



# OPEN Exploring the expression of DLL3 in gastroenteropancreatic neuroendocrine neoplasms and its potential diagnostic value

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Delta-like protein (DLL3) is a novel therapeutic target. DLL3 expression in gastroenteropancreatic neuroendocrine tumors (GEP-NECs) is poorly understood, complicating the distinction between well-differentiated neuroendocrine tumors G3 (NET G3) and poorly differentiated NEC. DLL3 immunohistochemistry (IHC) was performed on 248 primary GEP-NECs, correlating with clinicopathological parameters, NE markers, PD-L1, Ki67 index, and prognosis. Achaete-scute complex-like 1 (ASCL1) IHC was performed on some GEP-NECs. DLL3 IHC was conducted on 36 GEP-NETs, 29 gastric adenocarcinomas (GACs), and metastatic tumors (9 lymph node metastases and 19 distant metastases). DLL3 expression rates were 54.8% in GEP-NECs at the primary site, associated with small cell neuroendocrine carcinoma (SCNEC) ( $p < 0.001$ ), chemotherapy before baseline ( $p = 0.015$ ), and at least two NE markers ( $p = 0.048$ ). DLL3 expression in metastatic GEP-NECs was similar to that of primary tumors. Expression rates in NET G1, NET G2, NET G3, and GACs were 0%, 0%, 15.8%, and 0%, respectively, highlighting DLL3 as a powerful tool for identifying poorly differentiated NEC. DLL3 expression was related to ASCL1 in GEP-NECs, especially in SCNEC. It was not correlated with progression-free survival (PFS) or overall survival (OS), regardless of cutoff value (1%, 50%, 75%). In conclusion, DLL3 targeted therapy may offer potential for the treatment of poorly differentiated NEC of the digestive system, although further studies are needed to validate its efficacy.

**Keywords** Delta-like protein, Gastroenteropancreatic neuroendocrine tumors, Immunohistochemistry, Therapeutic target, ASCL1

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are highly heterogeneous tumors that constituting approximately 65% of all neuroendocrine neoplasms and rank as the second most common gastrointestinal cancer<sup>1,2</sup>. Improved imaging techniques and enhanced awareness have contributed to an increased incidence from 1.1/100,000 in 1973 to 6.9/100,000 in 2012<sup>3</sup>. According to the 2019 WHO grading system, GEP-NENs are categorized as neuroendocrine tumors (NETs), neuroendocrine carcinomas (NECs), or mixed neuroendocrine-non-neuroendocrine neoplasms (MiNENs) based on histological differentiation, mitotic count, and the Ki-67 proliferation index. Well-differentiated NETs are further stratified into Grade1 (G1), Grade2 (G2), and Grade3 (G3) subtypes, whereas NECs with poorly differentiated NECs are classified as small cell NEC (SCNEC) and large cell NEC (LCNEC)<sup>4</sup>. NECs are often non-functional, highly aggressive, and frequently diagnosed late with distant metastasis, resulting in dismal 5-year survival rates below 5%<sup>5</sup>.

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Owing to the advanced stage and poor prognosis of GEP-NECs, treatments primarily aim to extend survival and enhance quality of life. Within the neuroendocrine spectrum, GEP-NECs share similar molecular and transcriptional profiles with small cell lung carcinoma (SCLC). Therefore, GEP-NECs treatment was similar to that for SCLC. Systemic chemotherapy, typically platinum-based regimens such as etoposide plus cisplatin (EC) and irinotecan plus cisplatin (IC), is the first-line of treatment for metastatic GEP-NECs. Notably, a Ki-67 index > 55% reliably predicts the reactivity of platinum-based chemotherapy<sup>6</sup>. Although some studies have suggested efficacy for agents such as 5-fluorouracil, irinotecan, and oxaliplatin, there are still no standardized second-line regimens. Ongoing trials, such as the one using 5-fluorouracil + leucovorin + irinotecan offer promise (NCT03387592)<sup>7–9</sup>. Advances in molecular detection technology and basic experiments have led researchers to investigate the molecular pathways and interactions in neuroendocrine neoplasms, offering potential targets for GEP-NECs. For instance, sunitinib, a multitarget tyrosine kinase inhibitor primarily inhibiting vascular endothelial growth factor receptor and platelet-derived growth factor receptor, elicited positive responses in 11 of 20 patients with GEP-NECs in a phase II trial encompassing 6 NET G3 and 20 NEC cases<sup>10</sup>. Phase II and III clinical trials testing single- or multi-drug combinations targeting various pathways, including XPO1 (NCT02250885), PARP (NCT04209595), and HDAC (NCT05076786), are currently ongoing for GEP-NECs. Among them, the negative regulator of the Notch pathway, DLL3, has drawn significant attention as a potential therapeutic target for neuroendocrine neoplasms<sup>10,11</sup>.

The Notch pathway, known for its highly conserved signaling cascade, is a critical factor in cellular transformation, including cell proliferation, epithelial-mesenchymal transition, neuroendocrine cell differentiation, chemoresistance, and immune microenvironment modulation<sup>12</sup>. As an inhibitory ligand of the Notch pathway<sup>13</sup>, DLL3 interacts with various Notch receptors (Notch1–4) to facilitate malignant transformation. DLL3 expression is absent or minimal in normal cells and is predominantly localized within the Golgi apparatus and cytoplasmic vesicles. Conversely, in malignant NENs, DLL3 translocates to the cell membrane surface, inhibiting the Notch pathway<sup>14,15</sup>. *ASCL1*, an intrinsic transcription factor in normal cells, regulates DLL3 expression, guiding neuroendocrine cell differentiation and initiating SCLC<sup>16</sup>. *ASCL1* activation increases DLL3 expression, enhancing the inhibition of Notch1 signaling<sup>17</sup>. Both the inhibition of Notch1 and the upregulation of *ASCL1* contribute to NEN development<sup>18,19</sup>. The efficacy of DLL3-targeted therapy has been confirmed in SCLC. Rova-T, a classic antibody-drug conjugate (ADC) in a phase I clinical trial (NCT01901653), has shown efficacy against recurrent or refractory SCLC<sup>20</sup>. However, severe adverse reactions have halted further clinical investigations of Rova-T. Next-generation ADCs aim to enhance tumor cell uptake by refining drug linkers to mitigate toxicity and optimize pharmacokinetics for improved clinical utility<sup>21,22</sup>. Tarlatamab, a bispecific T cell engager (TCE) targeting DLL3 on tumor cells and CD3 on T cells, has demonstrated promising results in a phase I trial, with a 13% objective response rate (ORR) and 71% of patients with SCLC experiencing relief for ≥ 6 months<sup>23</sup>. Other candidate drugs, such as HPN328 and AMG 119, have also shown beneficial antitumor responses in clinical and preclinical stages<sup>24,25</sup>. Patients with DLL3-expressing SCLC or NEC are currently enrolled in the first human Phase I trial of BI 764,532 (TCE) (NCT04429087). Although DLL3-targeted therapies are designed for tumors expressing DLL3, the immunohistochemical positivity for DLL3 expression on tumor cells was not a required criterion for patient enrollment in the trials. Therefore, DLL3 immunohistochemistry does not have a predictive role in determining eligibility for the trials.

Although DLL3 expression was initially identified in SCLC, in vitro investigations have revealed its diverse oncogenic role; elevated expression promotes aggressive behavior through Snail overexpression<sup>26,27</sup>. Moreover, DLL3 is highly expressed not only in SCLC but also in lung LCNEC. In a retrospective study of pulmonary LCNEC, over 74% (70/94) of patients expressed DLL3<sup>28</sup>. DLL3 expression extends beyond lung cancer to various invasive malignancies, including prostate cancer, bladder SCNEC, malignant melanoma, glioblastoma, and medullary thyroid carcinoma<sup>29–33</sup>, suggesting its potential as a biomarker for neuroendocrine-origin malignancies. However, DLL3 expression in GEP-NECs is poorly understood. This study aimed to investigate DLL3 expression in GEP-NECs and analyze its clinicopathological correlation and the relationship between DLL3 expression and patient prognosis.

## Methods

### Patient selection

All available information in this retrospective study was sourced from the Peking University Cancer Hospital. Basic patient information spanning 2010 to 2023, including age, sex, primary tumor site, histological classification, TNM stage, chemotherapy prior to baseline, and some aspects of prognosis, were primarily collected from medical records. Telephone follow-up was specifically used to obtain detailed prognostic information, which may not have been fully documented in the medical records or required updates beyond what was originally recorded. TNM staging refer to the American Joint Committee on Cancer (AJCC) 8th edition<sup>34</sup>. After re-examination by two experienced pathologists, it was ensured that each case met the WHO definition of neuroendocrine tumors: the shape was organ-like, trabecular and palisade-like, the mitotic count and Ki67 index strictly adhered to the standards of different grades, and expressed at least one neuroendocrine marker (Syn, CgA, CD56). Cases that did not meet these conditions were excluded from the study. Cases that lacked sufficient tissue samples for further staining were also excluded. The study protocol received approval from the Medical Ethics Committee of Peking University Cancer Hospital (approval number 2023KT29.), and all patients provided informed consent before tissue sample utilization.

### Pathological material

The sample composition of this experiment is summarized in Supplementary Fig. 1. We obtained 248 tumor tissue samples from primary GEP-NECs (including surgery and biopsy samples), 19 distant metastatic GEP-NECs samples (all were liver metastases) and 9 lymph node metastases. Additionally, 36 GEP-NETs (8 G1, 9 G2,

19 G3) samples and 29 GACs samples were also collected. Each sample underwent formalin fixation and paraffin embedding (FFPE). Some cases had immunohistochemical staining results for three neuroendocrine markers, Ki67, and PD-L1, when included in the study. For cases lacking any markers, additional immunohistochemical staining was carried out on available tumor tissues. The assessment of neuroendocrine marker results follows the 5th edition WHO criteria<sup>35</sup>, the Ki67 index was evaluated as a percentage, and the expression of PD-L1 was evaluated by CPS (22C3, Agilent DaKo, Denmark, 1:50). PD-L1 expression was defined as the ratio of the number of immune-related cells (tumor cells, lymphocytes, macrophages) expressing PD-L1 to the number of all tumor cells, with CPS  $\geq 1$  defined as positive.

### Immunohistochemical staining

FFPE specimens were sectioned into 4- $\mu$ m-thick slices using a rotary microtome, followed by incubation in EDTA solution (pH = 8.4) at 95 °C for 36 min. A DLL3 antibody (clone SP347, Roche, ready-to-use) was used for staining with Ventana, whereas ASCL1 antibody (24B72D11.1, BD Biosciences, 1:100) was stained with Leica Bond III. The color reaction was achieved using diaminobenzidine (ZSGB-BIO, Beijing, China). Staining outcomes were evaluated using an optical microscope with the 20 $\times$  or 40 $\times$  magnification. Each slide was reviewed by two experienced pathologists. Only when both pathologists agree on the result was it adopted, in cases of disagreement, the specimens were re-evaluated and discussed to achieve a consensus. DLL3 and ASCL1 results were binarily categorized. For DLL3, positive staining was defined as a reaction in  $\geq 1\%$  of tumor cells, regardless of intensity, based on criteria established by other studies<sup>36–38</sup>. Any punctate, cytoplasmic and/or membranous staining was considered positive, as previously described<sup>20,28</sup>. DLL3-high was characterized by  $\geq 50\%$  positive tumor cells<sup>39</sup> and  $\geq 75\%$  positive tumor cells<sup>40</sup>. For ASCL1, tumor cell positivity was categorized as 0, 1, 2, or 3 (negative, faint, moderate, or strong, respectively). We adopted semi-quantitative algorithms with H-score, which was calculated by multiplying the percentage of positive tumor cells (%) by the corresponding staining intensity. An H-score  $\geq 50$  is defined as positive<sup>41</sup>.

### Statistical analysis

We conducted all analyses using SPSS software (IBM Corp., Armonk, NY, USA, version 25). The correlation between DLL3 expression and clinicopathological features was evaluated using the chi-squared test or Fisher's exact test. Median progression-free survival (PFS) and overall survival (OS) were determined using Kaplan–Meier analysis, and survival differences were assessed using log-rank tests based on DLL3 expression status. The statistical significance level was set at  $p < 0.05$ .

## Results

### Patient profile

Table 1 provides a summary of the baseline characteristics of patients diagnosed with GEP-NECs. Among the 248 primary GEP-NECs cases, the age range was 23 to 85 years, with a median of 62 years. The study included 182 (73.4%) males and 66 (26.6%) females. The total number of SCNEC, LCNEC, and MiNENs cases were 138 (55.7%), 101 (40.7%), and 9 (3.6%), respectively. Gastric NECs accounted for the largest proportion (148, 59.7%) in this cohort, followed by the esophagus (51, 20.6%), and pancreas (24, 9.7%). T1-stage patients accounted for 3.2% (8/248), while T2–T4-stage patients accounted for 43.1% (107/248). Patients with stage N0 and N1–N3 accounted for 11.7% (29/248) and 33.1% (82/248), respectively; 40.7% (101/248) of patients with M stage were M0 stage, and 5.2% (13/248) were M1 stage. Additionally, 64 (25.8%) patients received chemotherapy before baseline. The rates of Ki67 proliferation index in different intervals 25–50%, 51–75%, and 76–100% were 11.7% (29/248), 39.5% (98/248), and 35.1% (87/248), respectively. In 75.4% (187/248) of cases, at least 2 NE markers were positive, while in 12.5% (31/248) of cases  $< 2$  NE markers were positive. A positive reaction of PD-L1 was shown in 6.0% (15/248) of cases.

### Relationship between DLL3 expression and clinicopathological features

DLL3 staining was positive in the cytomembrane, cytoplasm, and punctate, in which cytoplasmic and membranous staining was diffuse, whereas intermittent perinuclear staining was punctate. However, we identified four cases in which nuclear positivity was observed alongside cytoplasmic and membranous staining, a pattern not previously reported in other studies. The nuclear positivity in these cases may represent a nonspecific or incidental reaction. Representative images are presented in Fig. 1a. Moreover, DLL3 has a heterogeneous expression pattern in GEP-NECs. Correlations between DLL3 expression and clinicopathological features are presented in Table 2. Positive expression was observed in 68.1% of SCNEC (94/138) compared to 38.6% of LCNEC (39/101) and 33.3% (3/9) of MiNENs ( $p < 0.001$ ). Of note, in MiNENs, the neuroendocrine component exhibited positive DLL3 expression, while the adenocarcinoma component showed negative expression (Fig. 1b). The expression rate of DLL3 was highest in the esophagus (68.6%, 35/51), followed by the colorectum (53.8%, 7/13), stomach (52.7%, 78/148), pancreas (41.7%, 10/24), and small intestine (25.0%, 2/8). DLL3 showed significant expression differences among different T-stage cases (T1 100.0% vs. T2–T4 53.3%,  $p = 0.009$ ). Patients who underwent chemotherapy prior to baseline demonstrated a higher prevalence of DLL3 expression compared to individuals who did not undergo previous therapy (67.2% vs. 50.5%,  $p = 0.015$ ). In the group with  $< 2$  NE positive markers, the expression rate of DLL3 was 38.7%, significantly lower than that in the group with  $\geq 2$  NE positive markers (56.7%,  $p = 0.048$ ). No associations were observed between DLL3 expression and sex, N stage, M stage, Ki67 index, or PD-L1 expression.

### DLL3 expression in metastatic tumors

The expression of DLL3 in metastatic tumors is shown in Supplementary Fig. 2. The staining pattern of DLL3 in metastatic lesions was similar to that in primary tumors. In lymph node metastasis and distant metastases, the

| Baseline characteristics     | N (%)       |
|------------------------------|-------------|
| Total                        | 248         |
| Age (median, range)          | (62, 23–85) |
| Gender                       |             |
| Male                         | 182 (73.4)  |
| Female                       | 66 (26.6)   |
| Histological classification  |             |
| SCNEC                        | 138 (55.7)  |
| LCNEC                        | 101 (40.7)  |
| MiNENs                       | 9 (3.6)     |
| Primary site                 |             |
| Esophagus                    | 51 (20.6)   |
| Stomach                      | 148 (59.7)  |
| Small intestine              | 8 (3.2)     |
| Colorectum                   | 13 (5.2)    |
| Pancreas                     | 24 (9.7)    |
| Other*                       | 4 (1.6)     |
| T stage                      |             |
| T1                           | 8 (3.2)     |
| T2-T4                        | 107 (43.1)  |
| NA                           | 133 (53.7)  |
| N stage                      |             |
| N0                           | 29 (11.7)   |
| N1-N3                        | 82 (33.1)   |
| NA                           | 137 (55.2)  |
| M stage                      |             |
| M0                           | 101 (40.7)  |
| M1                           | 13 (5.2)    |
| NA                           | 134 (54.1)  |
| Chemotherapy before baseline |             |
| Yes                          | 64 (25.8)   |
| No                           | 184 (74.2)  |
| Ki67 index (%)               |             |
| 20–50                        | 29 (11.7)   |
| 51–75                        | 98 (39.5)   |
| 76–100                       | 87 (35.1)   |
| NA                           | 34 (13.7)   |
| NE markers                   |             |
| < 2 NE markers positive      | 31 (12.5)   |
| ≥ 2 NE markers positive      | 187 (75.4)  |
| NA                           | 30 (12.1)   |
| PD-L1                        |             |
| Positive                     | 15 (6.0)    |
| Negative                     | 25 (10.1)   |
| NA                           | 208 (83.9)  |

**Table 1.** Baseline characteristics of patients with GEP-NECs. GEP-NECs gastroenteropancreatic-neuroendocrine carcinomas, SCNEC small cell neuroendocrine carcinoma, LCNEC large cell neuroendocrine carcinoma, MiNENs mixed neuroendocrine neoplasms. Others\* include three cases originating from the gallbladder and one case originating from the appendix. NE markers include Syn, CgA, CD56. NA\* not applicable.

positive rate of DLL3 was 44.4% and 52.6%, respectively. Chi-square analysis showed no significant difference in the expression of DLL3 between primary tumor, lymph node metastases, and distant metastases ( $p = 0.818$ ) (Fig. 2a).

### Explore the differential diagnosis value of DLL3

To explore the expression of DLL3 in well-differentiated GEP-NETs, we stained 36 cases of GEP-NETs. Among the GEP-NETs cases, none of the eight cases of NET G1 and nine cases of NET G2 exhibited positive DLL3 staining, while 3 out of 19 (15.8%) cases of NET G3 were positive for DLL3 staining. The DLL3 expression was also heterogeneous, and all of them were positive in the cytoplasm and membrane (Fig. 1c). Since the morphological overlap between NET G3 and NECs or poorly differentiated adenocarcinoma and NECs often puts pathologists in a dilemma with respect to differential diagnosis, we further assessed the value of DLL3 in differential diagnosis. Therefore, we performed DLL3 staining on additional GACs with different degrees of differentiation. The results show that no positive DLL3 staining was detected in 29 GACs cases, regardless of the degree of differentiation (Fig. 1c). DLL3 may serve as a useful differential diagnostic tool with a sensitivity of 54.8% and a specificity of 84.2% when differentiating NECs from NET G3, and a sensitivity of 54.8% and a specificity of 100.0% when differentiating NECs from GACs, with a cutoff value of 1% positive tumor cells. However, with the threshold raised to 50% positive tumor cells, the sensitivity declined to 31.9%, while the specificity increased to 94.7% when identifying NECs and NET G3 and to 100.0% when identifying NECs and GACs, reflecting the stricter standard (Table 3).

### ASCL1 expression

Almost all positive reactions for ASCL1 were located to the nucleus, with only one case showing an unusual punctate positivity around the nucleus (Fig. 3a). The expression status of 111 samples of ASCL1 and DLL3 is shown in Fig. 3b. In addition, it was observed that in samples with co-expression of DLL3 and ASCL1, tumor cells expressing ASCL1 also exhibited DLL3 expression, demonstrating a spatial consistency. (Fig. 3c). Similarly, its expression was also heterogeneous inside the tumor. Overall, ASCL1 expression was detected in 14.4% (16/111) of GEP-NECs cases. Interestingly, DLL3 expression rate differed between the ASCL1 positive and ASCL1 negative groups ( $p=0.002$ ) (Fig. 2b). In the ASCL1 positive group, the DLL3 positive rate was 87.5% (14/16), whereas 47.4% (45/95) showed positive DLL3 staining in the ASCL1 negative group. Similarly, DLL3 expression was higher in the ASCL1 positive group among GEP-SCNECs (100.0% vs. 56.8%,  $p=0.02$ ) (Fig. 2c). However, the differential expression of DLL3 was not significant in GEP-LCNECs (33.3% vs. 37.8%,  $p=0.687$ ) (Fig. 2d).

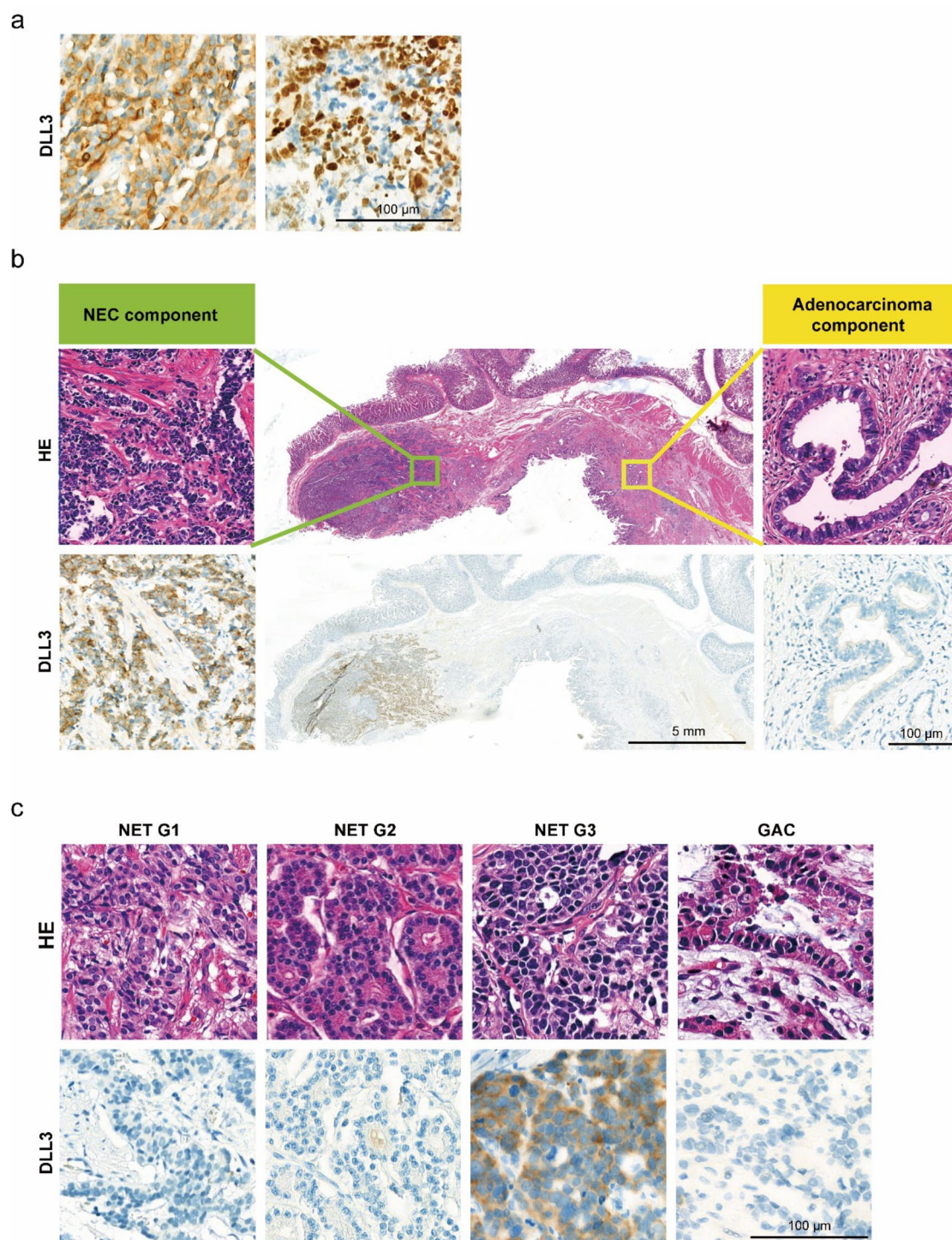
### Follow-up and prognosis

The prognosis information of 199 patients was obtained. The median follow-up time was 19.7 months (range: 0.8–140.4 months). Survival analysis was conducted for different cutoff values of DLL3 expression ( $N=199$ ) (Fig. 4). In the DLL3 positive group (with the 1% cutoff value), the median PFS and median OS were 12.3 months (95% CI: 7.9–16.6) and 24.4 months (95% CI: 20.2–28.6), respectively, while in the DLL3 negative group, the median PFS and median OS were 13.4 months (95% CI: 10.5–16.3) and 25.9 months (95% CI: 18.9–32.9), respectively. The log-rank test showed that there was no significant difference in the prognosis between the DLL3 negative group and the DLL3 positive group (Fig. 4a). Moreover, no significant difference in survival was observed between high and low DLL3 expression groups, regardless of whether the cutoff value defined by high DLL3 expression was 50% or 75% (Fig. 4b and c).

### Discussion

High levels of DLL3 expression are found in carcinoid subtypes of pulmonary NETs that exhibit significant dendritic cell infiltration, suggesting its potential as a viable target for focused intervention. Previous studies have reported a 65% DLL3 expression rate in 37 patients with LCNEC<sup>17</sup>. Analysis of clinical trial groups revealed DLL3 expression in over 70% of SCLC cases<sup>40,42</sup>. A high expression rate of DLL3 was also observed in patients with castration-resistant neuroendocrine prostate cancer<sup>29</sup>. A previous study reported a DLL3 expression rate of 76.9% in 13 cases of gastrointestinal-pancreatic NECs<sup>43</sup>. We first assessed DLL3 positivity in a large sample of GEP-NECs ( $n=248$ ) and found a positivity rate of 54.8%. Our study revealed different DLL3 expression rates between SCNEC and LCNEC, with SCNEC exhibiting a higher positivity rate than LCNEC. Regarding primary site distribution, the esophagus exhibited a high DLL3 expression rate, which was largely attributed to the high proportion of SCNEC in esophageal NECs. Similarly, this cohort showed a high expression rate of DLL3 in T1 stage patients, partly because SCNEC had an absolute advantage in the number of T1-stage patients (7/8). A single-cell transcriptome sequencing study of SCLC tumor cells demonstrated that chemotherapy resulted in decreased expression of therapeutic target genes, including *DLL3*<sup>44</sup>. However, our results showed that DLL3 has a higher expression rate in patients receiving chemotherapy before baseline treatment, which prompts future research to focus on whether chemotherapy affects the expression of the *DLL3* gene.

Several studies have adopted various definitions for DLL3-high expression. When defined as staining in  $\geq 50\%$  of tumor cells, the DLL3-high expression rates in SCLC range from 32% to 79.5%<sup>11,17,39</sup>. With a cutoff value of 75%, the positive rate of high expression was 70% in another study on SCLC<sup>40</sup>. Conversely, high expression rates of DLL3 in lung LCNEC are relatively low at 54%<sup>28</sup>. In this study, we applied 50% and 75% cutoff values to define DLL3-high expression in GEP-NECs, exploring the DLL3-high expression rate for the first time. Although these cutoff values have been frequently used in small cell lung cancer (SCLC), their application to GEP-NECs has not been previously reported. Our results showed a 31.8% expression rate of DLL3 with a cutoff value of 50% and a 25.4% expression rate of DLL3 with a cutoff value of 75%. Since GEP-NECs contains SCNEC and LCNEC, we analyzed the high expression rates in different subtypes (Supplementary Table 1). Irrespective of the cutoff value, the expression rate of DLL3 in SCNEC is always higher than that in LCNEC. Furthermore, the expression rate of DLL3 in the digestive system was lower than that in high-grade neuroendocrine tumors of the lung, regardless of the cutoff value. Recent studies in SCLC indicate significant improvements in ORR and PFS in patients with high expression of DLL3 (defined as positive staining in  $\geq 50\%$  of tumor cells); the high expression



**Fig. 1.** (a) DLL3 staining was positive in the cytomembrane, cytoplasm, and nucleus, in which cytoplasmic and membranous staining was diffuse, whereas intermittent perinuclear staining was punctate. The second picture also showed the heterogeneity of DLL3 staining. (b) In MiNENs, the neuroendocrine component exhibited positive DLL3 expression, whereas the adenocarcinoma component showed negative. (c) The staining of DLL3 was positive in NET G3, while negative in NET G1, NET G2, and GACs. NEC Neuroendocrine carcinoma. NET Neuroendocrine tumor. GAC Gastric adenocarcinoma.

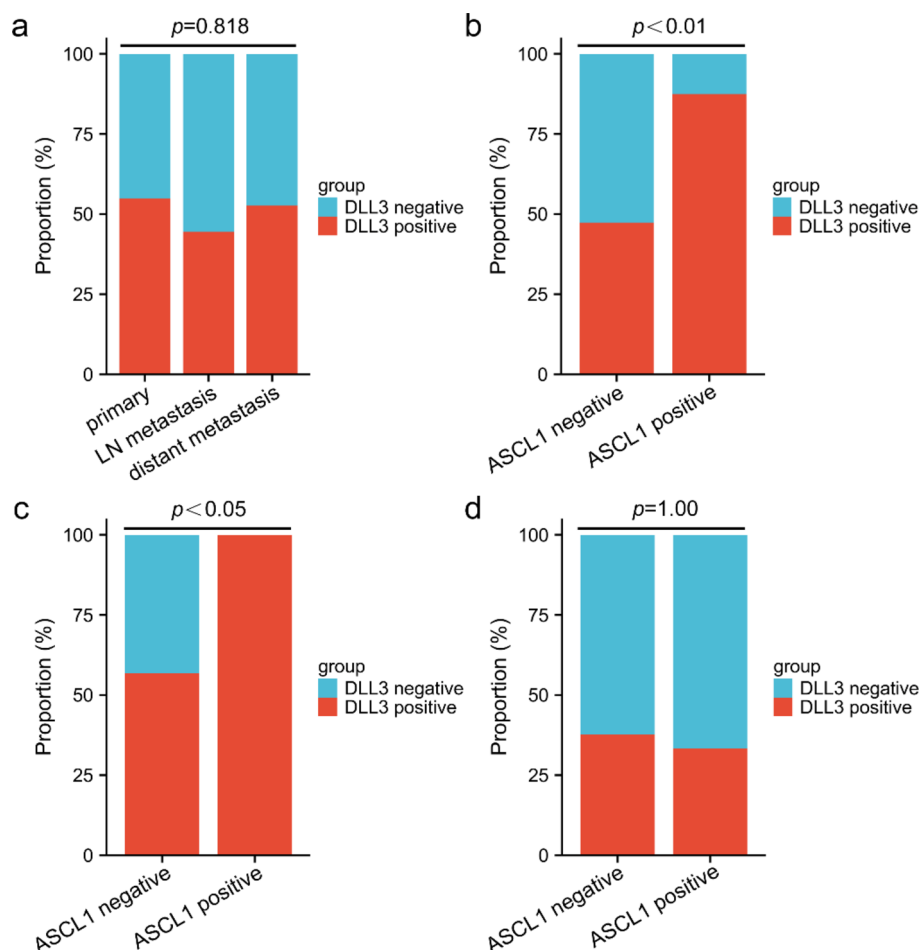
| Clinical characteristics     | DLL3 expression |           | P      |
|------------------------------|-----------------|-----------|--------|
|                              | Positive        | Negative  |        |
| Gender                       |                 |           | 0.527  |
| Male                         | 102 (56.0)      | 80 (44.0) |        |
| Female                       | 34 (51.5)       | 32 (48.5) |        |
| Histological classification  |                 |           | <0.001 |
| SCNEC                        | 94 (68.1)       | 44 (31.9) |        |
| LCNEC                        | 39 (38.6)       | 62 (61.4) |        |
| MINENs                       | 3 (33.3)        | 6 (66.7)  |        |
| Primary site                 |                 |           | 0.068  |
| Esophagus                    | 35 (68.6)       | 16 (31.4) |        |
| Stomach                      | 78 (52.7)       | 70 (47.3) |        |
| Small intestine              | 2 (25.0)        | 6 (75.0)  |        |
| Colorectum                   | 7 (53.8)        | 6 (46.2)  |        |
| Pancreas                     | 10 (41.7)       | 14 (58.3) |        |
| Other*                       |                 |           |        |
| T stage                      |                 |           | 0.009  |
| T1                           | 8 (100.0)       | 0 (0.0)   |        |
| T2-T4                        | 57 (53.3)       | 50 (46.7) |        |
| N stage                      |                 |           |        |
| N0                           | 16 (55.2)       | 13 (44.8) | 0.505  |
| N1-N3                        | 47 (57.3)       | 35 (42.7) |        |
| M stage                      |                 |           | 0.516  |
| M0                           | 58 (57.4)       | 43 (42.6) |        |
| M1                           | 7 (53.8)        | 43 (46.2) |        |
| Chemotherapy before baseline |                 |           |        |
| Yes                          | 43 (67.2)       | 21 (32.8) | 0.015  |
| No                           | 93 (50.5)       | 91 (49.5) |        |
| Ki67 index (%)               |                 |           | 0.758  |
| 20–50                        | 13 (44.8)       | 16 (55.2) |        |
| 51–75                        | 52 (53.1)       | 46 (46.9) |        |
| 76–100                       | 46 (47.1)       | 41 (52.9) |        |
| NE markers                   |                 |           | 0.048  |
| <2 NE markers positive       | 12 (38.7)       | 19 (61.3) |        |
| ≥ 2 NE markers positive      | 106 (56.7)      | 81 (43.3) |        |
| PD-L1                        |                 |           | 0.403  |
| Positive                     | 7 (46.7)        | 8 (53.3)  |        |
| Negative                     | 14 (56.0)       | 11 (44.0) |        |

**Table 2.** Correlation of DLL3 expression with clinicopathological characteristics. GEP-NECs gastroenteropancreatic-neuroendocrine carcinomas, SCNEC small cell neuroendocrine carcinoma, LCNEC large cell neuroendocrine carcinoma, MINENs mixed neuroendocrine neoplasms. Others\* include three cases originating from the gallbladder and one case originating from the appendix. NE markers include Syn, CgA, CD56. NA \* not applicable. P-value<0.05 was considered statistically significant.

group demonstrated significant benefits in confirmed objective response (35% versus 0%) and disease control (90% versus 60%) compared to the low expression group of DLL3<sup>17</sup>. Additionally, in another phase II clinical trial, the ORR was 14.3% in the DLL3 high-expression group, with high expression defined as positive staining in  $\geq 75\%$  of tumor cells<sup>40</sup>. In phase I/II studies of multiple cancer types, a higher remission rate was observed with high DLL3 expression<sup>45</sup>. However, some patients with DLL3 expression in  $< 50\%$  of tumor cells also exhibit stable disease for a prolonged duration<sup>20,45</sup>. Hence, in the following clinical phases, the assessment should focus on evaluating the efficacy in patients exhibiting any measurable DLL3 levels.

The field of immuno-oncology (IO) has revolutionized the landscape of cancer therapy, actively influencing patient outcomes<sup>46</sup>. PD-L1 has been identified as the first biomarker for anti-PD-1 therapy and is included in the prescribing information for pembrolizumab. Currently, other immune therapy efficacy-related markers include tumor mutational burden (TMB), mismatch repair system deficiency (dMMR), high microsatellite instability (MSI-H), neoantigens, mutations in antigen presentation pathways, and circulating tumor DNA (ctDNA) as indicators for selecting patients who may benefit from immunotherapy<sup>47</sup>. In a study on pancreatic ductal adenocarcinoma, high DLL3 expression was positively correlated with PD-L1/2 expression<sup>48</sup>. Another study in SCLC also observed cases with high DLL3 expression and negative NOTCH1 expression to have a higher PD-L1 expression rate, revealing a favorable prognosis in such SCLC patients<sup>49</sup>. However, our study did not find any correlation between DLL3 and PD-L1 expression in GEP-NECs. Although no significant correlation was observed, in our study cohort, 9 out of 40 (22.5%) patients co-expressed DLL3 and PD-L1, suggesting the feasibility of combined DLL3-targeted therapy and immunotherapy in some patients and providing a theoretical basis for developing new treatment strategies for patients with GEP-NEC, which requires further clinical validation.

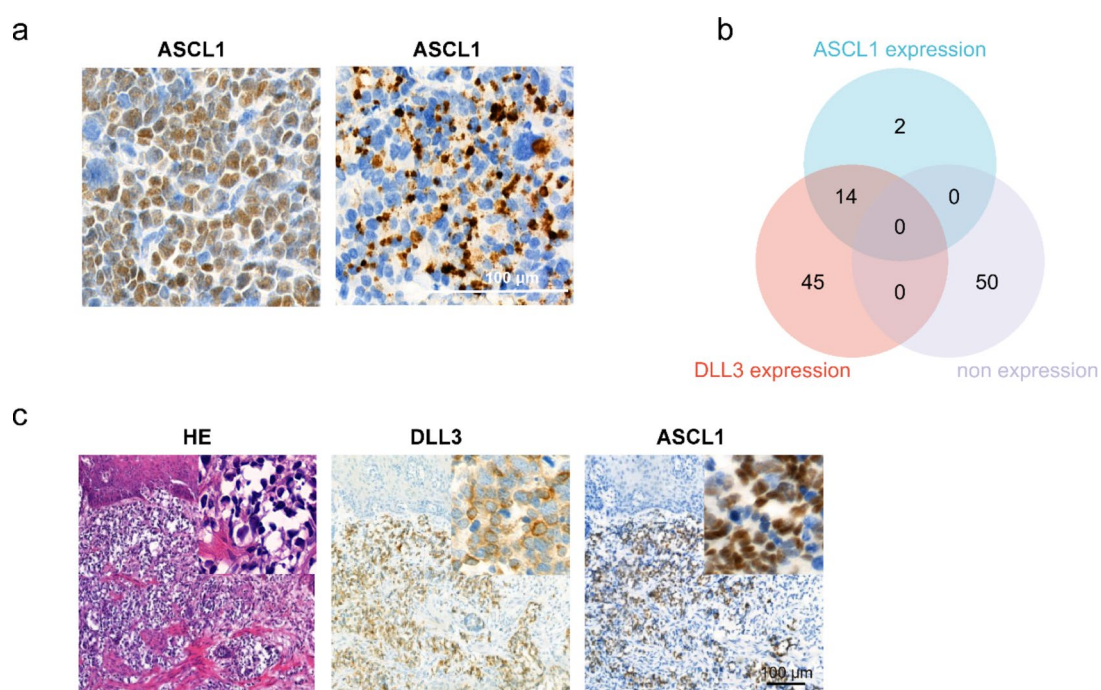
In recent studies, four subtypes of SCLC have been identified based on the unique expression patterns of four key transcripts: SCLC-A, SCLC-N, SCLC-P, and SCLC-Y<sup>50</sup>. Baine et al. confirmed higher DLL3 expression in ASCL1-high SCLC compared to other groups<sup>51</sup>. Wang et al. classified NECs into five subtypes (ASCL1, NEUROD1, HNF4A, POU2F3, and YAP1) across various tumor sites (including GEP)<sup>52</sup>. Although our study did



**Fig. 2.** (a) The expression rate of DLL3 in primary tumors and metastatic tumors. The expression rate of DLL3 between ASCL1 negative group and ASCL1 positive group in GEP-NECs (b), GEP-SCNEC (c), and GEP-LCNEC (d). GEP-NECs Gastroenteropancreatic neuroendocrine carcinomas. GEP-SCNEC Gastroenteropancreatic small cell neuroendocrine carcinoma, GEP-LCNEC Gastroenteropancreatic large cell neuroendocrine carcinoma.

|                 |               | NECs | NET G3 | Sensitivity | Specificity | <i>P</i> |
|-----------------|---------------|------|--------|-------------|-------------|----------|
| NECs vs. NET G3 | DLL3 positive | 136  | 3      | 54.8%       | 84.2%       | 0.003    |
|                 | DLL3 negative | 112  | 16     |             |             |          |
| NECs vs. NET G3 | DLL3 high     | 79   | 1      | 31.9%       | 94.7%       | <0.001   |
|                 | DLL3 low      | 169  | 18     |             |             |          |
|                 |               | NECs | GACs   |             |             |          |
| NECs vs. GACs   | DLL3 positive | 136  | 0      | 54.8%       | 100.0%      | <0.001   |
|                 | DLL3 negative | 112  | 29     |             |             |          |
| NECs vs. GACs   | DLL3 high     | 79   | 0      | 31.9%       | 100.0%      | <0.001   |
|                 | DLL3 low      | 169  | 29     |             |             |          |

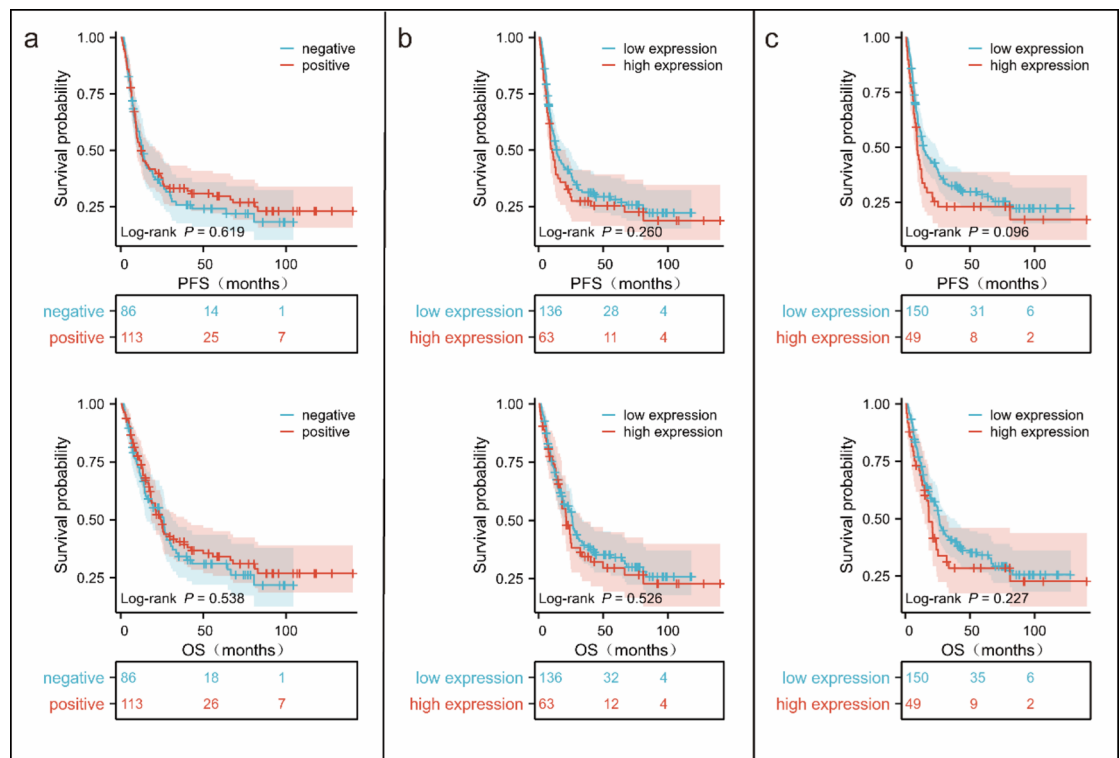
**Table 3.** Evaluation of DLL3 as a diagnostic marker for GEP-NECs. DLL3-high means positive tumor cells  $\geq 50\%$ . DLL3-low means positive tumor cells  $<50\%$  or no positive tumor cells. NECs neuroendocrine carcinomas, NET neuroendocrine tumors, GACs gastric adenocarcinomas. *P* value  $<0.05$  was considered statistically significant.



**Fig. 3.** (a) The positive reactions for ASCL1 were located in the nucleus, with only one case showing an unusual dot-like positivity around the nucleus. (b) The expression of ASCL1 and DLL3 in 111 cases. (c) In the samples with co-expression of DLL3 and ASCL1, the spatial distribution of tumor cells expressed by DLL3 and ASCL1 was consistent.

not further classify subtypes, we first verified the expression of ASCL1 and DLL3 in 111 available tumor tissues, showing a significantly higher DLL3 expression rate in the ASCL1-positive group (likely corresponding to the A subtype) compared to the ASCL1-negative group (likely corresponding to non-A subtypes) at the protein level, confirming a significant correlation. However, this correlation was only evident in small cell GEP-NECs and was not seen in large cell or mixed types, which was quite different from that observed with pulmonary neuroendocrine tumors. The correlation of DLL3 and ASCL1 expression was observed in both SCLC and LCNEC of the lung<sup>28,51,53</sup>. Nonetheless, these results suggest that patients with the ASCL1 subtype may benefit from DLL3-targeted therapy. Furthermore, the study by Wang et al. revealed that A and N types of GEP-NEC are classified as NE-high types<sup>52</sup>. Similarly, in our cohort, we found that among the 13 ASCL1-positive cases that underwent NE staining in available tissues, 10 cases were positive for  $\geq 2$  NE markers. Additionally, in tumors positive for  $\geq 2$  NE markers, the expression rate of DLL3 was higher than that of the subgroup of  $< 2$  NE markers, which is consistent with the results observed in pulmonary neuroendocrine carcinomas<sup>28</sup>.

It has been found that a classic pathway in the development of SCLC involves tumor initiation under the premise of bi-allelic mutations in *RB1* and *TP53*, driven by NOTCH signaling inactivation<sup>54</sup>. While the roles of the DLL3/Notch pathway and the loss of *RB1* and *TP53* mutations in the development of GEP-NECs have



**Fig. 4.** Kaplan-Meier survival curves for progression-free survival (PFS) and overall survival (OS) of GEP-NECs patients ( $n = 199$ ) according to DLL3 status with the cut-off value 1%(a), 50%(b), and 75%(c).

not been extensively studied, in high-grade neuroendocrine tumors of the digestive system, *TP53* and *RB* mutations are the most common genetic alterations, differing from the frequently occurring *MEN1*, *ATRX*/*DAXX*, and *mTOR* pathway activation in NET<sup>55</sup>. This may explain why DLL3 is negative in well-differentiated neuroendocrine tumors, but shows positive reactions in some high-grade neuroendocrine tumors, suggesting that part of digestive neuroendocrine tumors may share a similar carcinogenic pathway with SCLC. However, this speculation needs further confirmation in future studies.

DLL3 staining in all GACs cases showed negative results regardless of the degree of differentiation. Among the nine cases of MiNENs comprising a mixture of adenocarcinoma and NECs, no positive signal was detected in the adenocarcinoma component. Hence, DLL3 staining may serve as a tool to differentiate gastric NECs from GACs with 100% specificity, particularly in biopsy samples where limited tissue samples may hinder a definitive diagnosis. Based on the classification established by the 2010 World Health Organization, NENs were divided into highly differentiated NETs (NET G1 and NET G2) and poorly differentiated NECs<sup>56</sup>. Despite being highly proliferative, certain tumors progress slowly and have a favorable prognosis, while others follow an aggressive course. To address this heterogeneity, a subgroup termed NET G3 was introduced into the GEP-NENs classification system, representing an intermediate prognosis between NET G1/G2 and NEC<sup>57</sup>. Nevertheless, distinguishing NET G3 with a relatively favorable prognosis from poorly differentiated NECs remains challenging for pathologists. Currently, a comprehensive diagnostic approach combining clinical performance, morphology, immunohistochemistry, and molecular biomarkers is preferred. These biomarkers include RB, *DAXX*/*ATRX*, *SSTR2*, *CgA*, and *p53*<sup>58</sup>. No large-sample studies have confirmed the value of DLL3 in distinguishing NET G3 from NEC. A small-sample study on GEP-NENs found no DLL3 expression in NET G3 (0/5)<sup>43</sup>. Chen et al. utilized various methods, including WES, FISH, qPCR, and IHC, to identify DLL3 expression in three out of eight GEP-NET G3 cases, with no abnormal DLL3 expression in NET G2<sup>59</sup>. Our study marks the first discovery of DLL3 protein expression in GEP-NET G3 with a relatively large sample size, suggesting the differential diagnostic value of DLL3, and calling for further research on DLL3-targeting agents in NET G3.

Additionally, there was no significant difference in DLL3 expression among primary tumors, lymph node metastases, and distant metastases. Therefore, DLL3 staining in metastases appears to reflect the condition of primary tumors, suggesting the feasibility of applying DLL3-targeted therapy in advanced patients with distant organ metastases.

In a study on DLL3 expression in SCLC without targeted therapy, patient prognosis was found to be unrelated to DLL3 expression status, with high or low expression ( $\geq 50\%$  cutoff) showing no impact on survival<sup>11</sup>. Similar findings were observed in the GEP-NECs cohort in our study. However, another study encompassing SCLC, carcinoid, and atypical carcinoid cases noted that DLL3-high expression ( $\geq 50\%$  positive tumor cells) correlated with improved OS in SCLC ( $p = 0.049$ ), without adjusting for age, tumor dimension, and stage<sup>39</sup>. Conversely, a small-sample cohort study of GEP-NENs ( $n = 46$ ) observed significantly better PFS and OS in the DLL3-negative group, largely because of the predominance of NET G1/G2/G3 cases in their study cohort<sup>43</sup>. This study was the

largest GEP-NECs cohort to date and found that the expression of DLL3 has no relationship with the prognosis of patients with GEP-NECs.

This study had some limitations. Being a single-center study with a modest sample size, future research should involve larger cohorts to validate the differences in DLL3 expression across primary tumor sites and further assess the diagnostic performance between NET G3 and NECs in the digestive system. Although cytoplasmic and/or membranous staining was considered positive, as reported by other studies<sup>20,28</sup>, the presence of nuclear positivity remains poorly understood. This discrepancy highlights a limitation in our study, as the relationship between DLL3 staining type (cytoplasmic, membranous, punctate and nuclear) and therapeutic response to DLL3-targeted therapy remains unclear. Further investigation is required to elucidate whether different DLL3 staining patterns are associated with varying clinical outcomes. Moreover, the study's retrospective nature leads to potential biases stemming from progression in clinical practices over time. This highlights the necessity for prospective studies to more effectively clarify the influence of DLL3 expression on patient prognosis.

## Conclusion

Our study confirmed DLL3 expression in GEP-NECs and found that DLL3 expression was related to the SCNEC subtype and chemotherapy. The DLL3 expression rate in NET G3 supports the application of DLL3-targeted therapy in high-grade NETs of the digestive system. DLL3 IHC was conducive for distinguishing between NET G3 and NECs, as well as GACs and NECs. Finally, we confirmed the correlation between DLL3 and ASCL1 protein expression in GEP-NECs. Our study suggests that DLL3 expression is not a prognostic factor in patients with GEP-NECs.

## Data availability

The datasets generated and/or analysed during the current study are not publicly available due patient privacy but are available from the corresponding author on reasonable request.

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## Author contributions

M.L., J.L. and Y.S. provided the ideas. L.Y. and R.W. collected the data and followed up patients. X.M. carried out immunohistochemical staining on the tissues. L.Z., Z.W. and T.L. provided the DLL3 antibody. L.Y. analyzed the data, made all the figures and tables, and wrote the manuscript. K.J., Y.H., X.Z. and Y.S. revised the manuscript and all the figures and tables. Y.S. completed the submission. Y.S. provided funding acquisition. All authors read and approved the manuscript.

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## Declarations

### Ethics declarations

Human samples and clinical data were collected and used in accordance with the principles of the Declaration of Helsinki and approved by the Ethics committee of Peking University Cancer Hospital. All participants provided written informed consent, approval number 2023KT29.

### Competing interests

The authors declare no competing interests.

### Additional information

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