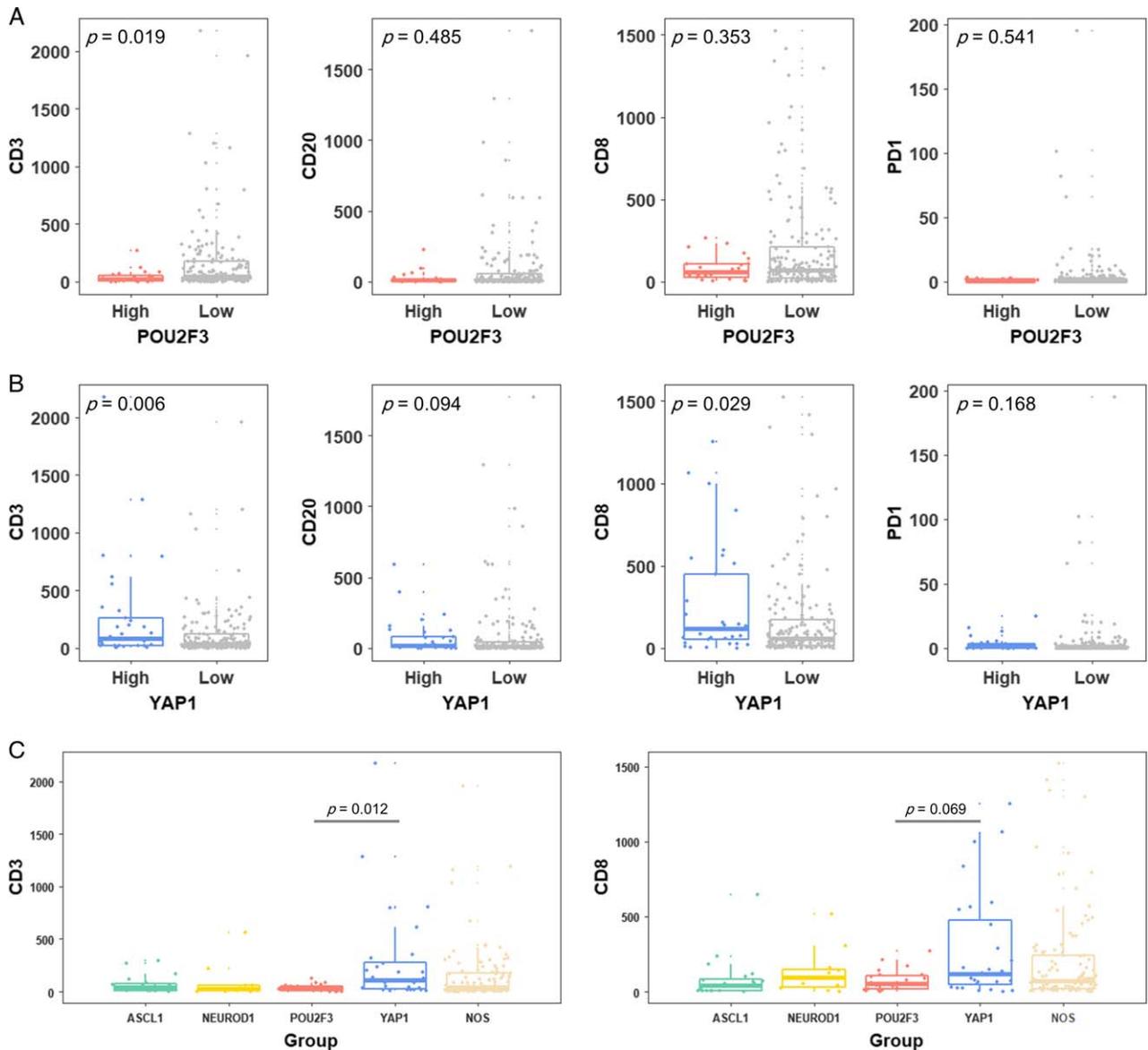
FIGURE 5. Immune microenvironment according to POU2F3 and YAP1 expression. A, No significant differences were noted in tumor-infiltrating lymphocytes according to POU2F3 expression, except for low CD3-positive T-cell density in POU2F3-positive tumors ($P = 0.019$). B and C, YAP1-positive tumors were characterized by inflamed microenvironment, represented by higher CD3-positive and CD8-positive T-cell infiltration.

EP-NEC/PDCs showing low expression of conventional NE markers, to allow better characterization of the disease.

To identify additional key oncogenic pathways or potential therapeutic targets of POU2F3-positive tuft cell carcinomas, we compared the gene expression patterns according to POU2F3 expression. The members of IGF1R signaling pathway included in the studied panel (*IGF1*, *IGF1R*, and *IGFBP3*) showed no significant differential expression according to POU2F3 phenotype. Interestingly, *PLCG2* and *BCL2* were notably upregulated in POU2F3-positive tumors, which was verified by IHC of the entire EP-NEC/PDC cohort. A recent study identified PLCG2 as a key

element of intracellular signaling in tuft-2 cells, a tuft cell subset[43]; this strengthens our hypothesis that POU2F3-positive EP-NEC/PDCs could arise through malignant transformation of tuft cells. In addition, high *PLCG2* expression in a subgroup of SCLC cells was found by single-cell transcriptome sequencing, and PLCG2-high subpopulation of SCLC was closely associated with increased invasiveness, prometastatic potential, stem-like features, and poor prognosis.[26] In line with this report, we found that the PLCG2 expression level in POU2F3-dominant subgroup was significantly associated with worse survival, further emphasizing the unique biology of POU2F3-positive tuft cell carcinomas and the role of PLCG2 in them.

In SCLCs, enrichment of *BCL2* was associated with the ASCL1-dominant SCLC subtype,[44] and was suggested to be a novel therapeutic target, because a higher BCL2 expressions level was associated with a better response to BCL2 inhibitors.[45,46] Therefore, our finding of a significantly high BCL2 expression level in POU2F3-positive tuft cell carcinomas suggests that, despite certain similarities between SCLC and EP-NEC/PDC, the unique features of EP-NEC/PDC should be studied further. In particular, further investigation of the use of BCL2 inhibitors for the treatment of a subset of EP-NEC/PDCs is strongly suggested. We also found significantly higher c-MYC expression levels in NEUROD1-dominant EP-NEC/PDCs, which is in line with previous findings from a study on SCLC, which demonstrated the potential efficacy of aurora kinase inhibitors for the treatment of MYC-driven SCLCs.[27] Taken together, our findings suggest that EP-NEC/PDCs are a disease group composed of heterogenous tumors, with distinct potential treatment options.

Because of enrichment of *PAX5*, and other immune-related gene sets in POU2F3-positive cases, we evaluated the immune microenvironment of EP-NEC/PDCs, and did not identify any evidence of inflamed microenvironment in POU2F3-positive tumors. Instead, the microenvironment of POU2F3-positive tumors resembled the "immune cold" microenvironment of SCLCs,[47] represented by no significant infiltration of TILs and minimal PD-L1 expression. By contrast, YAP1-dominant tumors had an inflamed tumor microenvironment, with higher infiltration of CD3-positive and CD8-positive T cells. This phenotype is similar to the SCLC-inflamed subtype proposed by Gay et al,[44] which was showed the greatest response to treatment with immune checkpoint blockade. Although the previous study did not identify an association between SCLC-inflamed and YAP1 expression level,[44] another one reported that YAP1-expressing SCLCs are characterized by T-cell inflamed gene expression signature.[48] In line with this, our study suggests that the YAP1-dominant EP-NEC/PDC is a distinct subgroup, whereas the existence of YAP1-dominant SCLCs is controversial.[19,25,44,48] The unique tumor microenvironment suggests that this subtype may demonstrate a good response to immune checkpoint blockade treatment.

Our EP-NEC/PDC cohort mainly included patients with available surgically resected samples, and excluded patients with inoperable diseases. In addition, due to the rarity of this disease, we used archival FFPE tissues; thus, our GEP analyses relied on panel-based profiling, and comprehensive assessment of POU2F3 target genes could not be performed. Therefore, our findings should be verified in future studies, particularly among patients with advanced and inoperable EP-NEC/PDCs.

In summary, we observed the POU2F3 expression in EP-NEC/PDCs arising from various organ systems, thereby extending our understanding of the clinical spectrum of POU2F3-positive tuft cell carcinomas. We showed that the molecular classification of SCLCs can be applied to EP-NEC/PDCs, and that POU2F3-dominant or YAP1-dominant subtypes are distinct subtypes of EP-NEC/PDCs. We found significantly high expression levels of *PLCG2* and *BCL2* in POU2F3-positive tuft cell carcinomas, which supports the novel cell-of-origin and suggests potential therapeutic vulnerability. The T-cell inflamed microenvironment of YAP1-dominant cases warrants further investigation. Taken together, despite the similarities between EP-NEC/PDCs and their pulmonary counterparts, our data suggest novel molecular pathologic features of EP-NEC/PDCs, emphasizing the need to focus on unique features of EP-NEC/PDCs.

REFERENCES

1. Frizziero M, Kilgour E, Simpson KL, et al. Expanding therapeutic opportunities for extra-pulmonary neuroendocrine carcinoma. *Clin Cancer Res.* 2022;28:1999–2019.
2. Frazier SR, Kaplan PA, Loy TS. The pathology of extrapulmonary small cell carcinoma. *Semin Oncol.* 2007;34:30–38.
3. Quinn AM, Blackhall F, Wilson G, et al. Extrapulmonary small cell carcinoma: a clinicopathological study with identification of potential diagnostic mimics. *Histopathology.* 2012;61:454–464.
4. Volante M, Birocco N, Gatti G, et al. Extrapulmonary neuroendocrine small and large cell carcinomas: a review of controversial diagnostic and therapeutic issues. *Hum Pathol.* 2014;45:665–673.
5. Dasari A, Mehta K, Byers LA, et al. Comparative study of lung and extrapulmonary poorly differentiated neuroendocrine carcinomas: a SEER database analysis of 162,983 cases. *Cancer.* 2018;124:807–815.
6. Cicin I, Karagol H, Uzunoglu S, et al. Extrapulmonary small-cell carcinoma compared with small-cell lung carcinoma. *Cancer.* 2007;110:1068–1076.
7. Brennan SM, Gregory DL, Stillie A, et al. Should extrapulmonary small cell cancer be managed like small cell lung cancer? *Cancer.* 2010;116:888–895.
8. Lee SM, Sung CO. Neuroendocrine carcinomas of the gallbladder. *Am J Surg Pathol.* 2020;44:1308–1321.
9. Howitt BE, Dong F, Vivero M, et al. Molecular characterization of neuroendocrine carcinomas of the endometrium. *Am J Surg Pathol.* 2020;44:1541–1548.
10. Wang G, Yuan R, Zhou C, et al. Urinary large cell neuroendocrine carcinoma. *Am J Surg Pathol.* 2021;45:1399–1408.
11. Walenkamp AME, Sonke GS, Sleijfer DT. Clinical and therapeutic aspects of extrapulmonary small cell carcinoma. *Cancer Treat Rev.* 2009;35:228–236.
12. Rudin CM, Poirier JT, Byers LA, et al. Molecular subtypes of small cell lung cancer: a synthesis of human and mouse model data. *Nat Rev Cancer.* 2019;19:289–297.
13. Huang Y-H, Klingbeil O, He X-Y, et al. POU2F3 is a master regulator of a tuft cell-like variant of small cell lung cancer. *Gene Dev.* 2018;32:915–928.
14. Matsumoto I, Ohmoto M, Narukawa M, et al. Skn-1a (Pou2f3) specifies taste receptor cell lineage. *Nat Neurosci.* 2011;14:685–687.
15. Gerbe F, Sidot E, Smyth DJ, et al. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature.* 2016;529:226–230.
16. Yamashita J, Ohmoto M, Yamaguchi T, et al. Skn-1a/Pou2f3 functions as a master regulator to generate Trpm5-expressing chemosensory cells in mice. *PLoS ONE.* 2017;12:e0189340.
17. Wang W, Epstein JI. Small cell carcinoma of the prostate. *Am J Surg Pathol.* 2008;32:65–71.
18. Perren A, Couvelard A, Scoazec J-Y, et al. ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: pathology—diagnosis and prognostic stratification. *Neuroendocrinology.* 2017;105:196–200.
19. Baine MK, Hsieh M-S, Lai WV, et al. SCLC subtypes defined by ASCL1, NEUROD1, POU2F3, and YAP1: a comprehensive immunohistochemical and histopathologic characterization. *J Thorac Oncol.* 2020;15:1823–1835.

20. Kim H, Chung J-H. PD-L1 testing in non-small cell lung cancer: past, present, and future. *J Pathol Transl Med*. 2019;53:199–206.

21. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. 2005;102:15545–15550.

22. Liberzon A, Birger C, Thorvaldsdottir H, et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst*. 2015;1:417–425.

23. Fabregat A, Sidiropoulos K, Viteri G, et al. Reactome pathway analysis: a high-performance in-memory approach. *BMC Bioinformatics*. 2017;18:142.

24. Nishimura D. BioCarta. *Biotech Softw Internet Rep*. 2001;2:117–120.

25. Pearsall SM, Humphrey S, Revill M, et al. The rare YAP1 subtype of SCLC revisited in a biobank of 39 circulating tumor cell patient derived explant models: a brief report. *J Thorac Oncol*. 2020;15:1836–1843.

26. Chan JM, Quintanal-Villalonga Á, Gao VR, et al. Signatures of plasticity, metastasis, and immunosuppression in an atlas of human small cell lung cancer. *Cancer Cell*. 2021;39:1479.e18–1496.e18.

27. Mollaoglu G, Guthrie MR, Böhm S, et al. MYC drives progression of small cell lung cancer to a variant neuroendocrine subtype with vulnerability to aurora kinase inhibition. *Cancer Cell*. 2017;31:270–285.

28. Megyesfalvi Z, Barany N, Lantos A, et al. Expression patterns and prognostic relevance of subtype-specific transcription factors in surgically resected small cell lung cancer: an international multicenter study. *J Pathol*. 2022;257:674–686.

29. Medvedovic J, Ebert A, Tagoh H, et al. Pax5: A master regulator of b cell development and leukemogenesis. *Adv Immunol*. 2011;111:179–206.

30. Yu P, Constien R, Dear N, et al. Autoimmunity and inflammation due to a gain-of-function mutation in phospholipase Cγ2 that specifically increases external Ca2+ entry. *Immunity*. 2005;22:451–465.

31. Yamada Y, Simon-Keller K, Belharazem-Vitacolonnna D, et al. A tuft cell–like signature is highly prevalent in thymic squamous cell carcinoma and delineates new molecular subsets among the major lung cancer histotypes. *J Thorac Oncol*. 2021;16:1003–1016.

32. Brady NJ, Bagadion AM, Singh R, et al. Temporal evolution of cellular heterogeneity during the progression to advanced AR-negative prostate cancer. *Nat Commun*. 2021;12:3372.

33. Deckmann K, Filipski K, Krasteva-Christ G, et al. Bitter triggers acetylcholine release from polymodal urethral chemosensory cells and bladder reflexes. *Proc Natl Acad Sci USA*. 2014;111:8287–8292.

34. Yamaguchi T, Yamashita J, Ohmoto M, et al. Skn-1a/Pou2f3 is required for the generation of Trpm5-expressing microvillous cells in the mouse main olfactory epithelium. *BMC Neurosci*. 2014;15:13.

35. Höfer D, Drenckhahn D. Identification of the taste cell G-protein, α-gustducin, in brush cells of the rat pancreatic duct system. *Histochem Cell Biol*. 1998;110:303–309.

36. Luciano L, Reale E. Brush cells of the mouse gallbladder. *Cell Tissue Res*. 1990;262:339–349.

37. Sato A, Miyoshi S. Fine structure of tuft cells of the main excretory duct epithelium in the rat submandibular gland. *Anat Rec*. 1997;248:325–331.

38. Beltran H, Rickman DS, Park K, et al. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. *Cancer Discov*. 2011;1:487–495.

39. Yamada Y, Sugimoto A, Hoki M, et al. POU2F3 beyond thymic carcinomas: expression across the spectrum of thymomas hints to medullary differentiation in type A thymoma. *Virchows Arch*. 2022;480:843–851.

40. Qu S, Fetsch P, Thomas A, et al. Molecular subtypes of primary SCLC tumors and their associations with neuroendocrine and therapeutic markers. *J Thorac Oncol*. 2022;17:141–153.

41. Rekhtman N. Lung neuroendocrine neoplasms: recent progress and persistent challenges. *Mod Pathol*. 2022;35:36–50.

42. Wu XS, He X-Y, Ipsaro JJ, et al. OCA-T1 and OCA-T2 are coactivators of POU2F3 in the tuft cell lineage. *Nature*. 2022;607:169–175.

43. Xiong Z, Zhu X, Geng J, et al. Intestinal Tuft-2 cells exert antimicrobial immunity via sensing bacterial metabolite N-undecanoylglycine. *Immunity*. 2022;55:686.e7–700.e7.

44. Gay CM, Stewart CA, Park EM, et al. Patterns of transcription factor programs and immune pathway activation define four major subtypes of SCLC with distinct therapeutic vulnerabilities. *Cancer Cell*. 2021;39:346.e7–360.e7.

45. Lochmann TL, Floros KV, Naseri M, et al. Venetoclax is effective in small cell lung cancers with high BCL-2 expression. *Clin Cancer Res*. 2018;24:360–369.

46. Gay CM, Tong P, Cardnell RJ, et al. Differential sensitivity analysis for resistant malignancies (DISARM) identifies common candidate therapies across platinum-resistant cancers. *Clin Cancer Res*. 2019;25:346–357.

47. Sabari JK, Lok BH, Laird JH, et al. Unravelling the biology of SCLC: implications for therapy. *Nat Rev Clin Oncol*. 2017;14:549–561.

48. Owonikoko TK, Dwivedi B, Chen Z, et al. YAP1 expression in SCLC defines a distinct subtype with T-cell–inflamed phenotype. *J Thorac Oncol*. 2021;16:464–476.