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Neuroendocrine neoplasia

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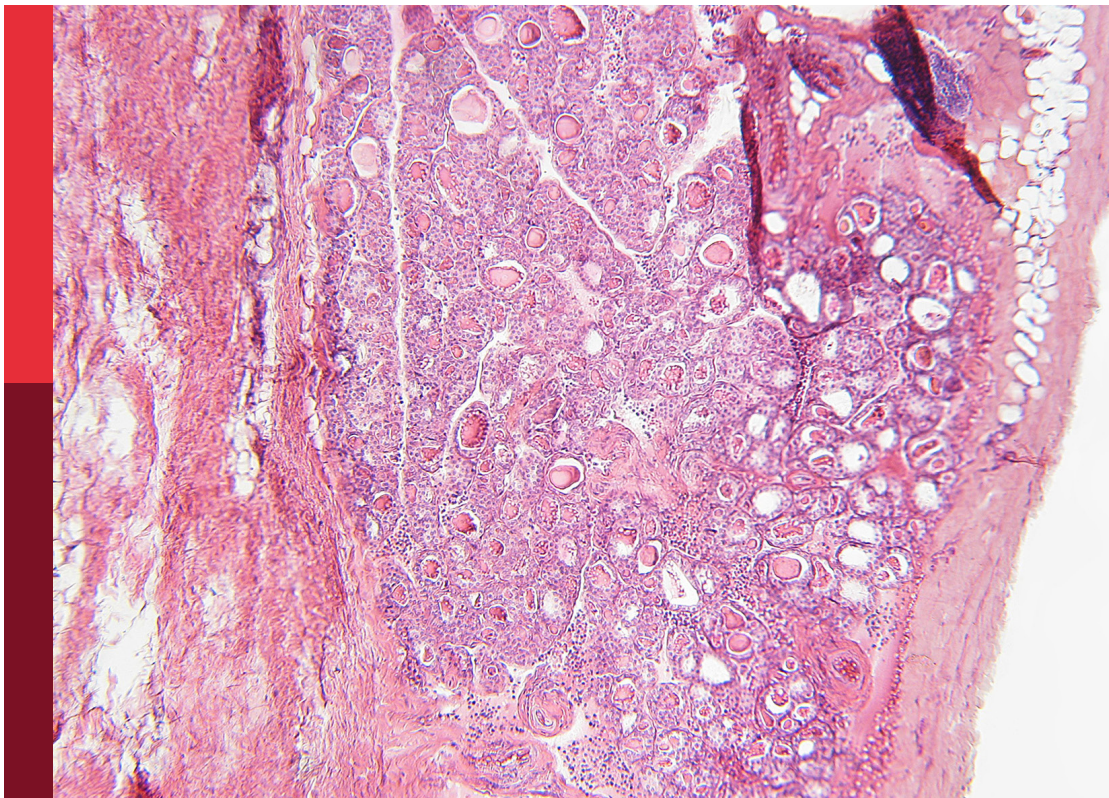

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Neuroendocrine neoplasia

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CASE REPORT
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Is Gangliocytic Paraganglioma Designated as a Subtype of Composite Paragangliomas and Originated From Pancreas Islet? A Case Report and Review of Literature

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Gangliocytic paraganglioma (GP) is quite rare, and origin and entity remain to be elucidated. A 51-year-old man presented with GP as a sessile polyp with a smooth surface that measured about 1 cm in diameter in the descending portion of duodenum. Pathological examination displayed that a neoplasm was predominantly located in the submucosa and infiltrated mucosa focally. The tumor consisted of epithelioid, ganglion-like, and spindle cells admixing in a haphazard way. The epithelioid cells resembled paraganglioma in cytological and architectural features. The ganglion-like cells were scattered and merged with the bland spindle cells in fascicular clusters, which resembled ganglioneuroma. Synaptophysin (Syn), microtubule-associated protein-2 (MAP-2), and chromogranin A (CgA) were positive in the epithelioid and ganglion-like cells in variety, and neurofilament (NF) staining highlighted the ganglion-like cells. S-100 and SOX-10 were positive in the spindle cell proliferation and around the epithelioid cells. Progesterone receptor (PR) was positive in the epithelioid cells. The polyp was resected, and no adjuvant therapy was given. The patient remained with no recurrence in 2 years' follow-up. Origin of GP is presumed to be related to pancreas islet. GP is distinguished from neuroendocrine tumor (NET) G1 and designated as paraganglioma-ganglioneuroma, a kind of composite paragangliomas.

Keywords: gangliocytic paraganglioma, duodenum, immunohistochemistry, progesterone receptor, paraganglioma-ganglioneuroma

INTRODUCTION

Paraganglioma (PGL) always involves the extra-adrenal ganglions among the sympathetic or parasympathetic chain. Gangliocytic paraganglioma (GP), a distinct type of PGL, is quite rare, and only up to 300 cases have been reported since it was first described in 1957. Origin and entity of GPs remain to be elucidated. We experienced a case of GP in the descending portion of the duodenum. Herein, we presented the case and discussed with review of literature.

CASE DESCRIPTION

A 51-year-old man had presented with abdominal discomfort for several years. Physical and experimental examinations revealed no significant differences. Gastrointestinal endoscopy was performed, and a sessile polyp with a smooth surface that measured about 1 cm in diameter was found in the descending portion of the duodenum (**Figure 1**). The lesion was excised then.

Microscopically, the tumor was predominantly located in the submucosa, infiltrated the lamina propria focally, and covered with duodenal mucosa (**Figures 2A, B**). It was composed of epithelioid cells, ganglion-like cells, and spindle cells (**Figure 2C**). The epithelioid cells were large in round or polygonal shape and arranged in nest or zellballen pattern. They had abundant eosinophilic cytoplasm and a round nucleus with inconspicuous nucleoli. Some were larger with granular cytoplasm and vesicular nuclei with prominent nucleoli. The ganglion-like cells were even larger with eosinophilic cytoplasm and out-standing eccentric vesicular nuclei and were always scattered and not easily distinguished from the epithelioid cells (**Figure 2D**). Both types of cells displayed mild cellular pleomorphism yet did not display mitosis. The spindle cells were bland and arranged in fascicular clusters. The three proportions were admixed in a haphazard way. Some distorted and enlarged glands were entrapped in the lesion. The tumor did not display inflammation, necrosis, and calcification.

Immunohistochemical study showed an extremely low index of Ki-67. The neoplastic proliferation was negative for cytokeratin (CK, AE1/AE3), epithelial membrane antigen (EMA), leukocyte common antigen (LCA), human melanoma black-45 (HMB45), Melan A, CD30, CD117, and discovered on GIST-1 (DOG-1). Synaptophysin (Syn) and microtubule-associated protein-2 (MAP-2) were diffusely positive for epithelioid cells and highlighted the ganglion-like cells (**Figure 3A**). The staining pattern of chromogranin A (CgA) was similar, whereas the number and intensity were limited (**Figure 3B**). The ganglion-like cells stood out in neurofilament (NF) staining (**Figure 3C**). Neu-N was negative in both epithelioid and ganglion-like cells. Progesterone receptor (PR) was positive in some epithelioid cells, whereas estrogen receptor (ER) was negative (**Figure 3E**). S-100 and SOX-10 were positive in the spindle cell proliferation and around the epithelioid cells (**Figure 3D**). CD34 was positive in the spindle cells and endothelia. Smooth muscle actin (SMA) and desmin stained the muscularis mucosa, which confirmed that the tumor infiltrated the mucosa (**Figure 3F**).

Integrating morphology and immunostaining, GP was the permanent pathological diagnosis. The patient received no adjuvant therapy and remained with no recurrence in 2 years' follow-up.

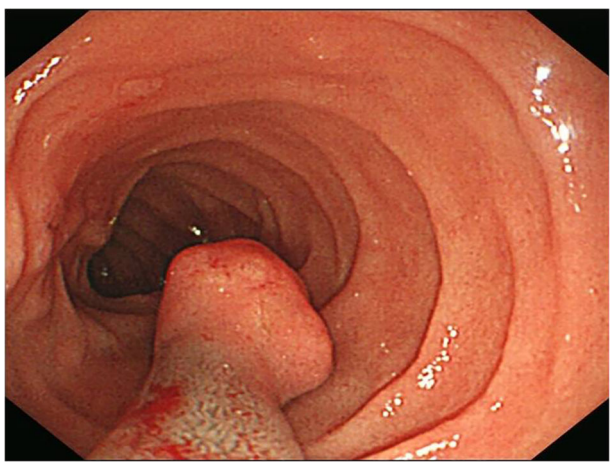

FIGURE 1 | A sessile polyp human melanoma black-45 (HMB45) measured about 1 cm in diameter with a smooth surface was found in gastrointestinal endoscopy.

DISCUSSION

GP is a rare neuroendocrine tumor (NET), and up to 300 cases have been reported until now. GPs affect individuals ranging from 15 to 84 years old with a mean of about 53 years old and are a little more prevalent in males with a male-to-female ratio of 1.5:1 (1). Nearly 90% of GPs were documented to be located in the duodenum, and involvement of other sites such as spinal cord, respiratory system, and digestive tract was also reported (2).

The presenting symptoms and complaints of GPs in digestive tracts include gastrointestinal bleeding, abdominal pain, anemia, and so on, which have no reliable diagnostic signs. Imaging examinations often demonstrate a mass lesion (3–5).

GPs range in size from 0.5 to 10 cm with an average of 2.5 cm in maximum diameter. In pathological investigation, they are always well-circumscribed and non-encapsulated, whereas some cases are infiltrative focally or even extensively. GPs in the duodenum are located in the submucosa, expanding to adjacent lamina propria or muscularis propria (2). Therefore, preoperative pathological diagnosis is difficult through endoscopic biopsy due to a relatively deep location, and definite diagnosis requires resection of the mass (6).

GP consists of three distinct cellular elements, including the epithelioid, ganglion-like, and spindle cells (7). The epithelioid cells have eosinophilic abundant cytoplasm with a round nucleus. They arrange in nest or zellballen pattern. They are positive for Syn and CgA, around which S-100 and SOX-10 are positive. They resemble PGL in cytological, architectural, and immunostaining features. Compared with the epithelioid cells, the ganglion-like cells are even larger and have more prominent nucleoli. They are always scattered or sometimes merged with the epithelioid or spindle cells individually or in small clusters. The immunophenotypes of the ganglion-like cells are similar with those of the epithelioid cells, whereas NF is positive uniquely. The bland spindle cells arrange in fascicular clusters and are positive for S-100 and SOX-10, which resemble neurofibroma. The neoplasm did not display mitosis and necrosis.

The proportion of the three cellular types is variable. In tumors predominant of spindle cells, the differential diagnosis includes spindle cell neoplasms, such as schwannoma and gastrointestinal stromal tumor (GIST) (8). The presence of epithelioid and ganglion-like cells, although perhaps rare, is the

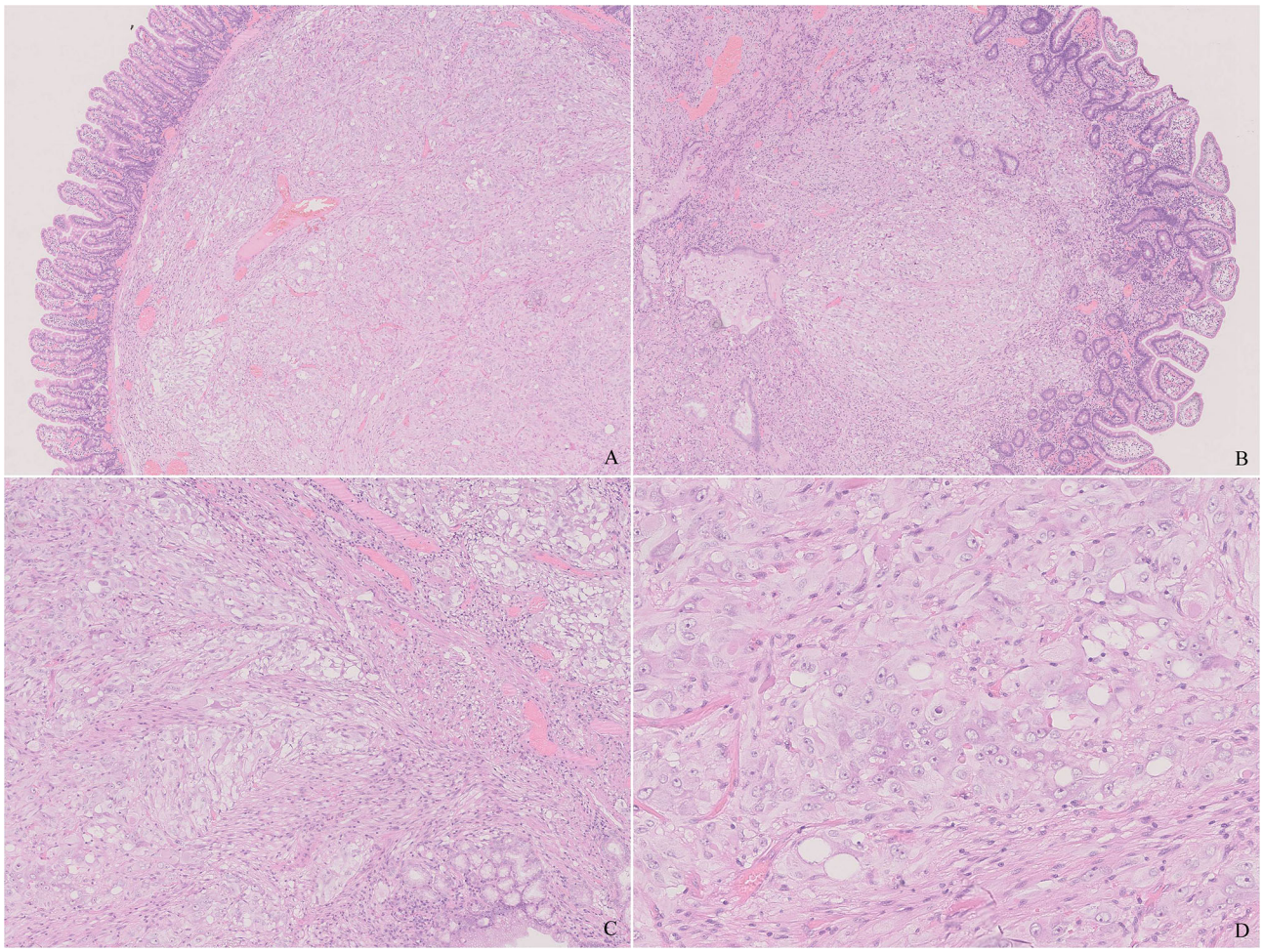

FIGURE 2 | Histology of the tumor. **(A)** The tumor was well-circumscribed and non-encapsulated and located in the submucosa. **(B)** The tumor infiltrated the lamina propria focally. **(C)** The tumor was composed of epithelioid, ganglion-like, and spindle cells. Note the muscularis mucosae in the upper right that are different from the neoplastic spindle cells. **(D)** The epithelioid cells have abundant eosinophilic cytoplasm and a round nucleus. Note a ganglion-like cell in the center, which was larger in shape, and a nucleus with prominent eccentric nuclei. [**(A, B)**, H&E ×20, **(C)**, H&E ×100, **(D)**, H&E ×400].

most important clue against schwannoma. Negative immunohistochemical expression for DOG-1 and CD117 provides compelling evidence against GIST.

In tumors predominant of ganglion-like or epithelioid cells, the differential diagnosis includes epithelial tumors, melanoma, and well-differentiated neuroendocrine neoplasms (NENs). The first two tumors are excluded by immunostaining of epithelial or melanic markers with relative ease.

The relationship between GPs and NENs is still in argument (9, 10). World Health Organization (WHO) classification of digestive system updated the classification and grading criteria for NENs in 2019. In the fourth edition, proliferation activity was nearly the only criterion, and NENs were classified into well-differentiated NETs including G1 and G2 and poorly differentiated neuroendocrine carcinomas (NECs) as G3. In the fifth edition, morphological characteristics such as atypia or necrosis are more emphasized besides proliferation activity, thus NETs and NECs are first distinguished. NETs are graded as G1, G2, and G3 according to proliferative activity. NECs are subtyped as small-cell NECs and large-cell NECs. In brief, the most significant difference between the two classifications is that NET G3 was considered to be synonymous with poor differentiation (i.e., NECs) in the fourth edition, while NET G3 and NECs are distinguished now. In addition, the cutoff and counting methods of proliferation index are adjusted. GPs have been classified among well-differentiated NETs by some authors (11). However, diversity of GPs is different from relative consistence of NETs in morphology. More significantly, GPs have a more indolent clinical behavior and favorable prognosis than NETs (12). Thus, in our opinion, GP is supposed to be differentiated from NET G1.

A multidisciplinary team (MDT) involving endoscopy, digestion, imaging, and pathology is always assembled for NENs in the gastrointestinal tract. An important clinical distinction among NENs relates to their hormonal functionality. Functions

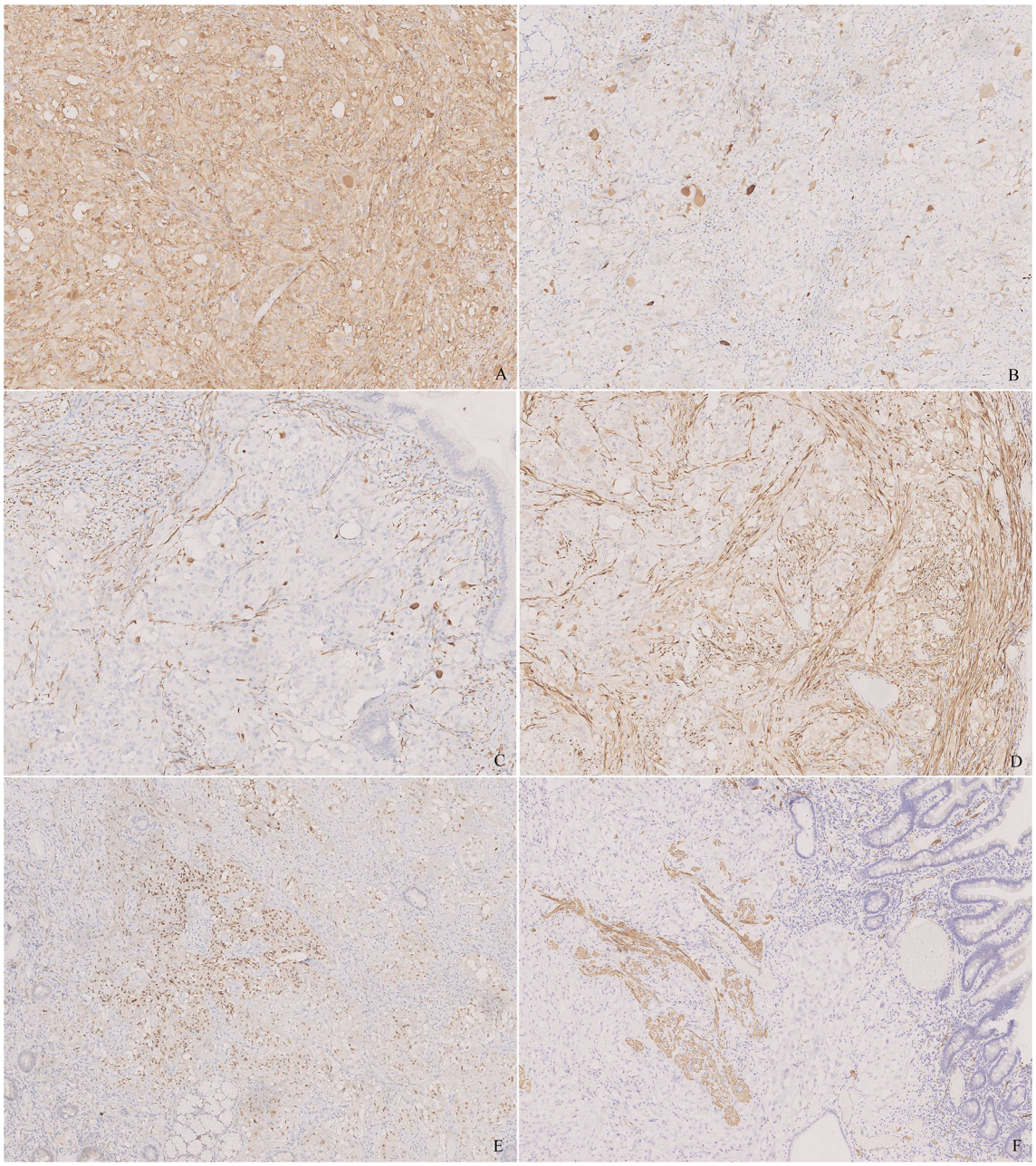

FIGURE 3 | Immunohistochemistry of the tumor. **(A)** Synaptophysin (Syn) was diffusely positive for epithelioid cells and highlighted the ganglion-like cells. **(B)** Chromogranin A (CgA) was positive for epithelioid cells and highlighted the ganglion-like cells, but the number of positive cells was less than that in Syn. **(C)** Neurofilament (NF) stained some ganglion-like cells and neoplastic spindle cells. **(D)** S-100 stained the sustentacular cells around the epithelioid cells and the neoplastic spindle cells. **(E)** Progesterone receptor (PR) was positive in some epithelioid cells. **(F)** Desmin stained the muscularis mucosa, which displayed that the tumor infiltrated the lamina propria. [**(A–F)**, ×100].

are determined by the abnormal production of hormones by NENs. Clinical non-functioning NENs may also produce hormones that do not result in clinical symptoms. Rare cases of GP were reported to have hormonal functions (13). In a sense, NENs in the duodenum can be subtyped into non-functioning NET, gastrinoma, somatostatinoma, enterochromaffin-cell carcinoid, NEC, and GP.

Entity of GPs remains a problem. As early as 2005, some authors pointed out that histological differences between pheochromocytoma-ganglioneuromas and GPs were not clear (14). Actually, GPs can be subdivided into two neoplastic components including PGL and ganglioneuroma. The occurrence of two or more synchronous tumors, which admixed so intimately

with each other as to be impossibly separated topographically, is supposed to be named for composite tumors. Therefore, GP is preferred as paraganglioma-ganglioneuroma, a subtype of composite PGLs. It is noteworthy that immature ganglion cells are supposed to be explored to exclude the possibility of paraganglioma-neuroblastoma, especially in young patients.

Succinate dehydrogenase (SDH) is known as a tumor suppressor gene that plays a role in PGL (15). *SDH* is involved in the mitochondrial tricarboxylic acid (TCA) cycle and composed of four subunits: *SDHA*, *SDHB*, *SDHC*, and *SDHD*. Inactivating mutations in the *SDH* genes contribute to PGL, and mutations in *SDHB* are the most frequent among the four subunits (16). It is not known whether mutations of *SDH* are involved in GPs due to rare cases. It is a pity that *SDH* was not studied in the case.

The novel expression of PR, which was also confirmed by some other recent studies, is worthy of attention (2). PR is supposed to be an alternative marker for differential diagnosis. GP is postulated to be originated from pancreas islet remnant or ectopia, since pancreatic islet cells also express PR (2). GP is always located in the submucosa, inducing hypothesis of origin from Meissner plexus (17). Origin of GP needs to be elucidated.

Although the clinical behavior of GP is usually benign, up to 10% of cases occurred with regional lymph node metastasis and only a few cases occurred with distant metastasis to bone, liver, or pelvic cavity (18–21). Age, tumor size, and depth of invasion appear to be related to metastasis (2). The immunohistochemical prognostic factors in NETs, such as Ki-67, P53, and Bcl-2, are not indicative of malignant potential. It is noted that all the three cellular components are supposed to be present in metastasis. The patients with metastasis have favorable prognosis with long survival periods, whereas only one case followed an aggressive clinical course and died of the disease (21). Endoscopic resection of duodenal GP appears enough for most cases, while additional surgery needs to be managed in cases of positive margins (22, 23). Adjuvant chemotherapy and irradiation appear to have no effect, even in cases with metastasis.

In conclusion, we presented a rare case of 51-year-old man with GP as a polyp in the descending portion of the duodenum. Pathological examination showed that a neoplasm was predominantly located in the submucosa and infiltrated the lamina propria. The tumor was composed of epithelioid, ganglion-like, and spindle cells. Syn, MAP-2, and CgA were positive in the epithelioid and ganglion-like cells in variety, and NF staining highlighted the ganglion-like cells. S-100 and SOX-10 were positive in the spindle cell proliferation and around the epithelioid cells. PR was also positive in the epithelioid cells. Origin of GP is presumed to be related to pancreas islet. GP is supposed to be distinguished from NET G1 and designated as paraganglioma-ganglioneuroma, a kind of composite PGL. Most GPs displayed benign clinical biological behaviors, and a few occurred in regional lymph nodes or distant metastasis. A large majority of cases follow a favorable prognosis, even with metastasis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the 7th Medical Center, Chinese PLA General Hospital. The patients/participants provided their written informed consent to participate in the study.

AUTHOR CONTRIBUTIONS

JL collected information and wrote the article. L-PW made the pathological diagnosis. P-SZ performed histological and immunohistochemical investigation. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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A MEN1 Patient Presenting With Multiple Parathyroid Adenomas and Transient Hypercortisolism: A Case Report and Literature Review

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Background: Multiple endocrine neoplasia type 1 (MEN1) is a hereditary endocrine syndrome caused by mutations in MEN1 tumor suppressor gene.

Case Presentation: A 53-year-old Chinese female was admitted to Division of Endocrinology, Tongji Hospital, for hypercalcemic crisis. Increased level of parathyroid hormone (PTH) was confirmed by laboratory tests, and imaging examination showed multiple parathyroid adenomas. Based on gene analysis, the patient was diagnosed as MEN1 associated hyperparathyroidism (HPT) by gene analysis with c.1378C>T (p.Arg460Ter) mutation in MEN1 gene. Her condition was complicated by transient hypercortisolism, mammary mass and uterine leiomyoma. After subtotal parathyroidectomy, PTH and serum calcium levels returned to normal.

Conclusion: HPT with multiple parathyroid adenomas is an indication of MEN1 gene mutation. Serum cortisol and its circadian rhythm can be abnormal in the presence of hypercalcemia and high PTH. These parameters can return to normal after parathyroidectomy.

Keywords: multiple endocrine neoplasia type 1, hyperparathyroidism, hypercortisolism, parathyroid adenomas, hypercalcemia

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INTRODUCTION

Multiple endocrine neoplasia (MEN) is characterized by the occurrence of two or more endocrine tumors in a single patient (1). There are four major types of MEN denoted as MEN1-4 (2). Each type of MEN is characterized by the occurrence of tumors in specific endocrine glands, inherited as an autosomal-dominant syndromes or may be sporadic (1).

The classic manifestation of MEN1 is co-occurrence of parathyroid, pancreatic islet, and anterior pituitary tumors. Other neoplasms may occur during the course of MEN1, including adrenal tumors, gastric tumors, skin and subcutaneous tumors, as well as breast cancer reported recently (3, 4). The incidence of MEN1 has been estimated to be 0.25% from randomly chosen postmortem studies, and to be 1-18% in patients with primary hyperparathyroidism (PHPT) (3). A diagnosis of MEN1 is established if a patient has one of three manifestations: 1) two or more main MEN1-

associated endocrine tumors, 2) one MEN1-associated tumor and a first-degree relative of a confirmed MEN1 patient, 3) a germline mutation in MEN1 gene (3).

Here, we report a complex case of MEN1 associated with symptomatic PHPT and a transient hypercortisolism. Multiple parathyroid adenomas raised our concerns regarding the diagnosis of MEN1. To our knowledge, dynamic change of cortisol level in MEN1 patient has not been reported before.

CASE DESCRIPTION

A 53-year-old Chinese female was referred to local hospital in April 2019 because of her sore left knee (**Supplementary Figure 1**). After admission, the patient was diagnosed with bone cyst of the left patella and osteoporosis based on X-ray and dual-energy X-ray absorptiometry (DEXA) scanning. The preoperative examinations showed a significant increase in serum calcium (4.03mmol/L), while a decrease of potassium level (2.9mmol/L). Thus, an operation proposed based on the primary diagnosis was canceled. Although the patient received fluid infusion and potassium supplementation, the serum potassium and calcium concentrations did not return to normal levels. Subsequently, the patient was transferred to our department for further clinical evaluation. The patient suffered from dry mouth, fatigue, and muscular weakness in the past year. There was no nausea, poor appetite, back pain, neurological alterations, and other discomforts. She didn't receive any medical treatment. In addition, the patient had a history of hypokalemia, hypertension and hysterectomy for uterine leiomyoma. She took anti-hypertension medications (calcium channel blockers) and oral potassium tablets intermittently. Her mother had a long history of hypertension and type 2 diabetes and her father had died of gastric cancer. There was no family history of electrolyte disturbances, psychosocial and hereditary disease.

On admission, the patients was conscious with a body temperature of 36.5°C, pulse rate of 96 beats/min, and blood pressure of 140/99mmHg. An oval-shaped mass with regular edges was palpable on the left side of the neck. There were no symptoms or signs of hypoglycemia, headache, vision loss, moon face, hirsutism, purple striae, or central obesity.

Laboratory examinations revealed notable elevations in parathyroid hormone (PTH) (1917.00 pg/mL, normal range 15-65 pg/mL) and calcium (4.14 mmol/L, normal range 2.15-2.50 mmol/L), indicating hypercalcemic crisis (**Table 1**). Based on these findings, the patient was diagnosed as PHPT. The patient also presented a hypercortisolism and loss of circadian rhythm (8am 221.00 μg/L, 4pm 287.70 μg/L, 12MN 281.40 μg/L, **Table 2**), while adrenocorticotrophic hormone (ACTH) level was normal. The serum cortisol could not be inhibited by low-dose overnight dexamethasone suppression test (DST) (**Table 2**) (5).

A 99mTc-methoxyisobutylisonitrile (MIBI) scan of the parathyroid showed three focal uptakes (one behind the left lobe of thyroid with the size of 34×25mm and two behind the right lobe of thyroid with the size of 10×10mm and 14×13mm, respectively), suggesting multiple parathyroid adenomas (**Figure 1A**).

Besides rehydration and potassium supplementation, the patient was treated with diuretics (furosemide) in the first 8 hours after admission. However, the serum calcium remained above 4 mmol/L. Subsequently, salmon calcitonin and bisphosphonate (zoledronic acid, 4mg, intravenous drip slowly) were given according to the guidance for Emergency management of acute hypercalcaemia in adult patients (6). The level of serum calcium gradually decreased from 4.33mmol/L to 2.37mmol/L (**Table 1**) and the eGFR maintained stable. The symptoms including thirsty, fatigue and knee soreness were relieved as well. Thereafter, the patient received subtotal parathyroidectomy (SPTX) and 3 glands were removed. Calcium (Calcium carbonate D3 tablets, 1800 mg/day) and vitamin D (Alfacalcidol Capsules, 0.75 ug/day) were supplemented after surgery (7). Meanwhile, the potassium replacement was stopped.

Postoperative pathological findings confirmed multiple parathyroid adenomas (**Figure 1B**). PTH levels decreased to normal range 3 days after surgery (**Table 1**). We re-evaluated the functions of endocrine glands after surgery. As shown in **Table 2**, cortisol level returned to normal range immediately after parathyroidectomy. The 2-day high-dose dexamethasone suppression test (HDDST, 2-mg dexamethasone every 6 hours) was performed and a suppression rate of serum cortisol more than 50% was observed (**Table 2**) (8). The concentrations of renin and aldosterone in addition to aldosterone-renin ratio (ARR) were tested after parathyroidectomy when the corresponded potassium level was normal, and no abnormal results were found. However, hemoglobin dropped to 73 g/L. After ruling out the cause of massive intraoperative blood loss and blood diseases, we suspected this drop may be attributed to the usage of zoledronic acid (9).

Because of the multiple parathyroid glands involvement, a DNA sequencing of MEN! Gene was performed. A heterozygous C to T change was identified at codon 460 in exon 10 according to the current human reference genome (GRCh37) (**Figure 1C**), which suggested a pathogenic mutation. Thus, this patient was diagnosed with MEN1. Subsequently, the MEN1 gene of her son

TABLE 1 | Electrolyte and PTH levels before and after parathyroidectomy.

Day	-7	-5	-3	-1	0	1	3	10	76	137
PTH (15-65 pg/mL)	1917.00	2055.00	/	2942.00	/	76.96	29.88	/	219.60	153.50
Ca (2.15-2.5mmol/L)	4.14	3.83	2.54	2.54	2.60	2.26	2.35	2.32	2.22	2.31
K (3.5-5.1mmol/L)	3.06	3.61	3.20	3.13	3.52	3.60	3.10	5.32	4.03	3.60
P (0.81-1.45mmol/L)	1.00	0.85	0.45	0.54	/	/	0.68	0.79	0.89	/

Ca, Calcium; K, Potassium; P, phosphorus; PTH, Parathyroid hormone; Day 0, date of surgery; /, not detected.

TABLE 2 | Laboratory examinations before and after parathyroidectomy.

		Pre-operation		Post-operation		1st Follow-up	2nd Follow-up
Blood routine tests	WBC (3.5-9.5X109/L)	9.77		5.45		/	/
	RBC (3.8-5.1X1012/L)	3.42		2.29		/	/
	Hb (115-150 g/L)	111		73		/	/
	PLT (125-350X109/L)	312		305		/	/
Liver function	ALT (≤33 U/L)	63		<5		/	/
	AST (≤32 U/L)	56		11		/	/
	ALP (35-105 U/L)	208		227		/	/
Renal function	BUN (2.6-7.5 mmol/L)	6.39		6		/	/
	Cr (45-84 μmmol/L)	135		136		/	/
	UA (142.8-339.2 μmol/L)	350		298		/	/
	eGFR (>90 mL/min/1.73m2)	38.6		38.3		/	/
Glucose and insulin tests	FPG (4.11-6.05 mmol/L)	6.02		/		/	/
	FINS (1.8-11.8 μIU/mL)	17.6		/		/	/
Sex hormone	PRG (0.00-0.78 ng/mL)	2.06		/		/	/
	FSH (16.74-113.59 mIU/mL)	82.48		/		/	/
	LH (10.87-58.64 mIU/mL)	51.85		/		/	/
	PRL (2.74-19.64 ng/mL)	25.15		/		/	/
	Estradiol (≤40 pg/mL)	33		/		/	/
	Testosterone (≤0.75 ng/mL)	0.31		/		/	/
	β-HCG (≤8.3 mIU/mL)	0.46		/		/	/
ACTH and Cortisol		1mg DXM suppression		High dose DXM suppression			
		Before	After	Before	After		
	ACTH (1.6-13.9 pmol/L)	2.89	2.21	9.85	0.72	6.16	4.37
	8a.m. Cortisol (60.2-184 μg/L)	221	131	116.5	22.87	113	154.2
	4p.m. Cortisol (26.8-105 μg/L)	287.7	/	/	/	/	/
	12MN Cortisol (μg/L)	281.4	/	/	/	/	/
Renin and	Renin (4.4-46.1 μIU/mL)	/		119.4		64.4	/
Aldosterone	Aldosterone (0-353 pg/mL)	/		153		104	/
concentrations	ARR	/		1.3		1.6	/
Adrenal medullary hormone	Metanephrine (≤0.21nmol/L)	0.18		/		/	/
	Normetanephrine (≤0.59 nmol/L)	0.36		/		/	/
Others	GH (0-10 ng/mL)	0.92		/		/	/
	IGF-1 (255±85 ng/mL)	183		/		/	/
	25-hydroxy vitamin D (>30 ng/mL)	7.1		/		/	/

ACTH, adrenocorticotrophic hormone; ALP, alkaline phosphatase; ALT, alanine transaminase; ARR, aldosterone-renin ratio; AST, aspartate transaminase; BUN, urea nitrogen; Cr, creatinine; DXM, dexamethasone; FPG, fasting plasma glucose; FINS, fasting insulin; FSH, follicular stimulating hormone; GFR, glomerular filtration rate; GH, growth hormone; Hb, hemoglobin; HCG, human chorionic gonadotropin; IGF-1, Insulin-like growth factor 1; LH, luteinizing hormone; PLT, blood platelet; PRL, prolactin; PRG, progesterone; RBC, red blood cell; UA, uric acid; WBC, white blood cell; /, not detected.

was also evaluated, and it was identified as wild type at this genetic locus (**Figure 1D**).

Radiological screening tests for MEN1-associated tumors were conducted at the meanwhile. Considering the eGFR of the patient, the non-contrast-enhanced magnetic resonance imaging (MRI) of pituitary was performed, which showed a slight decrease of the T1 signal of posterior pituitary (**Figure 1E**). The non-contrast-enhanced adrenal computed tomography (CT) scan demonstrated bilateral nodular enlargement, considering as hyperplasia or adenoma (**Figure 1F**). There were no abnormalities observed from CT of lung, pancreas, or gastrointestinal tracts, except for nephrolithiasis in both kidneys (**Supplementary Figure 2A**) and punctate high-density shadows in the left breast (**Supplementary Figure 2B**). Breast ultrasound and mammography were not conducted because of the objection of the patient.

The patient came for the first follow-up visit two and half months after surgery. Calcium and vitamin D were regularly taken with the unchanged dosage while potassium replacement has been stopped since the operation. As shown in **Tables 1**, **2**, PTH level increased again (219.60 pg/mL) but serum calcium levels along with ACTH, cortisol, potassium, renin and aldosterone concentrations were within normal range. It was noteworthy to mention that the PTH on the second follow-up visit 4.5 months after surgery decreased to 153.50 pg/mL with serum calcium 2.31 mmol/L (**Table 1**).

DISCUSSION

MEN1 is a rare autosomal dominant hereditary tumor syndrome caused by a germline mutation on chromosome 11q13 (3). MEN1 gene is a tumor suppressor gene, encoding the protein menin, which plays a role in regulating gene expression and cell proliferation through selectively mediate chromatin remodeling (2).

Patients with MEN1 can present with a wide variety of manifestations including PHPT, pituitary tumor, adrenal lesion, lipoma, myoma of uterus, gastroenteropancreatic neuroendocrine tumors (GEP-NET), and breast cancer (**Table 3**), among which PHPT is one of the most frequent presentations (10). In this case, the patient presented variable clinical manifestations including bilateral enlargements of adrenal glands, mammary mass, uterine

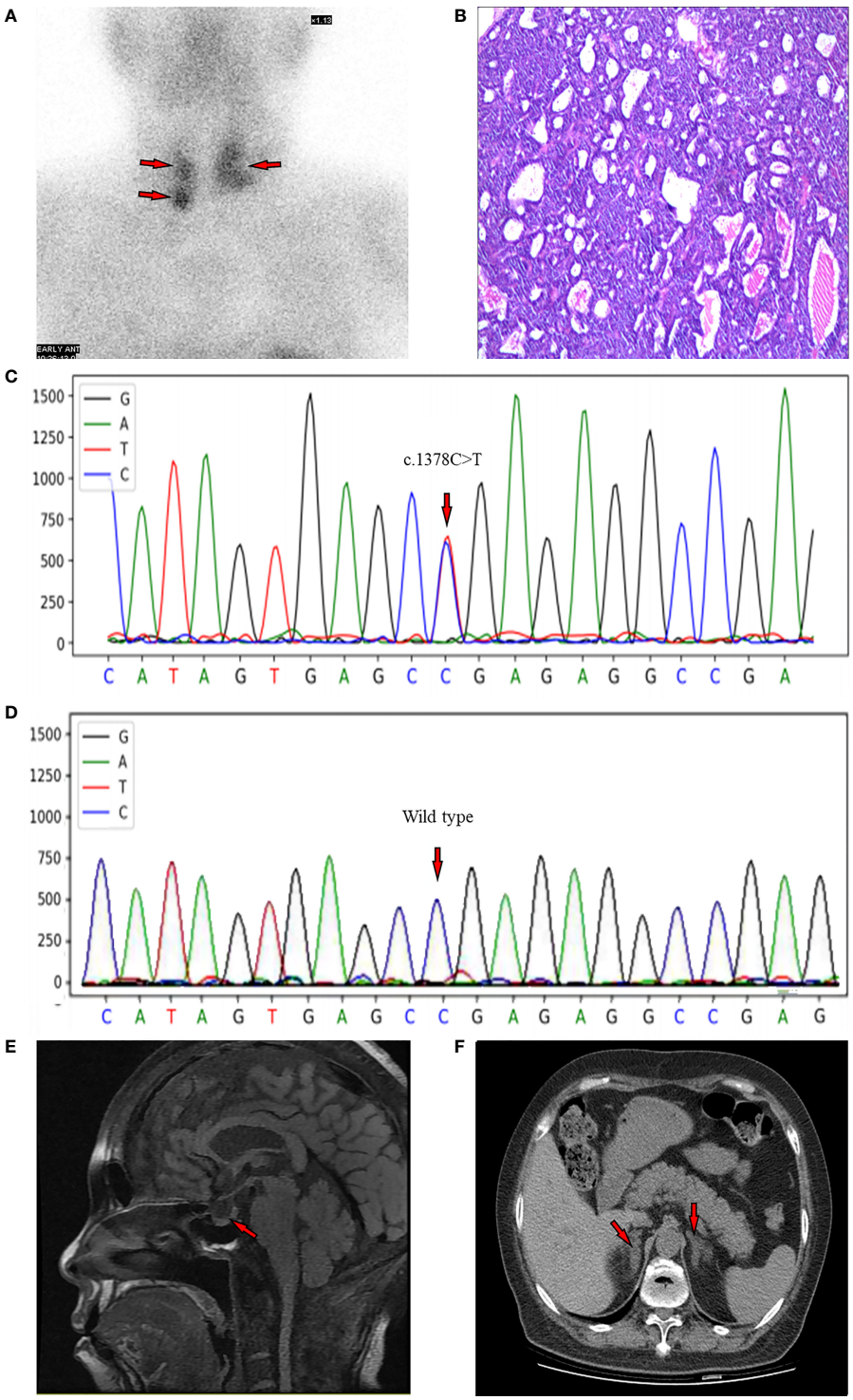

FIGURE 1 | **(A)** MIBI scan of the parathyroid with three focal uptakes. **(B)** Histopathologic image of tissue parathyroidectomy. **(C)** Result of sequencing of MEN1 gene of the proband. The red arrow indicates the mutation of c.1378C>T (p.Arg460Ter) in exon 10. **(D)** The genetic locus of the son of proband. **(E)** Non-contrast-enhanced MRI scan of pituitary with a slight decrease of the T1 signal of posterior pituitary. **(F)** CT scan of adrenal gland showing bilateral nodular enlargement.

TABLE 3 | Clinical concomitant manifestations of MEN 1.

MEN1-related lesion	Mean age 1 (years)	Mean age 2 (years)	n/included MEN1 (%)	First manifestation (%)	Malignancy n (%)	Functional endocrine gland n (%)	Reference
PHPT	38.6 ± 14.9	45.1 ± 18	405/436 (93%)	291 (67%)	/	405 (100%)	(10)
	/	/	32/33 (96.9%)	/	/	32 (100%)	(11)
	/	/	19/20 (95%)	/	/	/	(12)
	/	/	41/49 (83.7)	/	/	/	(13)
	39	/	8/9 (89%)	4 (44.4%)	/	/	(14)
Pituitary tumor	33.4 ± 14.7	38.7 ± 15.7	178/436 (41%)	56 (12.8%)	/	142 (80%)	(10)
	/	/	16/33 (48.5%)	/	/	/	(11)
	/	/	9/20 (45%)	/	/	7 (77.8%)	(12)
	/	/	20/49 (40.8%)	/	/	/	(13)
Adrenal lesion	40 ± 4	42 ± 4	15/436 (3.4%)	2 (0.5%)	/	2 (14%)	(10)
	/	/	12/33 (37%)	/	/	/	(11)
	/	/	7/20 (35%)	/	/	0 (0%)	(12)
	45	39.6	18/67 (26%)	/	4(22.2%)	8 (44.4%)	(15)
	42.7	35.8	21/38 (55%)	/	1(4.7%)	3 (14.3%)	(16)
	/	/	30/49 (61%)	/	/	2 (6.7%)	(13)
	52.2	35.9	9/16 (56.3%)	/	/	2 (12.5%)	(14)
	46.1 ±1.4	/	146/715 (20.4%)	9 (1.2%)	10(13.8%)	11 (15.3%)	(17)
Lipoma	45	52	130/436 (30%)	1 (0.25%)	/	/	(10)
	/	/	4/20 (20%)	/	/	/	(12)
Myoma of uterus	34	48	2/5 (40%)	/	0	/	(18)
GEP-NET	37.3 ± 14.5	44.6 ± 16.1	230/436 (53%)	81 (18.6%)	/	94 (41%)	(10)
	/	/	24/33 (72.7%)	/	/	/	(11)
	/	/	20/20 (100%)	/	/	/	(12)
Breast cancer	48 ± 8.8	/	44/865 (5.1%)	/	44 (100%)	/	(4)
	/	/	1/20 (5%)	/	1 (100%)	/	(12)

Mean age 1, the mean age at diagnosis of MEN1-related lesion; Mean age2, the mean age at the diagnosis of MEN1; MEN-1, multiple endocrine neoplasia type 1; PHPT, primary hyperparathyroidism; GEP-NETs, gastroenteropancreatic neuroendocrine tumors; /, not reported.

leiomyoma, and transient hypercortisolism, in addition to multiple parathyroid adenomas.

PHPT most commonly manifests a single benign parathyroid adenoma (80%). Multiglandular disease is only seen in approximately 15%-20% of patients (19). One observational study reported that multiple adenomas or hyperplasia developed in only 7% of PHPT patients (20). On the contrary, the probability of two or more abnormal parathyroids are significantly higher in MEN1-associated PHPT (56%) (20). Thus, multiple parathyroid adenomas in this PHPT patient raised our concerns on the diagnosis of MEN1.

The adrenal lesions including cortical adenomas, hyperplasia, multiple adenomas, nodular hyperplasia, cysts, or carcinomas, are also commonly seen in MEN1 patients (**Table 3**), the percentage of which ranged from 3.4% to 61% (10–17). However, hormonal hypersecretion is rare and most of the lesions are nonfunctional (17). Importantly, nonfunctional adrenal tumors in MEN1 patients may develop into hypersecretion carcinoma (16). Waldmann J et al. reported that one in twenty-one MEN1 patients with nonfunctional adrenal tumor developed cortisol and testosterone-secreting adrenocortical carcinomas within 9 months (16). The analysis of 24 published studies covering more than 2500 cases of adrenal incidentaloma, showed a 0.1% pooled risk of developing malignancy (21). In the current case, although bilateral adrenal glands were both enlarged, levels of adrenal hormones including renin, aldosterone, metanephrine and normetanephrine were normal. Interestingly, the cortisol level was elevated along with impaired circadian rhythm before parathyroidectomy and it could not be inhibited by 1-mg overnight DST (cutoff value: serum cortisol >1.8 μg/dL) (5). Howbeit, the increased serum cortisol rapidly returned to normal range after the operation (**Table 2**), indicating a transient hypercortisolism. Possibly, the activation of adrenal cortical function in this case may be caused by the chronic condition of PHPT. However, a close follow-up of adrenal glands is recommended.

Similar to this study, the transient hypercortisolism along with increased ACTH was also reported on in a patient with PHPT other than MEN1 (22). In an observational study conducted by Rajput et al., patients with PHPT also presented loss of circadian rhythm while their plasma ACTH and morning serum cortisol were in normal range (23). The structural similarity between 15-25 amino acid of PTH and 1-11 amino acid of ACTH (24) enables PTH in high concentration to stimulate the cortisol secretion (25). This assumption is further supported by an *in vitro* experiment, in which PTH and PTH-related peptide stimulated the secretion of cortisol from dispersed human adrenocortical cells, through adenylate cyclase (AC)/protein kinase A (PKA)- and phospholipase C (PLC)/protein kinase C (PKC)-dependent signaling pathways (26). Besides PTH, calcium is also able to exert an influence on ACTH and cortisol release (27). A human study by Fuleihan et al. suggested that an calcium infusion may result in an increase of baseline ACTH levels (28). Based on these studies, the transient ACTH-independent hypercortisolism in the current case may be attributed to the increased levels of PTH and calcium (23). Nevertheless, the fluctuation of cortisol level and

even the false-positive results of the 1 mg DST may also happen because of aging, hospitalization, psychiatric and stress (29). Further investigations in the pathological mechanisms and related cohort studies are necessary to disclose the root cause of the transient fluctuation in cortisol level in patients with MEN1.

There are several case reports identifying the hypokalemia in patients with PHPT (30–32) though the underlying pathogenesis was not clear. One mechanism assumption is based on renin-angiotensin-aldosterone system (RAAS) although the relationship between PTH and RAAS is still under debate. It has been reported that PTH and calcium can trigger the secretion of aldosterone *in vitro* as well as in animal models (26, 33, 34). On the contrary, a study included patients with PHPT before and after surgery demonstrated that PTH was weakly correlated with plasma renin activity but had no correlation with serum aldosterone (35). More recently, Maniero et al. showed a highly significant increase in the number of cases of HPT among patients with confirmed primary hyperparathyroidism (PA) (36), thus suggesting a bi-directional link between the adrenocortical zona glomerulosa and the parathyroid gland. The limitation in the current case is the lack of the preoperative values of renin and aldosterone. Nevertheless, it can be speculated that the hypokalemia of this patient may be related to the increased PTH and calcium levels, since the blood potassium could gradually return to normal without any potassium supplementation after parathyroidectomy.

Both *in vitro* and *in vivo* preclinical studies suggest that *MEN1* gene is implicated to the occurrence and development of breast cancer (37, 38). Several case reports (39) and human observational studies also support the conclusion that female MEN1 patients suffer increased risk for breast cancer, the standardized incidence ratio of which is ranged from 1.96 to 2.14 (4). A mammary mass was identified in this case (**Supplementary Figure 2**). Although the patient refused further examinations, cancer surveillance was recommended due to the potential risk of breast cancer in subjects with MEN1. In addition, McKeeby et al. reported the potential relationship between uterine leiomyoma and MEN1. Five of six uterine leiomyomata in two patients with MEN1 exhibited 11q13 loss of heterozygosity (LOH), indicating that smooth muscle tumors of uterus in MEN1 patients may develop through the inactivation of MEN1 gene (18).

According to the clinical practice guideline for MEN1 (3), a diagnosis of MEN1 may be established based on one of three criteria, defined from clinical, familial and genetic perspectives. MEN1 mutational analysis should be taken under the following situations: 1) an index case with two or more MEN1-related endocrine tumors; 2) first-degree relatives of an MEN1 mutation carrier; 3) in patients with suspicions or atypical for MEN1 (3). The last situation with multiple parathyroid glands involvement is an indication for MEN1 mutation testing (3), which might be neglected due to insufficient knowledge of MEN1. In the current case, although there were no evidence suggesting that the patient's first-degree relatives were MEN1 mutation carriers, MEN1was still highly suspicious because of multiple parathyroid adenomas. Accordingly, a genetic testing was performed and a mutation of c.1378C>T (p.Arg460Ter) in exon 10 was identified, which has been previously reported in MEN1 patients (40).

The therapeutic strategy was similar to that of the specific tumors in non-MEN1 patients. The MEN1 guideline (3) recommend 3.5 glands SPTX or total parathyroidectomy (TPTX) for MEN1-related PHPT; however, no clear conclusion on which option is better, considering the recurrent rate and hypoparathyroidism (41–43). In addition, one should note that the treatment effect in MEN1 patients may not be comparable to that of non-MEN1 patients, because multiple endocrine tumors may be larger, more aggressive, and poorly respond to the treatment. It has been reported that MEN1-related PHPT has a higher recurrence rate compared to PHPT in non-MEN1 patients (40-60% versus 4-16%) (3). Thus, periodic clinical surveillance is required, including biochemical test and imaging screening.

Limitation

The patient had hysterectomy a long time ago and we cannot verify the MEN1 mutation in her fibroid. Additionally, preoperative renin and aldosterone were not tested immediately because of the patient's poor health conditions and hypokalemia. Lack of these values makes it difficult to determine the role of RAAS in the pathogenesis of hypokalemia in the current case. Meanwhile, a lesion in left breast was identified during her hospitalization, but this patient refused further examination to evaluate the possibility of breast cancer.

CONCLUSION

In this case of PHPT, multiple parathyroid adenomas draw our attentions, which prompted us to test MEN1 gene mutation and to screen for other neuroendocrine tumors (NETs). The transient hypercortisolism may present in MEN1-associated PHPT, and return to normal after parathyroidectomy, along with decreases in serum calcium and PTH. In addition, MEN1 related tumors may grow at any time and convert from non-functional tumor to malignancy. Accordingly, follow-up with the biochemical and imaging screening for endocrine organs should be performed periodically and closely.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science & Technology.

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All the authors have contributed significantly. FC collected the clinical data, wrote the manuscript. QX summarized the relevant literature. WY and XY give suggestions about clinical investigations. All the work was done under the instructions of SS. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.802453/full#supplementary-material

Supplementary Figure 1 | The timeline of hospitalization and follow-up visits.

Supplementary Figure 2 | (A) Renal CT scan. **(B)** Chest CT scan.

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CASE REPORT
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Pasireotide for Refractory Hypoglycemia in Malignant Insulinoma- Case Report and Review of the Literature

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Malignant insulinomas are functional neuroendocrine tumors of the pancreas and the primary cause of tumor-related hypoglycemia. Malignant insulinoma is rare and has a poor prognosis. We report a case of metastatic malignant insulinoma in a 64-year-old female patient with severe and refractory hypoglycemia. After several ineffective locoregional and systemic therapeutic lines for the secretory disease, the introduction of pasireotide, a second-generation somatostatin analog, provided an improved clinical and secretory evolution both quickly and sustainably, with an excellent safety profile. Pasireotide is an effective and well-tolerated therapy in the treatment of refractory hypoglycemia in metastatic insulinoma.

Keywords: pasireotide, refractory hypoglycemia, neuroendocrine tumor, pancreatic neuroendocrine cancer, malignant insulinoma

INTRODUCTION

Insulinomas, functional neuroendocrine tumors of the pancreas, are the leading tumor-related cause of hypoglycemia with an estimated incidence of 1-3 per million per year (1) and 4% to 14% of insulinomas are characterized as malignant due to the presence of locoregional extension and/or metastatic spread. Metastatic insulinomas are almost well differentiated and the presence of liver metastases worsens their prognosis (2). Despite a large therapeutic arsenal including somatostatin analogs, debulking surgery, hepatic arterial embolization, percutaneous local tumor ablation, targeted therapies, Peptide Receptor Radionuclide Therapy (PRRT), and chemotherapy, malignant insulinoma still has a poor prognosis (3). Glycemic control and tumor volume control are the two therapeutic objectives.

We report a case of metastatic malignant insulinoma in a 64-year-old female patient with severe and refractory hypoglycemia despite having received several lines of treatment. The introduction of pasireotide, a second-generation somatostatin analog, has resulted in rapid and lasting control of blood glucose levels and a clinical benefit.

CASE DESCRIPTION

A 64-year-old woman with no significant past medical history presented, in February 2016, with recurrent episodes of non-fasting hypoglycemia with neuroglycopenic symptoms, which resolved with meal. She was in good general condition with a performance status of 0, a weight of 62 kg, and a height of 171 cm.

Thoracoabdominopelvic computed tomography (CT) and magnetic resonance imaging (MRI) of the liver revealed a hypervascular mass in the tail of the pancreas associated with liver bilobar metastatic spread. An endoscopic ultrasound found a 16 x 30 mm mass in the tail of the pancreas, demonstrated a paucicellular synaptophysin positive sample with a Ki-67 of less than 5%. A liver biopsy of a metastasis confirmed a well-differentiated grade 2 neuroendocrine tumor with Ki-67: 4%. In initial laboratory results, elevated Chromogranin A (CgA) levels of 2219 ng/mL (27-94), normal NSE levels of 12.5 ng/mL (<17), normal C peptide levels of 0.38 pmol/L (0.3-1.4) and normal insulin levels of 96 pmol/L (18-173) and glucose level of 0.30g/L (0.74- 1.06) were detected.

We initiated a somatostatin analog treatment with standard dose long acting-release (LAR) octreotide (30 mg). From the first injection of the drug an exacerbation of hypoglycemic crises occurred motivating the early discontinuation of octreotide after a unique dose. In July 2016, the patient was hospitalized for hypoglycemic coma. Diazoxide administration and trans-arterial chemoembolization (TACE) of the right hepatic lobe were performed, improving glycemic control with a significant reduction of hypoglycemic episodes.

In March 2017, attempted reintroduction, for tumor control, of another LAR somatostatin analog, lanreotide (120 mg every 4 weeks), rapidly lead to a recurrence of hypoglycemic episodes and resulted in definitive discontinuation of first-generation somatostatin analogs in June 2017. In August 2017, everolimus was introduced for glycemic control but had to be discontinued early, ten days later, due to grade 3 thrombocytopenia.

In September 2017, a second TACE was performed and resulted in blood glucose levels control. A third TACE in April 2018 was indicated for hepatic progressive disease. Hypoglycemic events quickly recurred in July 2018 requiring 30% glucose infusion.

In October 2018, ^{18}F-FDG positron emission tomography/computed tomography (^{18}F-FDG-PET/CT) revealed multiple hypermetabolic lesions of the liver (SUV_{max}=18.3), pancreas (SUV_{max}= 7.3), and lymph nodes (SUV_{max} =7.8) (**Figure 1).** From December 2018 to October 2019, everolimus was reintroduced at a reduced dose of 5 mg, due to the previous hematological toxicity, allowing radiological stable disease without control of hypoglycemia events.

After a fourth ineffective TACE, a hepatic debulking surgery was performed in June 2019. Hepatic histological analysis confirmed a well-differentiated grade 2 neuroendocrine tumor with an increased Ki-67: 18% (versus 4% in 2016). Immunostaining showed strong somatostatin receptor 2 (SSTR2) positivity and a weak SSTR5 expression (**Figure 2**)

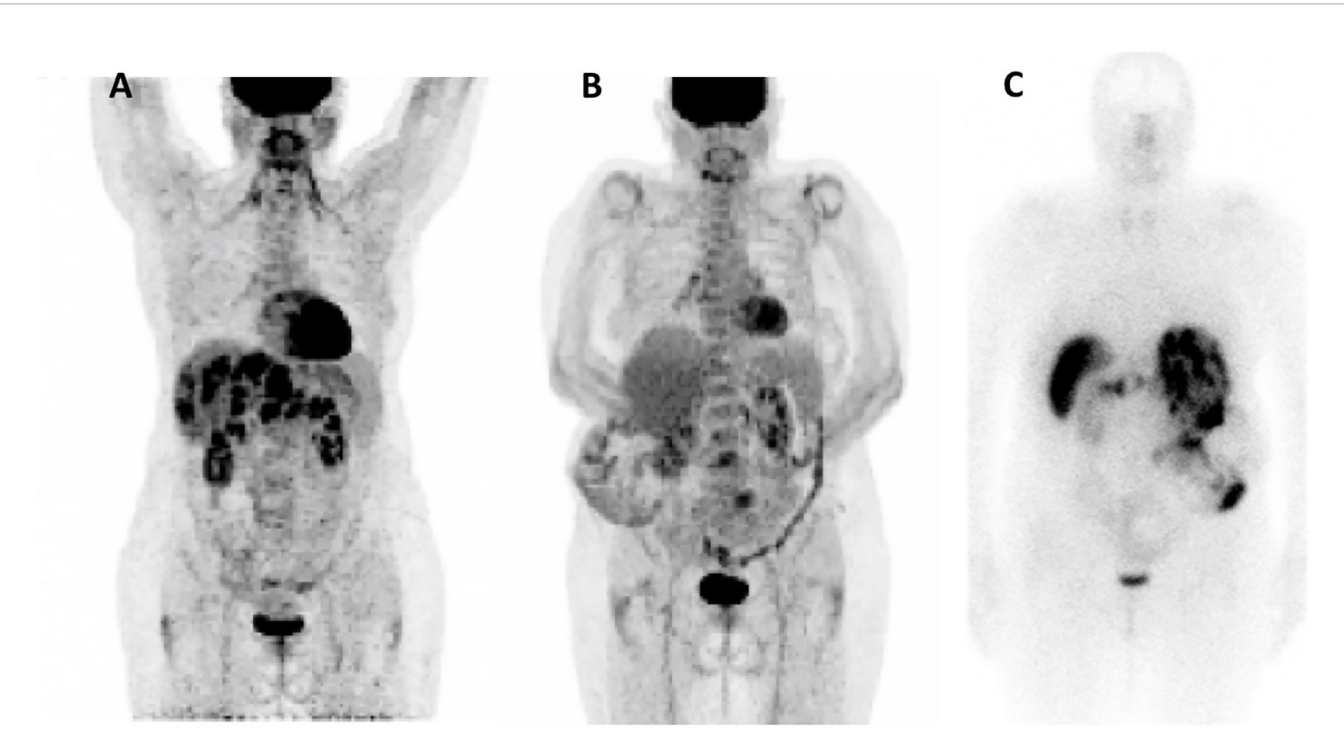

FIGURE 1 | Nuclear Imaging. **(A)** ^{18}F-FDG-PET/CT Oct 2018; **(B)** ^{18}F-FDG-PET/CT Dec 2020; **(C)** Octreoscan Dec 2020.

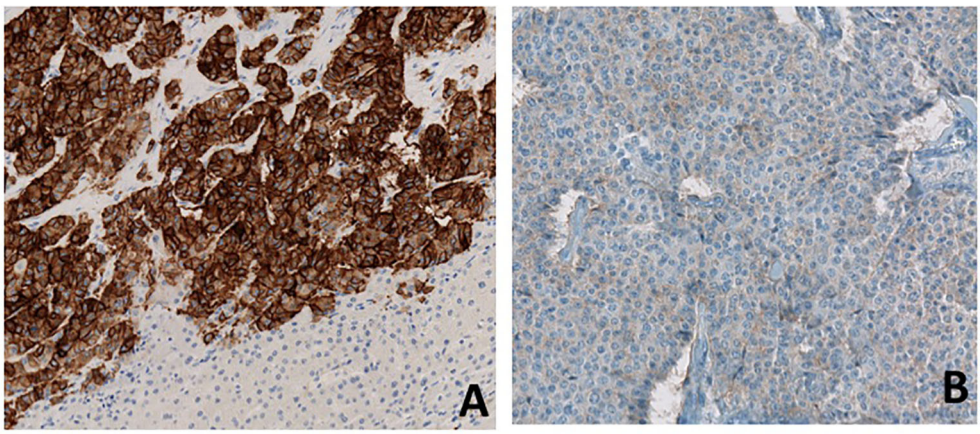

FIGURE 2 | Immunostaining for SSTR2 **(A)** and SSTR5 **(B)**. Immunostaining of metastatic hepatic lesion shows high expression of SSTR2 and low expression of SSTR5.

The patient was hospitalized from the end of October 2019 to the end of December 2019 for severe refractory hypoglycemia, seizures and a deterioration of the clinical status. After everolimus discontinuation, sunitinib was started at a dose of 37.5 mg/day. Despite continuous 30% glucose infusions and corticosteroid therapy, glycemic control was insufficient. Subcutaneous short-acting pasireotide at a dose of 0.9 mg every 12 hours was initiated, resulting in rapid clinical status improvement and a prompt decrease of the frequency and severity of hypoglycemic events counted by a continuous glucose monitoring (**Figure 3**).

Sunitinib was discontinued in June 2020 due to both recurrent hypoglycemia and an increase in CgA levels to 55173ng/ml with radiological stable disease. A capecitabine and temozolomide combination (CAPTEM) was administered while continuing pasireotide. Then, the patient was able to be managed at home with good glycemic control. Three months after starting the chemotherapy in association with pasireotide, a good tumor and secretory response was observed allowing progressive discontinuation of corticosteroids and glucose infusions. CAPTEM was continued until December 13, 2020 with dose reduction due to grade 3 thrombocytopenia. Short-acting

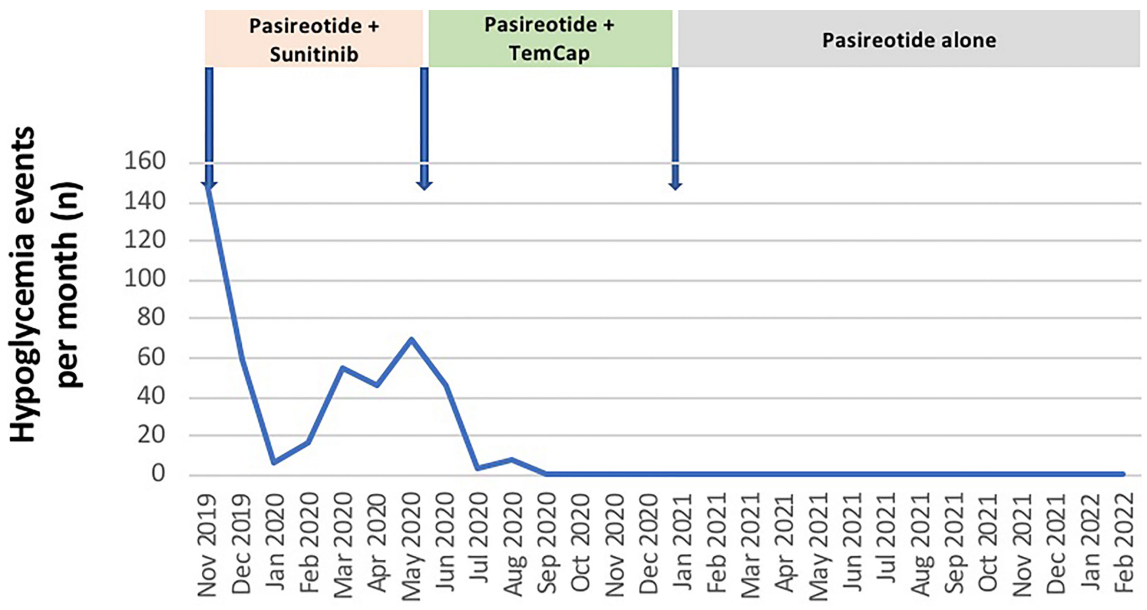

FIGURE 3 | Hypoglycemia events per month since beginning of Pasireotide.

pasireotide has been replaced by 60 mg LAR pasireotide in December 2020 to ensure better comfort. At the same time, a ^{18}F-FDG-PET/CT revealed a complete metabolic response compared to 2018 and Octreoscan showed intense hyperfixation of all secondary hepatic lesions in favor of SSTR2 expression (**Figure 1).** Tumoral response was assessed by hepatic MRI showing a partial response on December 2020 sustained through February 2022 (**Figure 4**). CAPTEM was discontinued after 6 cycles due to a good partial response and persistent grade 2 trombocytopenia. The anti-proliferative and anti-secretory treatments since diagnosis are detailed in **Figure 5**.

Pasireotide has been continued without interruption since its introduction. The patient has been treated as an outpatient for 26 months with monthly intramuscular injections of pasireotide LAR. Given the sustained glycemic control, the dose of pasireotide LAR could be progressively decreased from 60 mg to a dose of 20 mg in February 2022. Blood glucose levels have been completely normalized for over 18 months at this point, resulting in a significant improvement in quality of life.

DISCUSSION AND CONCLUSION

We report here a case of metastatic insulinoma with severe and refractory hypoglycemia that showed a good and durable secretory response to pasireotide, as well as good tumor response to the combination of CAPTEM and pasireotide.

Pasireotide is a second-generation multi-somatostatin receptor ligand with an affinity for four of the five SSTRs especially SSTR5, followed by SSTR2, SSTR3, and SSTR1. It is currently approved for the treatment of Cushing's disease (4) and acromegaly, with a safety profile similar to the first-generation somatostatin analogs, octreotide or lanreotide, but with an increased risk of hyperglycemia (5). In patients treated with pasireotide for Cushing's disease or acromegaly, the hyperglycemic effect may be explained by the different binding affinities to the different SSTR subtypes and by suppression of insulin secretion from normal pancreatic islet *via* SSTR5 activation.

Only a few cases of metastatic insulinoma treated with pasireotide have been described in the literature. Tirosh et al. described a case of metastatic insulinoma treated with pasireotide provided good glycemic control, but not antitumor efficacy, when compared to lanreotide and everolimus (6). Siddiqui et al. also reported a case of metastatic insulinoma presenting refractory hypoglycemia despite diazoxide and octreotide treatment with rapid control *via* pasireotide, which was finally stopped due to diabetes (7). Finally, Sileo et al. reported a case of benign insulinoma in a patient who was a poor candidate for surgery because of elderly and comorbidities and who achieved preoperative glycemic control with pasireotide, allowing for surgery of the pancreatic lesion in optimal clinical and biological conditions (8).

Unfortunately, pasireotide has not yet demonstrated antitumor efficacy in NETs, and it remains unknown whether pasireotide has greater antiproliferative effects than octreotide and lanreotide (9). The efficacy of combination therapy with pasireotide and everolimus in NETs is also controversial, with a reportedly higher response rate but without significant benefit in PFS compared to everolimus alone (10, 11). A phase II clinical trial (NCT01253161) assessed the clinical activity of pasireotide in treatment-naïve patients with metastatic NETs and showed that patients with low hepatic tumor burden, normal baseline chromogranin A, and high tumoral SSTR5 expression experienced the most favorable effect. SSTR 1-5 expression data was available for nearly all patients in this study (12). Thus, further study is required to determine the precise antiproliferative effect of pasireotide in NETs patients irrespective of SSTR expression. In our case, the patient strongly expressed SSTR2 on liver metastases but SSTR5 expression was low (**Figure 2**). However, the expression of SSTRs can be heterogeneous in neuroendocrine tumors and nothing can be concluded from the low positivity of SSTR5 on a single metastatic sample.

In this case, PRRT was not proposed initially due to the high hypermetabolism at ^{18}F-FDG-PET/CT in 2018. However, metabolic imaging data from 2020 would be encouraging to indicate PRRT in case of future progression since the disease strongly expresses SSTR2 and no longer shows FDG hypermetabolism (**Figure 1**).

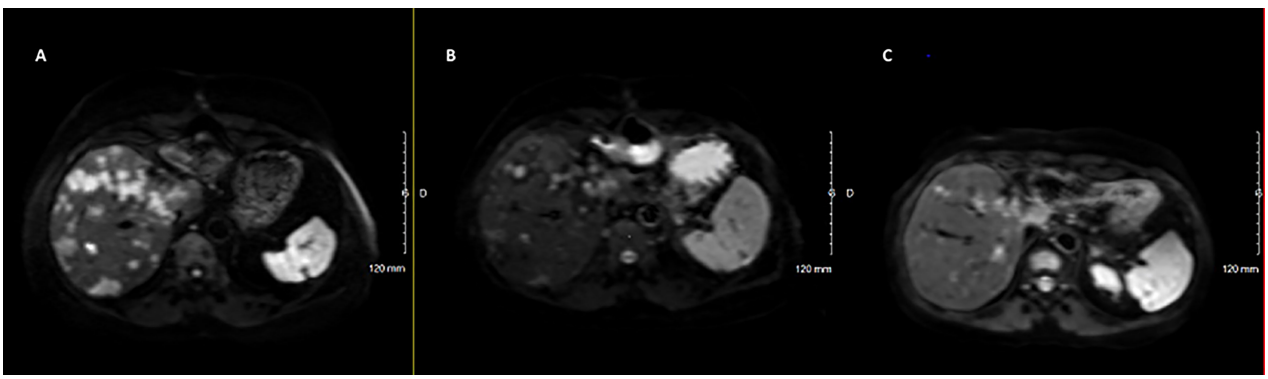

FIGURE 4 | Radiological (MRI Diffusion) to CAPTEM and pasireotide. **(A)** June 2020, before CAPTEM; **(B)** December 2020, after 6 cycles of CAPTEM; **(C)** February 2022, pasireotide alone.

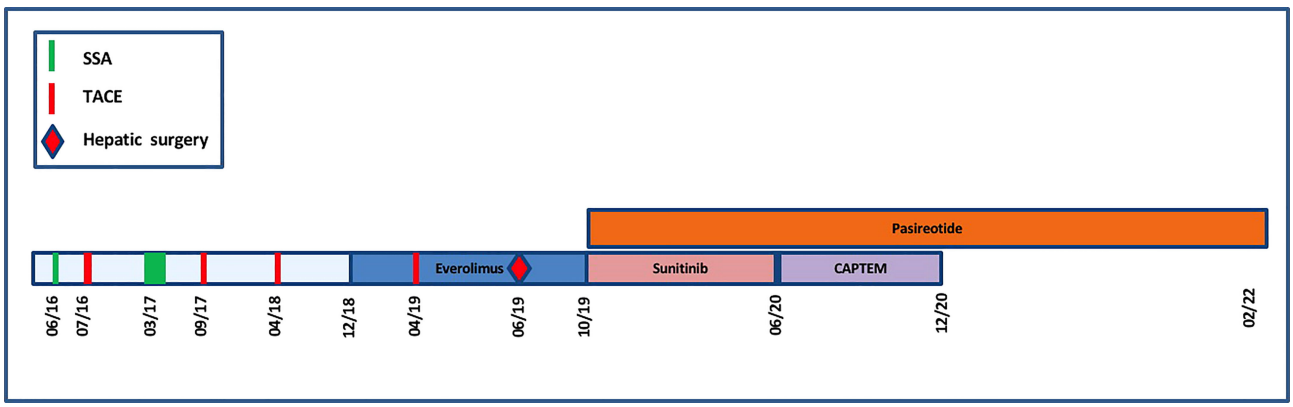

FIGURE 5 | Timeline representing the anti-proliferative and anti-secretory treatments since diagnosis.

The patient exhibited hepatic stable disease with the combination of sunitinib and pasireotide. The antitumor efficacy of sunitinib in PNETs has been proven with a majority of stable disease (13).

In our case, CAPTEM provided a prolonged partial response of the metastatic disease in accordance with literature data showing high and durable response rate in metastatic neuroendocrine tumors (14). The partial response was maintained 14 months after stopping chemotherapy while the patient was only receiving pasireotide. Then antitumor efficacy of pasireotide cannot be specifically assessed in this case.

CONCLUSION

Pasireotide provided rapid glycemic control in a patient with metastatic insulinoma who presented refractory hypoglycemia despite several prior lines of treatment. The combination with temozolomide-capecitabine resulted in liver tumor response with maintenance of excellent glycemic control. Pasireotide may be a therapeutic alternative in the treatment of metastatic insulinoma with refractory tumor induced-hypoglycemia.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SO-T and JM-Q wrote the first draft of the manuscript. SO-T, JM-Q, BC, FP and JE made contributions to the acquisition of the clinical data. SO-T and PN made critical revisions and approval final version. All authors contributed to the article and approved the submitted version.

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Predicting Progression, Recurrence, and Survival in Pancreatic Neuroendocrine Tumors: A Single Center Analysis of 174 Patients

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Introduction: The European Neuroendocrine Tumor Society, ENETS, reports variables of prognostic significance in pancreatic neuroendocrine tumors (PNET). However, studies have short follow-ups, and the optimal treatment remains controversial. We aimed to determine overall survival (OS), progression-free survival (PFS) after conservative treatment, and recurrence-free survival (RFS) after surgery and further to find predictors of aggressive PNET behavior to support treatment decisions.

Methods: 174 patients with PNET treated at Aarhus University Hospital from 2011 to 2021 were included in a retrospective cohort study. Patients were divided into surgically resected (*SUR*, n=91) and medically or conservatively treated (*MED*, n=83). Variables were tested in univariate and multivariate survival analysis. Median follow-up time was 3.4 years in the MED group and 4.5 years in the SUR group.

Results: The 5-year OS was 95% and 65% for the SUR and MED groups, respectively. The 5-year RFS in the SUR group was 80% whereas the 5-year PFS in the MED group was 41%. Larger tumor size, Ki67 index, tumor grade, and stage were predictive of shorter OS, RFS, and PFS. Further, chromogranin A was a predictor of OS. Larger tumor size was associated with higher stage and grade. Only 1 of 28 patients with stage 1 disease and size ≤2 cm developed progression on a watch-and-wait strategy during a median follow-up of 36 months.

Conclusion: This study supported the ENETS staging and grading system to be useful to predict OS, PFS, and RFS in PNET. Further, our data support that small, localized, low-grade PNETS can be followed with active surveillance.

Keywords: pancreatic neuroendocrine tumor (PNET), prognosis, survival, recurrence, ENETS

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs) are rare tumors, constituting 1%-2% of all pancreatic cancers and up to 10% of all NETs (1–3). The incidence has increased in the past 30 years and there has been a significant improvement in survival (3–6). PNETs are classified as functioning (F)- or non-functioning (NF) according to the potential hormone production. NF-PNETs comprise at least 70% and are discovered either incidentally or due to symptoms as a sign of advanced disease (7, 8).

Primary investigation of newly discovered tumors involves staging and grading. The European Neuroendocrine Tumor Society (ENETS) has proposed a staging system, which is widely recognized to predict survival (9). Micro-radical surgery is considered the only curative treatment and is associated with increased overall survival (OS). However, the management is complex, and the benefits of surgery must be weighed against the relatively high risk of perioperative morbidity (3, 10–12). Surgery is considered in early-stage disease but is often contraindicated in widely metastatic disease or in patients with a poor performance status (13, 14).

The incidental detection of asymptomatic PNETs is increasing along with the availability and sensitivity of imaging techniques. These pancreatic incidentalomas (PI) are small and resectable but often have indolent biology. Therefore, well-differentiated PNETs ≤2 cm are often managed conservatively (10, 15). However, approximately 10% of tumors ≤2 cm have lymph node involvement (16, 17), and a meta-analysis suggested a survival benefit for surgery even in smaller tumors (18).

Thus, our study aimed to investigate tumor characteristics in patients with PNETs related to both prognosis and aggressiveness based on the ENETS guidelines. Further, we wished to perform a subgroup analysis of localized PNETS ≤2 cm to test the current guidelines recommending a watch-and-wait strategy in small PNETS (19).

METHODS

In this single-center retrospective study, we identified 174 patients with PNET referred to Aarhus ENETS NET center of excellence from 2011 to September 2021. Ninety-one were surgically resected (SUR group) and 83 were medically treated and/or actively followed with a watchful wait strategy (MED group) according to the ENETS guidelines (19). The study was approved as a quality assurance project by The Central Denmark Region Committees on Health Research Ethics. Diagnosis was based on histology or somatostatin receptor-based imaging. See **Figure 1**.

Data were collected from the online record system, Electronic Patient Journal, in 2021 through the unique Civil Personal Registration numbers, given to all Danish citizens and residents (20). It was managed using REDCap electronic data capture tools (21) hosted at Aarhus University.

Patient and tumor characteristics, scan results, pathology, and biochemistry at diagnosis were collected. Tumors were staged according to the ENETS TNM-system (9) and graded based on the Ki67 index at diagnosis into grades 1 (<3%), 2 (3%-20%), and 3 (≥20%) as per 2019 WHO classification (22). Grade 3 comprised both well-differentiated NET-G3 and poorly differentiated neuroendocrine carcinomas, NEC-G3 (10, 11).

Outcome

Patients were followed until the time of death or until the end of follow-up on September 25, 2021. Median follow-up time was 3.4 years in the MED group and 4.5 years in the SUR group. The primary endpoints were OS, progression-free survival (PFS) in the MED group, and recurrence-free survival (RFS) in the SUR group.

Statistical Analysis

Data were analyzed using Stata 17 (*StataCorp, College Station, TX USA*). OS was time from diagnosis to death; PFS was time from diagnosis to progression, determined as clinical progression at a multidisciplinary NET tumor board meeting; and RFS was time from surgery to recurrence, or until the end of follow-up. Patients followed for less than 3, 5, and 10 years were censored in the respective survival analysis. OS, PFS, and RFS were calculated with Kaplan-Meier methodology, and log-rank tests compared categorical variables across subgroups. Cox proportional hazard models were used to estimate HR with 95% confidence interval for all significant variables in a univariate analysis and finally in a

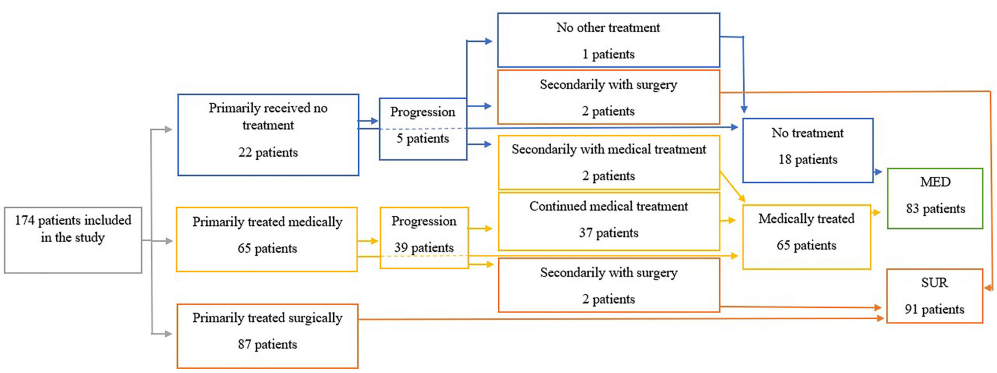

FIGURE 1 | The flowchart of the study. MED, group of patients treated medically or with watchful wait; SUR, surgically treated group.

multivariate analysis to identify independent predictors. Subgroups and association in-between variables were compared using simple t-tests. P-values ≤0.05 were considered statistically significant.

RESULTS

Of 174 patients, 48% were females and the average age was 65.8 ± 12 and 54.7 ± 13 in the MED and SUR group, respectively. Clinicopathological characteristics are summarized in **Table 1**.

In the overall MED group, 53% experienced progression and 29% died of PNET. In the SUR group, 16% had recurrence and 6% died of PNET. The 5-year OS was 65% in the MED group versus 95% in the SUR group ($p \leq 0.05$). Due to selection bias, the MED and SUR groups are not directly comparable. The MED group had more advanced disease, more co-morbidities, and higher age (**Table 1**).

Predictors of OS and PFS in the MED Group

The 83 patients in the MED group comprised 20 patients who received no initial treatment. The mean tumor size was 1.3 ± 10 cm. Further, the group comprised 63 patients, medically treated from the time of diagnosis. The mean tumor size was 4.6 ± 4 cm.

The 3-, 5-, and 10-year OS was 77%, 65%, and 38%, and the 3-, 5-, and 10-year PFS was 54%, 41%, and 33%, respectively (**Figure 2**).

F-PNET, Chromogranin A (CgA) ≥200pmol/L, high Ki67 index, G3-NEC, lymph node positivity, and tumor stage IV were significant negative predictors of OS (**Tables 2**, **3**). CgA, Ki67 index, and tumor stage IV were also significant in the multivariate analysis ($p \leq 0.05$).

Incidental discovery, size ≥ 2 cm, a higher Ki67 index, G2/G3-NET, G3-NEC, local infiltration, lymph node positivity, and tumor stages III and IV were significant negative predictors of PFS (**Tables 2**, **3**). In the multivariate analysis, tumor stage IV, Ki67 index, and G3-NEC were significant ($p \leq 0.05$).

Predictors of OS and RFS in the SUR Group

In the SUR group, the 3-, 5-, and 10-year OS were 99%, 95%, and 87%, and the 3-, 5-, and 10-year RFS was 85%, 80%, and 80%, respectively (**Figure 2**).

Ductus dilatation, high Ki67 index, and G3-NEC were significant negative predictors of OS (**Tables 2**, **3**). In the multivariate analysis, G3-NEC was significant ($p \leq 0.05$).

Size ≥ 2 cm, Ki67 index, G2-NET, G3-NEC, local infiltration, lymph node positivity, and tumor stages III and IV were significant negative predictors of RFS (**Tables 2**, **3**). In the multivariate analysis, tumor stage IV and lymph node positivity remained significant ($p \leq 0.05$).

Correlation Between Tumor Size, Stage, and Grade

Larger tumor size was related to lymph node positivity, metastatic disease, and higher grade ($p \leq 0.05$, **Table 4**).

TABLE 1 | Clinicopathological data in 174 patients with pancreatic neuroendocrine tumors divided into surgically treated (SUR group, n=91) and medically treated or non-treated (MED group, n = 83).

	MED group*n = 83*	SUR group*n = 91*
Patients, females, n (%)	40 (48)	43 (47)
Age at diagnosis, mean ± SD, Range	65.8 ± 12 (14;85)	54.7 ± 13* (22;81)
Charlson score of comorbidities, mean ± SD	2.3 ± 3	0.9 ± 1*
F-PNET, n (%)	6 (7)	20 (22)*
Insulin	1 (1)	15 (17)
Incidentaloma n (%)	56 (68)	49 (54)
CgA (pmol/L), mean ± SD	866.0 ± 3397	153.3 ± 157
Tumor diameter (cm), mean ± SD	3.7 ± 4	3 ± 2.4
Ductus dilatation on scan, n (%)	14 (17)	9 (10)
Ki67 index (%), mean ± SD	12.3 ± 19	10.7 ± 21
Tumor grade, n (%)		
NET-G1 (<3%)	30 (36)	55 (60)*
NET-G2 (3-20%)	22 (27)	20 (22)
NET-G3 (>20%)	4 (5)	4 (4)
NEC-G3	8 (10)	6 (7)
Tumor infiltration, n (%)	17 (21)	9 (10)*
Lymph node positivity, n (%)	36 (43)	20 (22)*
Tumor stage, n (%)		
I (T1N0M0)	31 (37)	42 (46)
II (T2-3N0M0)	4 (2)	26 (29)*
III (T4N0M0/TaN1M0)	10 (12)	16 (18)
IV (TaNaM1)	37 (45)	7 (8)*
Recurrence/progression, n (%)	40 (48)	15 (17)*
Death due to PNET, n(%)	24 (29)	5 (6)#
Follow-up time (years), mean (range)	3.4 (0.1-11)	4.5 (0.2-11)

**p ≤ 0.05, statistically significant t-test when comparing the baseline characteristics of the MED group to the SUR group #significant difference between the equality of survivor function in a log-rank test. CgA, Chromogranin A; F, functioning; NEC, neuroendocrine carcinoma; PNET, pancreatic neuroendocrine tumor.*

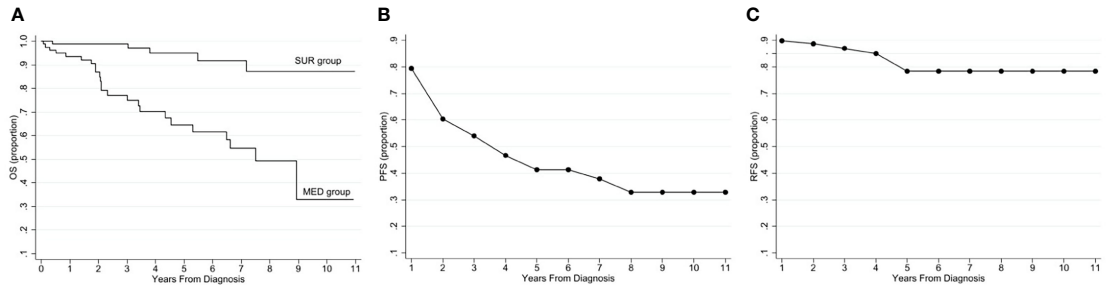

FIGURE 2 | Survival graphs on 174 patients with a primary PNET diagnosis. **(A)** Overall survival in the MED and SUR groups, **(B)** progression-free survival in the MED group, **(C)** recurrence-free survival in the SUR group. MED, group of patients treated medically or with watchful wait; OS, overall survival; PFS, progression-free survival; PNET, pancreatic neuroendocrine tumor; RFS, recurrence-free survival; SUR, group of surgically treated patients.

Subgroups

The frequency of lymph node positivity was 25% in PI versus 47% in symptomatic tumors ($p \leq 0.05$). The mean Ki67 index was 8% in PI and 16% in symptomatic tumors ($p \leq 0.05$). No other variables differed in the two groups. Overall, the F- and NF-PNET groups were identical, except that the patients with F-PNET were younger at diagnosis (54 years) compared to patients with NF-PNET (61 years) ($p \leq 0.05$).

TABLE 2 | Univariate analysis on determinants of mortality and 5-year survival (%) in 174 patients with pancreatic neuroendocrine tumors divided into surgically treated (SUR group, n = 91) and medically treated or non-treated (MED group, n = 83).

	PFS	OS (MED)	RFS	OS(SUR)
Overall survival	41	65	80	95[#]
Age				
<55	37	68	71	97
>55	42	65	90	92
Sex				
Male	50	68	72	90*
Female	31	63	88	100
Incidentaloma	54*	74	85	93
Symptomatic	22	54	77	96
F-PNET	44	33*	75	100
NF-PNET	17	70	90	93
Size				
<20mm	61	83	97	100
≥20mm	35*	62	68*	91
DD	47	51	60	97
No DD	47	73	81	71*
CgA (pmol/l).				
<200	53	78	75	92
≥200	24	50*	67	100
Grade				
NET-G1 (<3)	56	65	92	100
NET-G2 (3-20)	24*	68*	48*	89*
NET-G3 (>20%)	14*	100*	60*	100
NEC-G3	0*	8*	23*	58*
Tumor infiltration	29*	57	37*	100
Localized	44	67	84	91
N0	77	91	94	97
N1	11*	48*	49*	87
Tumor stage				
I	95	100	98	100
II	100	100	79*	92
III	52*	70*	43*	93
IV	7*	46*	55*	83

$p \leq 0.05$, comparing 5-year survivals within the variable categories in a log-rank test [#]$p \leq 0.05$, comparing the overall 5-year survivals in the MED and SUR groups. CgA, Chromogranin A; DD, ductus dilatation; F, functioning; NEC, neuroendocrine carcinoma; NF, non-functioning; OS, overall survival; PFS, progression-free survival; PNET, pancreatic neuroendocrine tumor; RFS, recurrence-free survival.

TABLE 3 | Kaplan-Meier survival analysis in 174 patients with pancreatic neuroendocrine tumors divided into surgically treated (SUR group, n = 91) and medically treated or non-treated (MED group, n = 83).

	OS (MED) HR (95% CI)	PFS HR (95% CI)	OS (SUR) HR (95% CI)	RFS HR (95% CI)
Incidentaloma		0.3 (0.2;0.6)*		
F-PNET	2.8 (1.03;7.7)*			
Tumor size				
≥20mm		3.1 (1.3;7.3)*		9.0 (1.2;68.7)*
Ductus dilatation			12.2 (2.0;73.0)*	
CgA≥200	2.9 (1.2;7.4)*			
Ki67 index**	1.02 (1.01;1.03)*	1.03 (1.02;1.05)*	1.1 (1.02;1.08)*	1.02 (1.01;1.04)*
Grade				
NET-G2 (3-20)	1.2 (0.5;3.2)	3.1 (1.4;6.9)*	3.1 (0.2;50)	9.3 (2.4;36.5)*
NET-G3 (>20)		4.6 (1.2;17.8)*	14.5 (0.9;233.5)	5.0 (0.5;48.3)
NEC-G3 (>20)	6.5 (2.2;19.2)*	16.0 (5.5;46.3)*	23.3 (2.1;260.6)*	20.4 (4.5;93.3)*
Tumor infiltration		2.1 (1.1;4.0)*		3.7 (1.2;11.6)*
Lymph node involvement				
N1	5.0 (1.7;14.7)*	11.1 (4.5;27.3)*		8.1 (2.4;26.7)*
Tumor stage				
II	0.0 (-)	0.0 (-)		6.9 (0.8;61.3)
III	0.0 (-)	11.4 (1.3;101.9)*		24.8 (3.0;202.5)*
IV	8.5e+9 (2.5e+9;2.1e+10)*	48.4 (6.6;355.6)*		22.1 (2.3;212.4)*

**p ≤ 0.05, statistically significant Hazard Ratio when comparing within categories **continuous variable. CgA, Chromogranin A; CI, confidence interval; F, functioning; HR, hazard ratio; NEC, neuroendocrine carcinoma; NF, non-functioning; OS, overall survival; PFS, progression-free survival; PNET, pancreatic neuroendocrine tumor; RFS, recurrence free survival.*

Insulinomas and Repeated Analysis After Exclusion

The SUR group comprised 15 insulinomas, and among these, there were no deaths. Only one patient experienced progression. Insulinomas have an excellent prognosis and all analyses were repeated after exclusion of these. Ki67 index and NEC-G3 were still significant predictors of survival in the non-insulinoma SUR group ($p \leq 0.05$). Ki67, G1- and NEC-G3, tumor infiltration, lymph node positivity, and tumor stage were still predictors of recurrence ($p \leq 0.05$). The 5-year OS and RFS in the non-insulinoma SUR group were reduced to 94% and 76%, respectively.

Tumors ≤2 cm

Fifty-nine patients with tumors ≤2 cm had localized G1 disease; 31 in the SUR group and 28 in the MED group. All 28 patients in the MED group were followed with a watch-and-wait strategy, and the group comprised 16 patients who were followed without treatment and 12 patients who were followed on Somatostatin Analogues. During a median follow-up of 36 months only one in 28 patients in the MED group experienced progression while 1 in 31 patients in the SUR group experienced recurrence during a median follow-up of 56.5 months. The SUR and MED groups differed in age, PI, and F-PNET (**Table 5**).

TABLE 4 | The significance of mean tumor size on tumor grade and stage in pancreatic neuroendocrine tumors.

	Mean size (cm)
Lymph node positive	5
Lymph node negative	2.4*
Stage IV	4.9
Stage I-III	2.9*
G3	5.9
G1/G2	3.5*
Infiltration	6.9
Local	2.7*

**p ≤ 0.05, significant difference in size when comparing predictors of aggressive tumor behavior.*

DISCUSSION

This large single-center cross-sectional study from an ENETS center of excellence demonstrated that high TNM-stage is a significant predictor of both RFS in surgically treated patients and PFS and OS in patients treated medically or with no treatment. This is in agreement with previous studies demonstrating that stage IV disease is the strongest predictor of a poor prognosis regardless of any other variable (23–25). We further demonstrated that high tumor grade is a strong, negative predictor of OS. Both tumor stage and grade are widely used for prognostic assessment, and the ENETS classification system for PNET has been evaluated previously (23, 25–29). In line with Ekeblad et al. and Scarpa et al. (23, 26), we found no significant difference between stage I and II disease (**Table 2**). This was also the case for G1 and G2 tumors and this may be caused by type 2 error. Meanwhile, Ki67 was a significant predictor of OS both in the MED and in the SUR groups. This is supported by Panzuto et al. (30) who also found that an increase in the Ki67 index was associated with poorer survival.

Brooks et al. showed that surgery in PNET is an independent predictor of OS (31). Our findings support these data as we demonstrated a higher 5-year OS after surgery (95% versus 65%). However, our study is non-randomized and retrospective, and the selection of the patients biases our results. Surgery was performed in younger patients with lower grade and stage

TABLE 5 | Clinicopathological data in 59 patients with stage I pancreatic neuroendocrine tumors ≤2 cm divided into surgically treated (SUR group, n = 31) and medically treated or non-treated (MED group, n = 28).

	MED group *n = 28*	SUR group *n = 31*
Gender, female, n (%)	16 (57)	12 (39)
Age at diagnosis, mean ± SD	67 ± 14	57 ± 10*
Incidentaloma n (%)	26 (93)	16 (52)*
F-PNET, n (%)	0 (0)	13 (41)*
Tumor diameter (cm), mean ± SD	1.3 ± 0.4	1.4 ± 0.5
Ki67 index (%), mean ± SD	1.1 ± 0.3	0.9 ± 0.5
CgA (pmol/L), mean ± SD	205 ± 372	148 ± 203
Recurrence/progression, n (%)	1 (3)	1 (3)
Death due to PNET, n (%)	1 (3)	1 (3)

**p ≤ 0.05, statistically significant t-test when comparing the baseline characteristics of the MED group to the SUR group. CgA, Chromogranin A; F, functioning; PNET, pancreatic neuroendocrine tumor.*

tumors and results should be interpreted with caution and with this selection bias in mind. F- and NF-PNETs are suggested to differ in aggressiveness and hence have a different prognosis (7, 23, 25, 26, 28). We were unable to demonstrate this. Insulinomas have an excellent prognosis after surgery, and we therefore, tried to exclude these and repeat all analyses. The 5-year OS remained excellent in the SUR group and only minor changes in the RFS were observed.

A recent meta-analysis demonstrated that post-surgery recurrence was higher in patients with high grade tumors, lymph node involvement, or vascular invasion (32). This is in accordance with our study, demonstrating that patients with higher stage and grade have a decreased RFS after surgery and therefore warrant closer follow-up.

In agreement with previous literature, PIs had a more indolent behavior compared to those that were symptomatic at diagnosis. The PFS in our study was longer, and they were more likely to be lymph node negative and have a low grade. This coheres with the fact that they are discovered early (8, 10, 16, 17, 24).

Tumors ≤2 cm

The 2016 ENETS Consensus Guidelines (19) suggest a conservative approach in non-metastatic, NF-tumors ≤2 cm. Our findings support that tumor size is a predictor of aggressive behavior. We demonstrated that larger tumor size was predictive of both higher stage and grade but also the presence of lymph node metastasis. Smaller size was also associated with lower RFS after surgery (**Table 3**). This all agrees with previous studies (23, 25–27, 32).

Betinni et al. found that tumors ≤2 cm predicted a non-indolent behavior and therefore advocated against surgery (29). Kuo et al. and Haynes et al. put this into perspective and demonstrated that the natural history is variable and the course difficult to predict (4, 16). Overall, they showed that PI can display aggressive behavior despite small size. Further, a meta-analysis from 2017 demonstrated survival benefits in tumors ≤2 cm (18).

In our study, 59 patients had stage I tumors ≤2 cm and 53% underwent surgery. The SUR group comprised more functioning tumors and younger patients than the MED group. Only 3% of the resected tumors ≤2 cm showed recurrence. This supports a recent study from Sallinen et al. who demonstrated an excellent disease-free survival after surgery (33). As long-term results after surgery are excellent, the outcome of non-operative management of tumors ≤2 cm is of paramount interest. The 28 patients in the MED group were followed with a watch-and-wait strategy. Only one patient (3%) experienced progression. Although the total number is limited, our findings support a conservative approach in accordance with the 2016 ENETS Consensus Guidelines (19).

In conclusion, in this large cohort of PNETs we demonstrated that high TNM-stage, tumor grade, Ki67 index, size, CgA, and symptomatic discovery are negative prognostic predictors of survival. Further, the surgically treated group had the highest survival, and we support the guidelines recommending surgery when predictors of aggressive tumor behavior are present. Further, we believe that a watch-and-wait strategy with active surveillance can be followed in patients with low grade, low stage NF-PNET ≤2 cm (19).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SK: conceptualization, methodology, formal analysis, investigation, resources, writing – original draft, writing – review and editing. GD: conceptualization, methodology, writing – review and editing, supervision, project administration. HG: conceptualization, methodology, writing – review and editing, supervision. NH: writing – review and editing. PK-N: methodology, writing – review and editing. AK: methodology, writing – review and editing. All authors contributed to the article and approved the submitted version.

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The content of the manuscript has previously appeared in a thesis by SK.

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Prognostic Factors of Small Non-Functional Pancreatic Neuroendocrine Tumors and the Risk of Lymph Node Metastasis: A Population-Level Study

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Background: Small non-functional neuroendocrine tumors (NF-PNETs) are a heterogeneous subset of tumors with controversy regarding their optimal management. We aimed to analyze the prognostic factors of patients with small NF-PNETs and create a risk score for lymph node metastasis (LNM).

Methods: Data of 751 patients with NF-PNETs ≤ 2 cm were obtained from the Surveillance, Epidemiology, and End Results (SEER) database. Multivariate survival analysis was performed to analyze the prognostic factors. Logistic regression was used to identify risk factors for LNM.

Results: Of the 751 patients, 99 (13.2%) were confirmed to have LNM. In multivariate survival analysis, LNM (hazard ratio [HR], 2.12; 95% CI, 1.04–4.32, p = 0.040) was independently associated with disease-specific survival. Logistic regression identified that tumor location in the head of the pancreas (odds ratio [OR], 4.33; 95% CI, 2.75–6.81; p < 0.001), size ≥ 1.5–2 cm (OR, 1.84; 95% CI, 1.17–2.87; p = 0.009), and grade III–IV (OR, 7.90; 95% CI, 1.79–34.90; p = 0.006) were independent risk factors of LNM. According to the OR value, the risk of LNM was scored as follows: a score of 1 for tumors located in the body/tail of the pancreas and 4 for those located in the head; a score of 1 for tumors <1 cm and 2 for those ≥1.5–2 cm; and a score of 1 for tumors with grade I–II and 8 for those with grade III–IV. Finally, the median score for this cohort was 4, with an interquartile range of 3–6. Therefore, patients were classified as three groups based on the risk score system: a total score of 1–3 for low risk, 4–6 for intermediate risk (OR, 2.98; 95% CI, 1.59–5.60; p = 0.001), and 7–14 for high risk (OR, 8.94; 95% CI, 4.50–17.7; p < 0.001), with an incidence of LNM 5.0%, 13.5%, and 31.8%, respectively (p < 0.001).

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Conclusion: Surgical resection with regional lymphadenectomy is recommended for small NF-PNETs with malignant potential of LNM. A risk score for LNM based on tumor grade, location, and size may preoperatively predict LNM of small NF-PNETs and guide clinical practice.

Keywords: small tumors, non-functional, pancreatic neuroendocrine tumors, lymph node metastasis, prognosis, SEER

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs) are a group of rare tumors with a variety of clinical manifestations and biological behaviors (1). PNETs can be divided into functional and non-functional tumors. Non-functional PNETs (NF-PNETs) are defined as those without a clinical symptom of hormone overproduction (2). In the past few decades, because of the widespread use of high revolution image technology and elevated attention from both clinicians and radiologists, there is an increased detection of NF-PNETs, especially for small NF-PNETs (≤2 cm) (3, 4).

Surgical resection is the mainstay treatment for PNETs; however, the management of small NF-PNETs remains controversial. Several studies have demonstrated the safety and feasibility of conservative management for asymptomatic sporadic small NF-PNETs (5–7). Considering the relatively high risk of morbidity and mortality in pancreatic surgery as well as the indolent course of small NF-PNETs, both National Comprehensive Cancer Network (NCCN) and European Neuroendocrine Tumor Society (ENETS) guidelines suggest that patients with an asymptomatic small tumor may be selectively observed (2, 8). Nevertheless, results from some surgical cohorts showed that 10%–15% of small NF-PNETs had the malignant potential with regional and distant metastases for which surgery is recommended (9–12). Moreover, although patients with small NF-PNETs generally have a good prognosis after surgery, 4%–6% of recurrences or tumor-related deaths have been observed (9, 13–15). Due to the overall rarity and heterogeneity, the prognostic factors of small NF-PNETs are not well defined.

Lymph node metastasis (LNM) has been proved to be a robust prognostic factor for PNETs. However, data regarding the prognostic value of lymph node status in small NF-PNETs were limited and with contrasting results. Vega et al. found that LNM was an independent predictor of poor disease-specific survival (DSS) and overall survival (9). Data from the National Cancer Database (NCDB) showed that LNM significantly decreased the disease-free survival (DFS) in patients with small NF-PNETs (mean survival: 115 vs. 95 months, $p < 0.0001$) (14). Conversely, in a European study with 210 resected small NF-PNETs, the presence of positive lymph nodes was not associated with DFS (15). In addition, the indication for regional lymphadenectomy for small NF-PNETs has also been debated. NCCN guidelines recommend that lymphadenectomy may be performed in tumors with a size >1 cm (8). According to the ENETS guideline, lymphadenectomy is confined within tumors larger than 2 cm (2). Recently, a multicenter study reported that patients with NF-PNETs measuring 1.5–2.0 cm had a much higher risk of LNM than patients with tumors < 1.5 cm (17.9% vs. 8.7%, $p = 0.013$) for whom lymphadenectomy should be considered (13). To date, tumor size is a major determinant of lymphadenectomy. In terms of the present controversy, more factors are needed to predict LNM and help choose an appropriate strategy for these patients.

Therefore, the primary aim of this study was to analyze the prognostic factor in patients with small NF-PNETs without distant metastasis. The second aim was to explore the risk of LNM and create a risk score based on preoperative factors.

METHODS

Study Population and Materials

The data of patients with NF-PNETs were obtained from the Surveillance, Epidemiology, and End Results (SEER) database between 2000 and 2018 by SEER stat software (version 8.3.9), and the reference number was 18464-Nov2020. NF-PNETs were classified according to the International Classification of Diseases for Oncology, Third Edition (ICD-O-3) codes: 8150 (pancreatic endocrine tumor), 8240 (carcinoid tumor), 8241 (enterochromaffin cell carcinoid), 8242 (enterochromaffin-like cell tumor), 8243 (goblet cell carcinoid), 8246 (neuroendocrine carcinoma), and 8249 (atypical carcinoid tumor). The inclusion criteria were known grade, known location (head, body, tail), surgery performed, tumor size ≤2 cm, known lymph node status, at least one lymph node were examined, known death cause, and complete survival data. The exclusion criteria were synchronous distant metastasis (M1). The flowchart of patient selection is shown in **Figure 1**. Variables analyzed in this study included age, sex, race, year of diagnosis, tumor location, tumor grade (I, well differentiated; II, moderately differentiated; III, poorly differentiated; and IV, undifferentiated), tumor size, lymph node status, number of positive and examined lymph nodes, and type of surgery.

This international database study is exempt from institutional review board (local ethics committee of the Sichuan University, West China Hospital) approval.

Statistical Analysis

Categorical variables were summarized using counts and percentages and compared using Pearson's χ^2 test or Fisher's exact test, as appropriate. DSS was evaluated using the Kaplan–Meier method. Univariate survival analysis was performed by

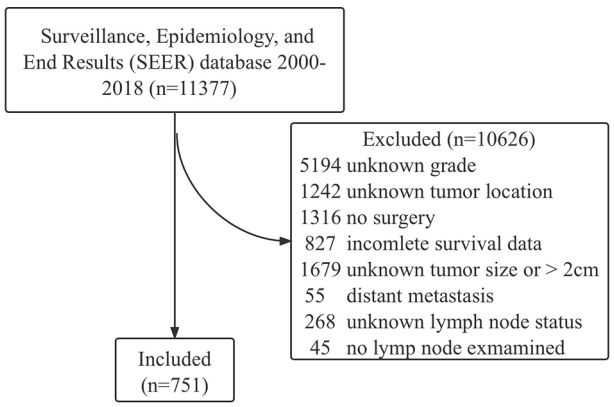

FIGURE 1 | Flowchart of patient selection.

log-rank test. Multivariate survival analysis was conducted by Cox proportional hazards model, with results expressed as hazard ratio (HR) and 95% CI. Univariate and multivariate logistic regression analyses were used to identify preoperatively available factors associated with LNM, with results expressed as odds ratio (OR) and 95% CI. Based on the OR value of multivariate logistic regression analysis, a risk score for preoperatively predicting LNM was established. All statistical analyses were performed by SPSS 26.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (version 8.2.1). A p-value <0.05 was considered statistically significant.

TABLE 1 | Baseline characteristics of small non-functional pancreatic neuroendocrine tumors.

Characteristics	Total, N (%)	Lymph node metastasis		p
		Presence, N (%)	Absence, N (%)	
Sex				0.501
Female	365 (48.6)	45 (45.4)	320 (49.1)	
Male	386 (51.4)	54 (54.6)	332 (50.9)	
Race				0.055
White	585 (77.9)	72 (72.7)	513 (78.7)	
Black	103 (13.7)	21 (21.2)	82 (12.6)	
Others	63 (8.3)	6 (6.1)	57 (8.7)	
Age, years				0.626
<60	328 (43.7)	41 (41.4)	287 (44.0)	
≥60	423 (56.3)	58 (58.6)	365 (56.0)	
Tumor location				<0.001
Head	237 (31.6)	61 (61.6)	176 (27.0)	
Body and tail	514 (68.4)	38 (38.3)	476 (73.0)	
Tumor size				0.020
<1.5 cm	393 (52.3)	41 (41.4)	352 (54.0)	
1.5–2 cm	358 (47.7)	58 (58.6)	300 (46.0)	
Type of resection				0.153
Local resection	27 (3.6)	6 (6.1)	21 (3.2)	
Formal resection	724 (96.4)	93 (93.9)	631 (96.8)	
Partial pancreatectomy	533 (71.0)	54 (54.5)	479 (73.5)	
Pancreaticoduodenectomy	130 (17.3)	28 (28.3)	102 (15.6)	
Total pancreatectomy	61 (8.1)	11 (11.1)	50 (7.7)	
Number of lymph nodes examined				0.006
1–5	257 (34.2)	22 (22.2)	235 (36.1)	
6–10	180 (24.0)	22 (22.2)	158 (24.2)	
≥11	314 (41.8)	55 (55.6)	259 (39.7)	
Grade				<0.001
I–II	720 (95.8)	72 (72.7)	648 (99.4)	
III–IV	31 (4.1)	27 (27.3)	4 (0.6)	

RESULTS

Patient Characteristics

In the SEER database, a total of 751 patients with small NF-PNETs without distant metastasis were identified for analysis (**Table 1**). There was no sexual difference, and most of the patients were white. Of these, 423 patients (56.3%) with age ≥ 60 years were classified as old people. The majority of patients (68.4%) had a tumor located in the body/tail of the pancreas. The median tumor size was 1.4 cm; thus, the cutoff value was defined as 1.5 cm. The most common surgery type was partial pancreatectomy (71%), followed by pancreaticoduodenectomy (17.3%), total pancreatectomy (8.1%), and local resection (3.6%). The percentages of patients with 1–5, 6–10, and ≥11 examined lymph nodes were 34.2%, 24.0%, and 41.8%, respectively. Most of the patients (95.8%) had a well or moderately differentiated (grade I–II) tumor, while 31 patients (4.1%) had a poorly differentiated or undifferentiated (grade III–IV) tumor.

Of the 751 patients, 99 (13.2%) were confirmed to have LNM. Baseline characteristics for patients with and without LNM were compared (**Table 1**). The proportion of tumors located in the head of the pancreas was significantly higher in patients with LNM. Patients with LNM were more likely to have a large tumor size (p = 0.020), advanced tumor grade (p < 0.001), and large numbers of lymph nodes examined (p = 0.006). Patients with tumor size measuring 1.5–2 cm had a much higher prevalence of LNM compared with those with tumor size <1.5 cm (16.2% vs. 10.4%; p = 0.020). However, no difference was found in the incidence of LNM between patients with tumor size <1 and 1–1.5 cm (14/154, 9.1% vs. 27/239, 11.2%; p = 0.485).

Survival Analysis

In the 751 patients with small NF-PNETs, 41 disease-specific deaths (5.5%) were observed. The 1-, 5-, and 10-year DSS rates were 97.3%, 94.7%, and 87.1%, respectively. The 5-year DSS rate for patients with and without LNM was 86.7% and 93.4% (p < 0.001, **Figure 2A**). Moreover, tumor location in the head of the pancreas and old age (≥ 60 years) were associated with a poor DSS (p = 0.003 and p = 0.007, respectively; **Figures 2B, C**). Univariate survival analysis identified that sex, age, tumor location, number of lymph nodes examined, and LNM were associated with DSS (**Table 2**). In multivariate survival analysis, LNM (HR, 2.12; 95% CI, 1.04–4.32, p = 0.040) combined with age (HR, 2.43; 95% CI, 1.20–4.93; p = 0. 014) and tumor location (HR, 2.24; 95% CI, 1.15–4.35; p = 0.017) were independently associated with DSS in patients with small NF-PNETs.

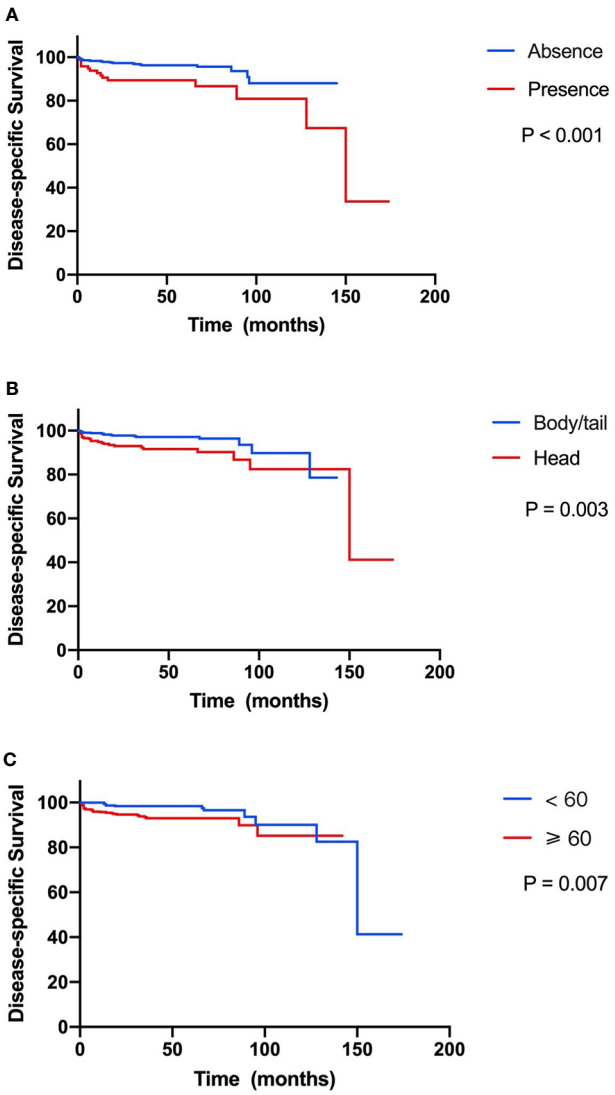

FIGURE 2 | Kaplan–Meier curve of disease-specific survival stratified by prognostic factors. **(A)** Presence versus absence of lymph node metastasis. **(B)** Tumor located in head versus body/tail. **(C)** Age ≥60 versus <60 years.

Risk Factors for Lymph Node Metastasis

In a univariate logistic regression analysis (**Table 3**), factors including race, tumor location, tumor size, and tumor grade were associated with LNM. Multivariate analysis identified that tumor location in the head of the pancreas (OR, 4.33; 95% CI, 2.75–6.81; p < 0.001), size ≥ 1.5–2 cm (OR, 1.84; 95% CI, 1.17–2.87; p = 0.009), and grade III–IV (OR, 7.90; 95% CI, 1.79–34.90; p = 0.006) were independent risk factors of LNM. According to the OR value of multivariate analysis, the risk of LNM was scored as follows: a score of 1 for tumors located in the body/tail of the pancreas and 4 for those located in the head; a score of 1 for tumors < 1.5 cm and 2 for those ≥ 1.5–2 cm; and a score of 1 for tumors with grade I–II and 8 for those with grade III–IV. Finally, the median score for this cohort was 4, with an interquartile range of 3–6. Therefore, patients were classified into three groups based on the risk score system (**Table 4**): a total score of 1–3 for low risk, 4–6 for intermediate risk (OR, 2.98; 95% CI, 1.59–5.60; p = 0.001), and 7–14 for high risk (OR, 8.94; 95% CI, 4.50–17.7; p < 0.001). Patients in the high-risk and intermediate-risk groups were nearly 9 and 3 times more likely to develop LNM compared with those in the low-risk group (**Figure 3A**, p < 0.001). As shown in **Table 4**, the influence of the number of examined lymph nodes on LNM was also evaluated. Only patients with ≥11 examined lymph nodes were associated with LNM. For patients with 1–5, 6–10, and ≥11 examined lymph nodes, the incidence of LNM was 8.6%, 12.2%, and 17.5% (**Figure 3B**, p = 0.006), respectively.

TABLE 2 | Univariate and multivariate analyses of factors associated with disease-specific survival.

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p
Sex, male	1.94	1.03–3.64	0.048	1.75	0.89–3.41	0.103
Race						
White		1				
Black	0.96	0.38–2.44	0.932			
Others	0.57	0.19–1.77	0.438			
Age, ≥60 years	2.47	1.32–4.62	0.007	2.43	1.20–4.93	0.014
Tumor location, head of pancreas	2.47	1.27–4.77	0.003	2.24	1.15–4.35	0.017
Tumor size, 1.5–2 cm	1.83	0.78–5.56	0.166			
Type of resection, formal resection	1.20	0.19–7.42	0.855			
Number of lymph nodes examined						
1–5		1				
6–10	1.75	0.62–4.94	0.271			
> 10	2.94	1.45–5.98	0.023	1.99	0.91–4.76	0.094
Lymph node metastasis, presence	2.70	1.18–6.22	<0.001	2.12	1.04–4.32	0.040
Grade, III–IV	3.90	0.40–38.3	0.243			

HR, hazard ratio.

TABLE 3 | Logistic regression analysis of the risk factors for lymph node metastasis.

Characteristics	Univariate analysis			Multivariate analysis		
	OR	95% CI	p	OR	95% CI	p
Sex, male	1.12	0.72–1.71	0.627			
Race						
White		1			1	
Black	1.83	1.06–3.13	0.029	2.18	0.98–3.97	0.055
Others	0.75		0.520	0.86	0.34–2.15	0.746
Age, ≥60 years	1.16	0.76–1.77	0.502			
Tumor location, head of pancreas	4.32	2.80–6.74	<0.001	4.33	2.75–6.81	<0.001
Tumor size, 1.5–2 cm	1.66	1.08–2.55	0.02	1.84	1.17–2.87	0.009
Grade, III–IV	14.00	3.43–56.76	<0.001	7.90	1.79–34.90	0.006

OR, odds ratio.

DISCUSSION

In this population-level study of 751 patients with resected NF-PNETs less than 2 cm, the overall 1-, 5-, and 10-year DSS rates were 97.3%, 94.7%, and 87.1%, respectively. The incidence of LNM was 13.2 (99/751). Multivariate survival analysis demonstrated that LNM combined with age and tumor location were independently associated with DSS. In a further logistic regression analysis, tumor location in the head of the pancreas, size 1.5–2 cm, and grade III–IV were independent risk factors for LNM. We further created a risk score for LNM of small NF-PNETs based on the OR values from logistic regression analysis and divided all patients into low-, intermediate-, and high-risk groups. The incidence of LNM in low-, intermediate-, and high-risk groups was 5.0%, 13.5%, and 31.8%, which was significantly different and increased with the risk level (**Figure 3A**, $p < 0.001$).

TABLE 4 | The risk for lymph node metastasis (LNM) stratified by risk score and number of examined lymph nodes.

Features	Number of patients	Events of LNM	Univariate logistic regression analysis		
			OR	95% CI	p
Risk score					
Low risk (1–3)	262	13		1	
Intermediate risk (4–6)	379	51	2.98	1.59–5.60	0.001
High risk (7–14)	110	35	8.94	4.50–17.77	<0.001
Number of examined lymph nodes					
1–5	257	22		1	
6–10	180	22	1.49	0.80–2.78	0.213
≥11	314	55	2.27	1.34–3.84	0.002

OR, odds ratio.

Small NF-PNETs are a heterogeneous subset of tumors with controversy regarding their optimal management. On the one hand, surgery has been considered a cornerstone for the management of NF-PNETs. A retrospective analysis from NCDB reported a significantly elevated 5-year overall survival of 82.2% in patients who underwent curative resection compared with a 5-year survival of 27.6% in patients who did not undergo surgery (11). Results from a meta-analysis also showed that an aggressive surgical policy for small NF-PNETs was associated with longer survival, while a watch-and-wait policy did not provide a benefit (16). On the other hand, in terms of the

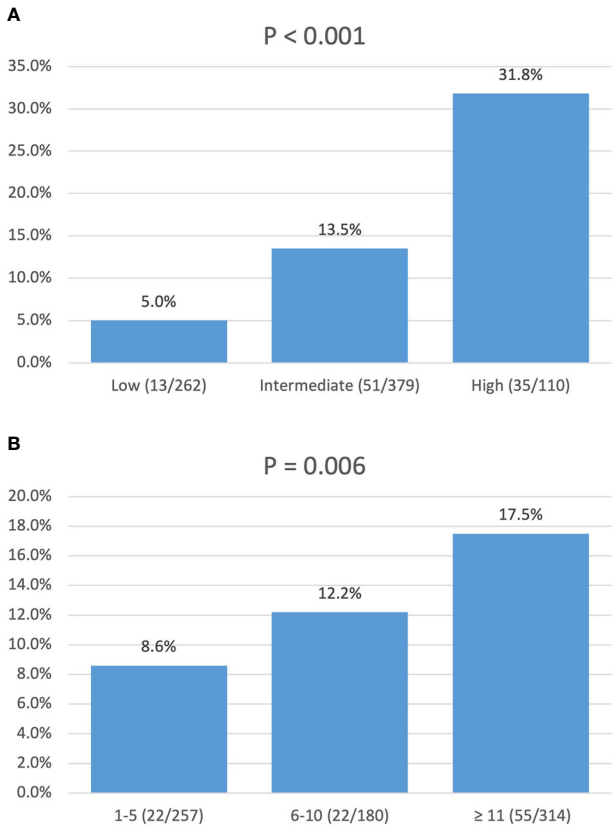

FIGURE 3 | Incidence of lymph node metastasis: **(A)** stratified by risk score; **(B)** stratified by number of lymph nodes examined.

severe and relatively high incidence of complications in pancreatic surgery and the indolent course of small NF-PNET, conservative management has been recently proposed as a possible option. Two meta-analyses (5, 6) and several retrospective studies (7, 17, 18) have shown that a surveillance approach can be safely applied to selective patients with small asymptomatic NF-PNETs. Currently, the main conundrum for the management of small NF-PNETs is to identify patients with a high risk of malignancy.

The standard of selecting patients for surgery versus surveillance needs to be carefully evaluated. A retrospective study has demonstrated that small asymptomatic NF-PNETs have an unpredictable clinical course, and a subset of them may show aggressive behavior (12). A European multicenter study found that the presence of biliary or pancreatic duct dilatation and WHO grade 2–3 was associated with the recurrence of small NF-PNETs, and surgical resection was advocated in patients with these signs (15). In order to ensure the safety of conservative management, the NCCN guideline recommends that observation can be considered for small (<1 cm), low-grade, incidentally discovered tumors (8). Consistently, surveillance is preferred for low-grade, asymptomatic tumors with no suspicious malignancy in ENETS guidelines (2). In the present study, we found that lymph node status, age, and tumor location were independently associated with DSS in patients with small NF-PNETs, which implies that patients with a high risk of LNM, old age, and tumor located in the head of the pancreas may not be good candidates for observation. Nevertheless, for patients with old age or tumors located in the head of the pancreas, the morbidity of surgery should be taken into consideration. Enucleation (and regional lymphadenectomy), which has been proved to achieve the completable oncological outcomes compared with formal resections by previous studies (19–21) and our present data ($p = 0.885$), may be applied to these patients, when appropriate.

The reported incidence of LNM in small NF-PNETs varies from 2.6% to 27.5% (13–15, 22, 23). In our data, after the exclusion of patients with distant metastasis, the incidence of LNM was 9.9% in 751 patients with at least one examined lymph node. In line with most previous studies (9, 13, 14, 23), LNM was associated with the poor prognosis of small NF-PNETs in our study, suggesting the necessity of surgical resection and regional lymphadenectomy for patients with a high risk of LNM. Factors including age (9), tumor size (13), Ki-67 index (23), and lymphovascular invasion (14) have been considered predictors of LNM. In the present study, we focused on the factors that are available preoperatively. Of all the assessed factors, tumor grade III–IV was most highly

associated with LNM (OR 7.90), although the proportion of these patients was relatively low. Tumor grade has been considered the main determinant of the malignancy of NF-PNETs, which is associated with not only the metastasis potential (9, 14) but also the long-term survival (15, 24). With the support of developed image technology, fine-needle aspiration biopsy (guided by endoscopic ultrasound (EUS), US, or CT) is recommended to evaluate the tumor grade preoperatively. Tumor location is another associated factor for LNM, with an OR of 4.33. Similar to our finding, Mei et al. found that tumors located in the pancreatic head were more likely to have LNM compared with those in the body/tail (42.8% vs. 30.9%, $p < 0.001$) and were associated with poor survival (25). The different embryological origins of the head and body/tail of the pancreas may partly contribute to these differences. As proved by previous studies (13, 14), tumor size was also related to the LNM of small NF-PNETs in this study. There was a significant difference in incidence of LNM (10.4% vs. 16.2%, $p = 0.02$) between tumors <1.5 cm and ≥1.5–2 cm. However, no difference was found in the incidence of LNM between patients with tumor size <1 and 1–1.5 cm. These results may give a reference to determine the therapeutic management of tumors between 1 and 2 cm. Based on three preoperatively available factors including tumor grade, location, and size, we created a risk score for LNM, which may be utilized to guide clinical practice and help choose an optimal strategy for the management of small NF-PNETs.

Although previous studies (26, 27) and our present data have shown that the detection rate of LNM is increasing with the number of examined lymph nodes, the minimal number of lymph nodes to be harvested for accurate nodal staging remains unclear. During pancreaticoduodenectomy, the number of harvested lymph nodes is generally adequate for an appropriate nodal staging. For distal pancreatectomy, Lopez-Aguiar et al. found that 7 or more lymph nodes should be examined for accurate staging (28), while a minimal number of 12 lymph nodes was suggested by Guarneri et al. (29). Moreover, the role of lymphadenectomy in organ-preserving surgeries such as enucleation and central pancreatectomy is undefined. In this study, we found that patients with ≥11 examined lymph nodes were more likely to have at least one positive node. However, more high-quality prospective studies are needed to determine the minimal number of lymph nodes in surgery for PNETs.

The strength of our findings is the population-level and long-term survival outcomes. However, there were several limitations in our study. The main limitations were the retrospective nature and the possible selection bias. All patients enrolled in this study have undergone surgery, which may lead to an overestimate of the malignant potential of small NF-PNETs. Furthermore, the SEER database does not collect information on recurrence; therefore, the primary end-point was DSS in our study. In addition, some tumor-related variables such as the Ki-67 index, vascular and perineural invasion, and postoperative complications are not available.

CONCLUSION

Patients with small NF-PNETs have a favorable prognosis after surgery; however, a subset of them may show the malignant potential of LNM. Lymph node status combined with age and tumor location was associated with DSS in patients with small NF-PNETs. Surgical resection with regional lymphadenectomy is recommended for small NF-PNETs with malignant potential of LNM. A risk score for LNM based on tumor grade, location, and size may preoperatively predict LNM of small NF-PNETs and guide clinical practice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

QT: conception, data collection, manuscript drafting, and editing. XW: conception, data analysis, manuscript drafting, and editing. YCL: data collection and analysis, and manuscript editing. YYL: data collection and analysis, and manuscript editing. XL: resources, supervision, and manuscript review and editing. NK: conception, resources, and manuscript review and editing. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

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Distant organ metastasis patterns and prognosis of neuroendocrine cervical carcinoma: a population-based retrospective study

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Background: Neuroendocrine carcinoma of the cervix (NECC) is a rare pathological form of cervical cancer. The prognosis of NECC with distant organ metastases is unclear. In our study, the patterns and prognosis of distant organ metastasis of NECC were investigated.

Methods: Data were obtained from the surveillance epidemiology and end results (SEER) database from 2000 to 2018. Cox regression, Kaplan–Meier and log-rank analyses were conducted.

Results: NECC was prone to single and multi-site metastases. The median overall survival (OS) was greatly decreased in patients with distant metastasis ($P < 0.0001$). Other characteristics such as age ≥60 years, poorer grade, higher T stage, those without surgery, no radiotherapy, and no chemotherapy were predictors of poor prognosis.

Conclusions: Metastasis is an independent prognostic factor for patients with NECC. Surgery, radiotherapy, and chemotherapy give an overall survival advantage for patients with distant organ metastases.

KEYWORDS

neuroendocrine cervical carcinoma, SEER database, metastasis, prognosis, survival

Background

Neuroendocrine carcinoma of the cervix (NECC) is a rare pathological type of cervical cancer, accounting for 1.4% of all cervical malignancies, and it has a poor prognosis (1). The fifth edition of the WHO 2020 divides neuroendocrine tumors are into highly differentiated neuroendocrine tumors and poorly differentiated neuroendocrine carcinoma (NEC). Among them, poorly differentiated NEC include small cell NEC and large cell NEC, with small cell NEC being the most common, accounting for about 80%, followed by large cell NEC (12%) and other histological types such as undifferentiated neuroendocrine neoplasms (8%) (1, 2).

The biology of NECC differs from that of squamous or adenocarcinoma of the cervix in that it exhibits a very aggressive biological behavior with a strong propensity for lymphatic and hematogenous spread. Local and distant recurrence is more common in NECC than in other pathological types of cervical cancer (3). Small cell NEC of the cervix is usually diagnosed at an advanced stage (4, 5), usually with metastasis to extra-pelvic organs such as the liver and lung as the first diagnosis (6). Its median overall survival (OS) is always less than 2 years (7, 8). At all stages of the disease, women with endocrine tumors have a worse survival rate compared to squamous cell carcinomas (5).

Due to the rarity of the disease, the optimal treatment for NECC remains uncertain. Current clinical experience mainly refers to the multimodal treatment of small cell lung cancer. The most common primary treatment modality for NECC is radical surgery combined with chemotherapy. Cohen et al. shown that adjuvant chemotherapy or chemoradiotherapy improved survival in patients with all stage of small cell NEC (7). The role of radiotherapy in NECC remains controversial, and Dong's study showed that the combination of radiotherapy and surgery has a significant survival advantage in metastatic NECC (9). Due to the low incidence of NECC, fewer studies have been conducted on metastatic NECC, especially on distant organ metastases. In this paper, we focus on NECC patients with distant organ metastases from the surveillance epidemiology and end results (SEER) database to study the prognosis of different distant organ metastatic sites, and then to study the effect of surgery, radiotherapy, and chemotherapy on metastatic NECC.

Methods

Study cohorts

The data for this retrospective cohort study were obtained from the SEER database [Incidence-SEER 18 Regs Research Data, Nov 2020 Sub (2000–2018 varying)]. The SEER database collects information on cancer from 18 United states registries, representing close to 28% of the US population, and provides an oversized quantity of elaborated analysis data (10). Patients' chemotherapy and radiotherapy records also are licensed to be used by the SEER workshop. The SEER*Stat software system (version 8.3.9, National Cancer Institute, Washington, USA) was used to access the data from the SEER database.

Data collection

The NECC study data and relevant clinical information were obtained from the SEER project (1). Patients with first malignant primary site cervical cancer (Site record International Classification of Diseases for Oncology-3 (ICD-O-3)/WHO 2008: Cervix Uteri) diagnosed between 2000 and 2018 were identified from the SEER database (2). International Classification of Diseases for Oncology (ICD-O-03) histology codes of 8002, 8012-8014, 8031, 8041, 8043-8045, 8154, 8158, 8240-8246, 8249 and 8574 (3). Patients with unknown age, race, follow-up time, and metastatic status were excluded from participation. The flow chart for patient choice is shown in Figure 1.

Demographic characteristics were collected: age at diagnosis, race, marital status, AJCC stage, grade, tumor size, cancer metastasis, surgery of primary site, radiotherapy, chemotherapy, and survival status. These were designated as risk and/or prognosis factors for more analysis of NECC patients with metastatic NECC. The primary endpoint was OS.

Statistical analysis

Descriptive analyses were performed on all participants. Categorical variables were expressed as proportions (%). Variables were compared using Pearson's chi-square tests. Univariable and multivariable Cox regression analyses were used to assess the independent association between metastasis status and OS. Survival curves were plotted by Kaplan–Meier and log-rank analyses.

All the analyses were performed with the statistical software packages R (http://www.R-project.org, The R Foundation) and Free Statistics software versions 1.5. A two-tailed test was performed and $p < 0.05$was considered statistically significant.

Results

Demographic and clinical characteristics for neuroendocrine cervical carcinoma

A total of 66,468 patients were diagnosed with cervical cancer between 2000 and 2018 according to the inclusion criteria, excluding patients with unknown age and survival time. A total of 1,025 neuroendocrine cervical cancer was collected by excluding patients with non-neuroendocrine type of pathology. After excluding patients with unknown distant metastasis status, a total

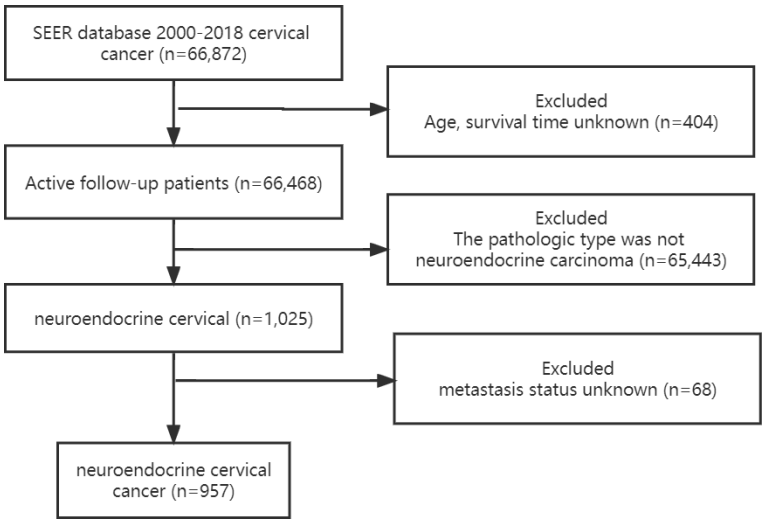

FIGURE 1
Flowchart of the neuroendocrine cervical cancer patient's selection.

of 957 cases remained. Detailed characteristics of the cohort of patients with neuroendocrine cervical cancer with and without distant metastases are listed in Table 1. The proportion of distant metastases was higher in middle-aged (41-60 years) and older (≥61 years) women than in young women (P<0.001). Other characteristics were different in patients with or without distant metastasis such as T, N, AJCC stage, tumor size, and metastatic organs including bone, brain, liver, and lung (P<0.001). A higher percentage of patients without distant metastases underwent primary site surgery (59.8% vs 20.7%, P<0.001), primary site radiation therapy (66.4% vs 44.9%, P<0.001), and chemotherapy (80.7% vs 74.5%, P<0.05).

Frequency of organ metastasis

The distribution of distant metastatic sites is shown in Table 2. 142 patients were able to extract data describing specific metastatic sites from the SEER database since 2010. Single-site metastases accounted for 55.6% of the cases, with liver metastases being the most common site of metastasis (20.4%), followed by lung (19.7%) and bone (13.4%) metastases. A total of 44.4% of patients had multi-organ metastases. Metastases to three organ, lung, liver, and bone, were more common than other multi-organ metastases (11.3%).

Cox proportional hazards regression analysis

Univariate and multivariate Cox regression was used to distinguish potential prognostic factors for OS (Table 3) and CSS (Supplementary Table S1) in patients with NECC with or without distant metastases. In the univariate analysis, we identified patients in the age group 41-60 years and above, white race, single status, higher T, N, M and AJCC stages, tumor size≥4cm, number of organ metastasis. In the group without distant organ metastases, 59.8% of patients received surgery, 66.4% received radiotherapy, and 80.7% received chemotherapy. However, in the distant metastasis group, only 20.7% of patients underwent surgery, 44.9% received radiotherapy, and 74.5% received chemotherapy. In the multivariable COX regression analysis, distant metastasis remained a risk factor for poor prognosis (HR: 1.85, 95%CI: 1.01-3.38, P<0.05). Other characteristics such as age older than 60 years old (HR: 1.66, 95%CI:1.32-2.07, P<0.001), worse grade, higher T stage, those without surgery (HR: 1.9, 95%CI: 1.54-2.35, P<0.001), no radiotherapy (HR: 1.29, 95%CI: 1.08-1.55, P=0.006), and no chemotherapy (HR: 2.7, 95%CI: 2.21-3.3, P<0.001) were predictors of poor prognosis. A similar survival trend was also observed for CSS (Supplementary Table S1).

Stratified analyses to evaluate the relationships between distant metastases and mortality in the different subgroups. Distant metastasis NECC have poor prognosis in each subgroup (Supplementary Table S2).

Effects of treatment on NECC patients with or without metastasis

OS curves were showed in Figure 2. The median overall survival months were considerably lower in patients with distant metastases (30 months vs 7 months, P<0.0001) (Figure 2A).

TABLE 1 Demographic and clinical characteristics for neuroendocrine cervical carcinoma patients diagnosed with and without distant metastasis (2000–2018).

Subject characteristics	Total n (%) n = 957	M0 n (%) n = 565	M1 n (%) n = 392	*P* value
Age(years)				<0.001
≤40	304 (31.8)	226 (40)	78 (19.9)	
41-60	400 (41.8)	220 (38.9)	180 (45.9)	
≥61	253 (26.4)	119 (21.1)	134 (34.2)	
Years of diagnosis (years)				0.25
2000-2004	226 (23.6)	139 (24.6)	87 (22.2)	
2005-2009	214 (22.4)	135 (23.9)	79 (20.2)	
2010-2014	249 (26.0)	144 (25.5)	105 (26.8)	
2015-2018	268 (28.0)	147 (26)	121 (30.9)	
Race				0.317
black	140 (14.6)	84 (14.9)	56 (14.3)	
white	689 (72.0)	398 (70.4)	291 (74.2)	
other	128 (13.4)	83 (14.7)	45 (11.5)	
Marital status				0.061
married	416 (43.5)	251 (44.4)	165 (42.1)	
single	507 (53.0)	288 (51)	219 (55.9)	
unknown	34 (3.6)	26 (4.6)	8 (2)	
Grade				0.387
I	3 (0.3)	2 (0.4)	1 (0.3)	
II	17 (1.8)	12 (2.1)	5 (1.3)	
III	428 (44.7)	261 (46.2)	167 (42.6)	
IV	199 (20.8)	120 (21.2)	79 (20.2)	
unknown	310 (32.4)	170 (30.1)	140 (35.7)	
T stage				<0.001
T1	369 (38.6)	316 (55.9)	53 (13.5)	
T2	202 (21.1)	137 (24.2)	65 (16.6)	
T3	203 (21.2)	89 (15.8)	114 (29.1)	
T4	54 (5.6)	19 (3.4)	35 (8.9)	
Tx	129 (13.5)	4 (0.7)	125 (31.9)	
N stage				<0.001
N0	433 (45.2)	356 (63)	77 (19.6)	
N1	398 (41.6)	189 (33.5)	209 (53.3)	
Nx	126 (13.2)	20 (3.5)	106 (27)	
AJCC Stage				<0.001
I	252 (26.3)	252 (44.6)	0 (0)	
II	80 (8.4)	80 (14.2)	0 (0)	
III	213 (22.3)	213 (37.7)	0 (0)	
IV	409 (42.7)	19 (3.4)	390 (99.5)	
unknown	3 (0.3)	1 (0.2)	2 (0.5)	
Tumor Size				<0.001
<4cm	180 (18.8)	147 (26)	33 (8.4)	
≥4cm	363 (37.9)	207 (36.6)	156 (39.8)	
unknown	414 (43.3)	211 (37.3)	203 (51.8)	
Bone metastasis				<0.001
no	449 (46.9)	291 (51.5)	158 (40.3)	
yes	64 (6.7)	0 (0)	64 (16.3)	
unknown	444 (46.4)	274 (48.5)	170 (43.4)	
Brain metastasis				<0.001

(Continued)

TABLE 1 Continued

Subject characteristics	Total n (%) n = 957	M0 n (%) n = 565	M1 n (%) n = 392	*P* value
no	499 (52.1)	291 (51.5)	208 (53.1)	
yes	14 (1.5)	0 (0)	14 (3.6)	
unknown	444 (46.4)	274 (48.5)	170 (43.4)	
Liver metastasis				<0.001
no	436 (45.6)	291 (51.5)	145 (37)	
yes	75 (7.8)	0 (0)	75 (19.1)	
unknown	446 (46.6)	274 (48.5)	172 (43.9)	
Lung metastasis				<0.001
no	430 (44.9)	291 (51.5)	139 (35.5)	
yes	81 (8.5)	0 (0)	81 (20.7)	
unknown	446 (46.6)	274 (48.5)	172 (43.9)	
Primary surgery				<0.001
yes	419 (43.8)	338 (59.8)	81 (20.7)	
no	538 (56.2)	227 (40.2)	311 (79.3)	
Radiotherapy				<0.001
yes	551 (57.6)	375 (66.4)	176 (44.9)	
no	406 (42.4)	190 (33.6)	216 (55.1)	
Chemotherapy				0.027
yes	748 (78.2)	456 (80.7)	292 (74.5)	
no	209 (21.8)	109 (19.3)	100 (25.5)	

The distant metastasis (M1) and no distant metastasis (M0) arms of the study were stratified based on the age of diagnosis, race, marital status, differentiation grade, AJCC stage, T stage, N stage, tumor size, treatments received (surgery, radiotherapy, chemotherapy). The two groups were subject to Pearson's Chi-square statistical analysis.

However, there was no statistically significant difference in median OS for metastases from different organs such as bone, brain, liver, lung and metastases from more than one site (P>0.05) (Figure 2B).

To investigate the therapeutic effect of treatment on patients with NECC, we compared the survival effects of surgery, radiotherapy, and chemotherapy on patients with and without metastases. Patients who were treated with surgery had median

TABLE 2 Frequencies of different metastasis sites and combination metastasis (n=142).

Metastatic site		Number	Percentage (%)
One site	Bone	19	13.4
	Brain	3	2.1
	Liver	29	20.4
	Lung	28	19.7
	Total	79	55.6
Two sites	Lung and liver	14	9.9
	Lung and bone	13	9.2
	Lung and brain	1	0.7
	Liver and bone	8	5.6
	Liver and brain	1	0.7
	bone and brain	1	0.7
	Total	38	26.8
Three sites	Lung and liver and bone	16	11.3
	Lung and liver and brain	2	1.4
	Lung and bone and brain	3	2.1
	Liver and bone and brain	2	1.4
	Total	23	16.2
Four sites	all	2	1.4

TABLE 3 Univariable and multivariable Cox regression analysis of overall survival in neuroendocrine cervical cancer patients with metastasis in SEER database (2000–2018).

Subject characteristics	Univariable		Multivariable	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
M stage				
M0	Ref	1.0	Ref	1.0
M1	3.58 (3.06,4.2)	<0.001**	1.85 (1.01~3.38)	0.045*
Age(years)				
≤40	Ref	1.0	Ref	1.0
41-60	1.67 (1.38,2.02)	<0.001**	1.2 (0.98~1.47)	0.084
≥61	2.95 (2.4,3.62)	<0.001**	1.66 (1.32~2.07)	<0.001**
Race				
black	Ref	1.0	Ref	1.0
white	0.72 (0.58,0.89)	0.002	0.96 (0.77~1.2)	0.717
other	0.66 (0.5,0.88)	0.004*	0.86 (0.64~1.16)	0.325
Marital status				
married	Ref	1.0	Ref	1.0
single	1.29 (1.1,1.51)	0.001**	1.03 (0.87~1.21)	0.766
unknown	0.83 (0.52,1.32)	0.426	0.89 (0.55~1.43)	0.626
Grade				
I	Ref	1.0	Ref	1.0
II	0.25 (0.06,1.14)	0.074	0.22 (0.05~1.02)	0.054
III	0.33 (0.08,1.32)	0.117	0.21 (0.05~0.85)	0.029*
IV	0.33 (0.08,1.35)	0.124	0.21 (0.05~0.86)	0.03*
unknown	0.36 (0.09,1.46)	0.153	0.18 (0.04~0.76)	0.019*
T stage				
T1	Ref	1.0	Ref	1.0
T2	2.11 (1.69,2.63)	<0.001**	1.49 (1.1~2.02)	0.011**
T3	3.63 (2.93,4.49)	<0.001**	1.78 (1.34~2.38)	<0.001**
T4	5.95 (4.32,8.21)	<0.001**	2.25 (1.45~3.5)	<0.001**
Tx	5.24 (4.12,6.67)	<0.001**	1.67 (1.2~2.34)	0.003*
N stage				
N0	Ref	1.0	Ref	1.0
N1	2.1 (1.77,2.49)	<0.001**	1.15 (0.91~1.46)	0.243
Nx	3.77 (3,4.74)	<0.001**	1.2 (0.9~1.61)	0.222
AJCC Stage				
I	Ref	1.0	Ref	1.0
II	1.84 (1.29,2.61)	<0.001**	1.11 (0.68~1.8)	0.674
III	2.52 (1.96,3.26)	<0.001**	1.64 (1.11~2.41)	0.013*
IV	6.15 (4.89,7.73)	<0.001**	1.51 (0.76~3.01)	0.242
unknown	5.99 (1.47,24.39)	0.012*	1.83 (0.38~8.8)	0.45
Tumor Size				
<4cm	Ref	1.0	Ref	1.0
≥4cm	1.93 (1.52,2.47)	<0.001**	0.97 (0.74~1.26)	0.806
unknown	2.07 (1.63,2.63)	<0.001**	0.89 (0.68~1.17)	0.416
Number of metastasis				
No metastasis	Ref	1.0	Ref	1.0
1 site metastasis	3.22 (2.42,4.28)	<0.001**	1.14 (0.83~1.57)	0.402
≥1 sites metastasis	3.82 (2.82,5.18)	<0.001**	1.62 (1.15~2.27)	0.005*
unknown	1.45 (1.21,1.73)	<0.001**	1.19 (0.97~1.45)	0.095

(Continued)

TABLE 3 Continued

Subject characteristics	Univariable		Multivariable	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Primary site surgery				
yes	Ref	1.0	Ref	1.0
no	3.04 (2.57,3.59)	<0.001**	1.9 (1.54~2.35)	<0.001**
Radiotherapy				
yes	Ref	1.0	Ref	1.0
no	1.38 (1.18,1.61)	<0.001**	1.29 (1.08~1.55)	0.006*
Chemotherapy				
yes	Ref	1.0	Ref	1.0
no	2.07 (1.74,2.47)	<0.001**	2.7 (2.21~3.3)	<0.001**

HR, Hazard Ratio; CI, Confidence Interval; Ref, reference.
*P<0.05; **P<0.001.

survival months of 112 months compared to 19 months for patients who were not treated with surgery in the M0 group (*P*<0.0001) (Figure 3A). Even among people with distant metastases, the median survival was 13 months in the surgery group and 5 months in the no-surgery group, respectively (*P*<0.0001) (Figure 3B). In the group of patients without metastases, there was no statistical difference in OS between those who received radiotherapy and those who did not (*P*>0.05) (Figure 3C). However, In the distant metastasis group, there was a statistical difference in OS between the radiotherapy and no radiotherapy groups (median of 13 months vs 5 months, *P*<0.05) (Figure 3D). when patients received chemotherapy, there was a significant difference in the median OS in both the no metastasis (37 months vs 15 months, P=0.0005) Figure 3E) and the metastasis groups (9 months vs 1 month, *P*<0.0001) compared with the no-chemotherapy group (Figure 3F).

Discussion

Compared to adenocarcinoma and squamous cell carcinoma, patients with small cell NEC have the worst prognosis in both the early and advanced stages of cancer (11). Due to its highly aggressive nature, lymph node metastases and distant metastases often occur at an early stage. In this SEER study, 42.7% of patients were diagnosed with stage IV. However, due to the rare incidence of NECC, there are very few studies on distant organ metastatic NECC. Our study showed that distant organ metastasis was more likely to occur in patients older than 40 years, with T3 or T4 stage, tumor size ≥4cm, and with lymph node metastasis N1 at diagnosis.

The metastasis pattern of NECC differs from that of other pathological types of cervical cancer. Previous studies have found that lung metastasis and bone metastasis are the most

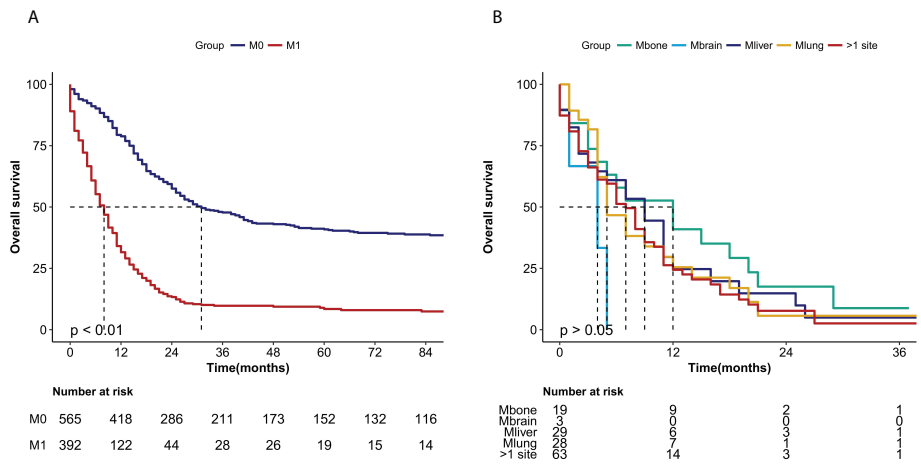

FIGURE 2
Kaplan-Meier curves of the overall survival in NECC cancer stratified by **(A)** metastasis (M0: no metastasis, M1 metastasis) (*P* < 0.0001) and **(B)** different sites of metastasis (M bone, M brain, M liver, M lung, M >1 site) (*P* > 0.05).

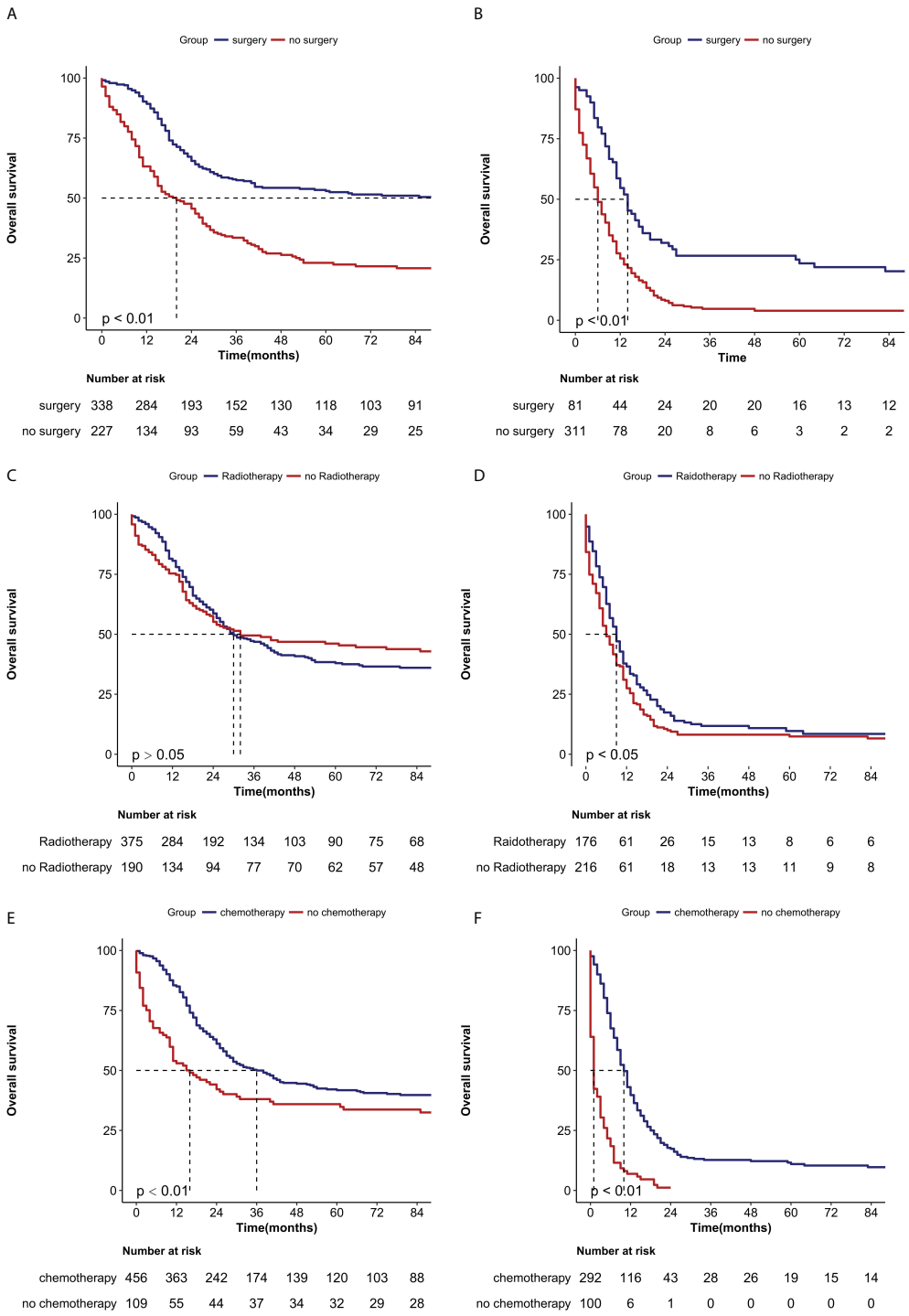

FIGURE 3
Kaplan-Meier curves of the overall survival in NECC with no metastasis or metastasis when stratified by treatment. **(A)** Surgery in no metastasis patients ($P < 0.0001$); **(B)** Surgery in metastasis patients ($P < 0.0001$) **(C)** Radiotherapy in no metastasis patients ($P > 0.05$), **(D)** Radiotherapy in metastasis patients ($P < 0.05$), **(E)** Chemotherapy in no metastasis patients ($P < 0.001$) **(F)** Chemotherapy in metastasis patients ($P < 0.0001$).

common organs of metastasis in squamous cell carcinoma of the uterine cervix (12). Bone, brain, liver, and bone marrow are the most common distant metastatic sites for small cell NEC (4, 13). With our limited data from the SEER database after 2010, the most common single metastatic site for NECC was the liver (20.4%), followed by the lung (19.7%). What's more, multisite metastases were very common in patients with NECC, with 44.4% of patients in our study presenting with metastases from more than one site. 26.8% of patients presented with metastases from two sites, and 17.6% of patients presented with metastases from three or more organs. The median OS for single bone metastases was longest but less than 12 months, compared with a median OS of 6 months for multiple metastases. The worst prognosis is for brain metastases alone, with a median OS of less than 5 months. However, due to the small sample size, we need further studies to confirm this conclusion.

For patients without distant organ metastases, primary site surgery and radiotherapy are mostly used, whereas chemotherapy is mostly used for patients with distant organ metastases. Cohen et al. summarized the results of 188 patients in which the use of adjuvant chemotherapy or chemoradiotherapy was associated with high survival rate in patients with early small cell NEC (7). External beam radiotherapy coupled with brachytherapy has been shown to improve median survival in locally advanced NECC (14). Lin et al. showed that radical surgery should be recommended for early-stage small cell NEC and a combination of radiotherapy and brachytherapy should be used for patients with advanced disease (15). However, Hou's study showed that radical surgery versus primary RT did not affect the survival of high-grade NECC (16). Studies on the treatment of distant organ metastatic NECC are limited. In a study of patients with relapsed NECC, the use of the combination of topotecan, paclitaxel, and bevacizumab (TPB) improved progression-free survival whit a trend toward improved OS (17). In our study, of those NECC patients with distant metastases, 20.7% received primary site surgery, 44.9% received radiotherapy, and 74.5% received chemotherapy. Furthermore, primary site surgery, radiotherapy, and chemotherapy improved OS in patients with metastatic NECC.

Since this study is a retrospective study based on the SEER database, there are some limitations in our study. First, based on the SEER database, inherent selection bias is inevitable, and we could only try to control for confounding factors. Second, metastatic sites other than brain, bone, liver, and lung were not included, and the specific organ transfer sites were unknown before 2010. In addition, there is no standard chemotherapy regimen for NECC, Cisplatin/carboplatin and etoposide (EP) are by far the most used treatment regimens (1, 18). However, the lack of specific chemotherapy information in the SEER database, we were unable to evaluate specific chemotherapy regimen. Similarly, we lack information on specific sites and methods of radiotherapy as well as targeted therapies and immunotherapy for NECC. We believe that patients with metastatic NECC can benefit from systemic chemotherapy and aggressive local treatment such as surgery, and radiotherapy. Hence, more randomized trials for patients with distant organ NECC are needed.

Conclusions

Our results showed that NECC is prone to a single-site and multi-site metastases, with the most common site of metastasis being the liver, followed by the lung. Median OS was less than 12 months in all metastatic patients, but metastases at different sites or multi-organ metastases did not affect OS. Metastases, age, T stage, surgery, radiotherapy, and chemotherapy were independent prognostic factors for patients with NECC. Surgery, radiotherapy, and chemotherapy all provide benefits to patients with distant organ metastases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

QL designed this study, analyzed the data, and wrote the manuscript. HY and JY contributed to the analysis of the data QYL reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.924414/full#supplementary-material

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Novel biomarkers predict prognosis and drug-induced neuroendocrine differentiation in patients with prostate cancer

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Background: A huge focus is being placed on the development of novel signatures in the form of new combinatorial regimens to distinguish the neuroendocrine (NE) characteristics from castration resistant prostate cancer (CRPC) timely and accurately, as well as predict the disease-free survival (DFS) and progression-free survival (PFS) of prostate cancer (PCa) patients.

Methods: Single cell data of 4 normal samples, 3 CRPC samples and 3 CRPC-NE samples were obtained from GEO database, and CellChatDB was used for potential intercellular communication, Secondly, using the "limma" package (v3.52.0), we obtained the differential expressed genes between CRPC and CRPC-NE both in single-cell RNA seq and bulk RNA seq samples, and discovered 12 differential genes characterized by CRPC-NE. Then, on the one hand, the diagnosis model of CRPC-NE is developed by random forest algorithm and artificial neural network (ANN) through Cbioportal database; On the other hand, using the data in Cbioportal and GEO database, the DFS and PFS prognostic model of PCa was established and verified through univariate Cox analysis, least absolute shrinkage and selection operator (Lasso) regression and multivariate Cox regression in R software. Finally, somatic mutation and immune infiltration were also discussed.

Results: Our research shows that there exists specific intercellular communication in classified clusters. Secondly, a CRPC-NE diagnostic model of six genes (*HMGN2, MLLT11, SOX4, PCSK1N, RGS16* and *PTMA*) has been established and verified, the area under the ROC curve (AUC) is as high as 0.952 (95% CI: 0.882–0.994). The mutation landscape shows that these six genes are rarely mutated in the CRPC and NEPC samples. In addition, NE-DFS signature (*STMN1* and *PCSK1N*) and NE-PFS signature (*STMN1, UBE2S and HMGN2*) are good predictors of DFS and PFS in PCa patients and better than other clinical features. Lastly, the infiltration levels of plasma cells, T cells CD4 naive, Eosinophils and Monocytes were significantly different between the CRPC and NEPC groups.

Conclusions: This study revealed the heterogeneity between CRPC and CRPC-NE from different perspectives, and developed a reliable diagnostic model of CRPC-NE and robust prognostic models for PCa.

KEYWORDS

single-cell RNA-seq, castration-resistant prostate cancer, neuroendocrine, cellular communication, prognosis

Introduction

Prostate cancer has become the second most common cancer in men worldwide, and androgen deprivation therapy (ADT) plays an indispensable impact on the treatment of PCa. On the one hand, enzalutamide, as an androgen receptor inhibitor, competes and replaces the natural ligand of androgen receptor by closely binding with the ligand binding domain of androgen receptor. At the same time, it also inhibits the translocation receptor of androgen from entering the nucleus and impairs the transcriptional activation of androgen response target genes (1). On the other hand, abiraterone weakens androgen receptor signaling by consuming adrenal and intra-tumoral androgens (2). Nevertheless, due to complex mechanisms such as lineage plasticity and phenotype switching, cytokine dysregulation (3). Prostate cancer cells can adapt to androgen deprivation and restore androgen receptor signaling, eventually progressing to CRPC, even CRPC-NE, which is a lethal subtype of PCa with extremely poor survival rate (4–6). In addition, the use of AR inhibitors is accompanied by an increase in the incidence rate of highly invasive AR negative prostate cancer. The percentage of AR negative tumors in mCRPC patients increased from 11% (1998-2011) to 36% (2012-2016) after the introduction of effective androgen receptor signaling inhibitors (such as enzalutamide and abiraterone) (7). Almost all men will eventually develop castration resistant prostate cancer (CRPC) after ADT (8), Furthermore, the most common situation is that during drug treatment, nearly 25% CRPC gradually trans-differentiate into NEPC (9), called t-NEPC, but neuroendocrine prostate cancer can also presented *de novo*.

Presently, NEPC is divided into different subtypes according to different morphological characteristics: 1. Adenocarcinoma with neuroendocrine (NE) differentiation; 2. Paneth cell NE differentiation; 3. Carcinoid; 4. Small-cell carcinoma; 5. Large-cell NE carcinoma; and 6. Mixed NE carcinoma-acinar adenocarcinoma (10). Zou et al. have shown that focal neuroendocrine differentiation (NED) and ultimately well differentiated neuroendocrine prostate cancer are directly produced by trans-differentiation of luminal adenocarcinoma cells (11), which indicates that in the process of CRPC patients treated with androgen deprivation, luminal cells inside could experience trans-differentiation, resulting in luminal/NE intermediate cells. Previous studies have shown that prostate basal cells express basal keratins *KRT5, KRT14* and key transcription factors *TP63 (*12); Luminal or secretory cells express keratins *KRT8, KRT18*, androgen receptors, and secretory proteins consisting of prostate specific antigen (PSA) and prostatic acid phosphatase (13). An increasing number of neuroendocrine prostate cancer markers (such as *CHGB, ENO2, LMO3, EZH2, SOX2* and *SIAH2*) are being identified (14, 15). It has been reported that in mouse and adult prostate, cells with co-expression markers of basal cells and luminal cells (such as the co-expression of *KRT5/KRT14* and *KRT8/KRT18/KRT19*) are called intermediate cells, representing either pluripotent prostate stem cells or intermediate cells between basal stem cells and luminal progenitor cells (16), supplying a solid support to classify and annotate cells.

Great importance should be attached to develop diagnostic signatures for CRPC with NE characteristics. Zhang et al. has successfully identified four novel biomarkers for the diagnosis of NEPC, including *NPTX1, PCSK1, ASXL3*, and *TRIM9* (17) *via* Bulk-RNA sequencing data, in our study, by combining single-cell RNA seq with Bulk-RNA seq, the CRPC-NE diagnostic model *via* machine learning algorithm was successfully built, and the prostate cancer prognosis model was also constructed and validated triumphantly.

Materials and methods

Data collection and procession of Sc-RNA seq and Bulk-RNA seq

Attaching great attention on neuroendocrine prostate cancer, the sample inclusion criteria are as follows: (1) the patients must have developed resistance to castration therapy; (2) Gene expression data must be available for both CRPC and NEPC tumors; (3) The diagnostic information must be clear. The single-cell RNA sequencing information of GSE176031 (18) as well as GSE137829 (19) were obtained *via* GEO database (https://www.ncbi.nlm.nih.gov/geo/), The former provides

with 4 normal samples (8038 cells) taken from radical prostatectomies, The single-cell transcriptome information of NEPC and CRPC were obtained from the other one, including 3 CRPC samples (7119 cells) and 3 NEPC samples (16384 cells). Harmony algorithm was not used to remove batch effects so as not to eliminate the inherent differences between samples. Then CRPC and CRPC-NE clusters were separated according to well-acknowledged cell markers, We used CellChat (v1.4.0) R package to analyze the intercellular communication among annotated clusters (20), and calculated 102 differentially expressed genes (DEGs) (logFC > 0.5 & pvalue < 0.05) between CRPC and CRPC-NE by "FindMarkers" function in Seurat (v4.1.1) R package (21–24). These genes were then used for GO and KEGG analysis.

The Bulk transcriptome RNA-seq data and corresponding clinical data, consisting of SU2C/PCF Dream Team(n=208) (25), Multi-Institute Cohort (n=49) (26) were download from Cbioportal Database (https://www.cbioportal.org/) and used to identify genes upregulated in CRPC-NE samples compared with CRPC samples after quality control, 41 samples were excluded due to inadequate information in SU2C/PCF Dream Team cohort. Only 12 genes highly expressed in both single-cell transcriptome data and Bulk-RNA data were selected for the establishment of CRPC-NE diagnosis model. The workflow of the diagnostic model is presented in Figure 1. Additionally, TCGA PanCancer data (27) from Cbioportal Database and 138 PCa samples in GSE21035 (28) were explored in order to construct prognosis model for DFS as well as PFS. The workflow of the prognosis model is demonstrated in Figure 2. Genes mapped to multiple probes were calculated by their average values. The batch effects of Bulk RNA-seq data were modified through "ComBat" function in sva (v3.44.0) package (29). The clinicopathological information of enrolled samples is listed in Table 1.

Single–cell RNA–seq analysis

The Seurat package (v 4.1.1) was utilized to generate the object and filtered out cells with poor quality. Then, we

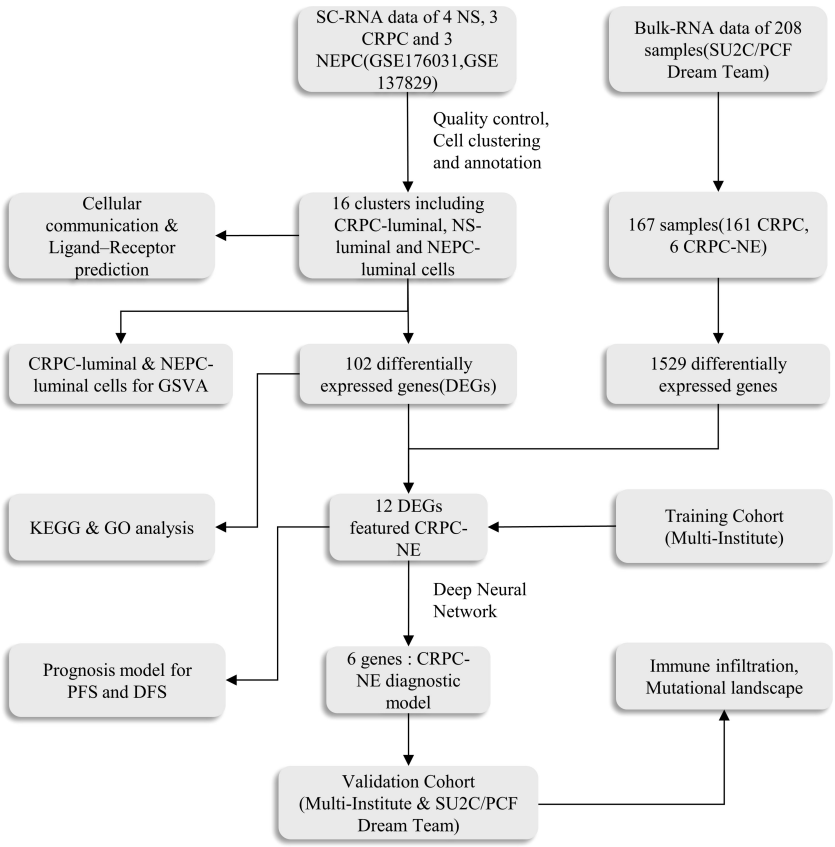

FIGURE 1
The main workflow of the study. SC-RNA, single-cell RNA; NS, normal sample; CRPC, castration-resistant prostate cancer; NEPC, Neuroendocrine prostate cancer;DEGs, differentially expressed genes; GSVA, Gene Set Variation Analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic; DFS, disease free survival; PFS, progression free survival.

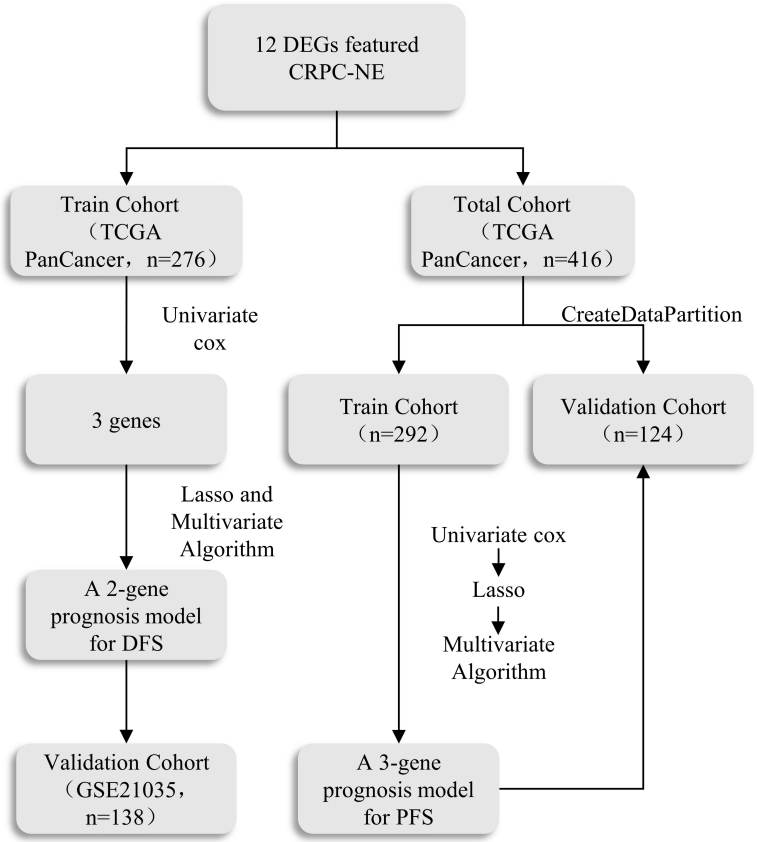

FIGURE 2
The scheme of the prognosis model.

conducted standard data preprocessing, where we calculated the percentage of the gene numbers, cell counts and mitochondria sequencing count. Genes with less than only 3 cells detected and disregarded cells with less than 50 detected gene numbers were excluded. We filtered out cells with fewer than 500 or more than 4,000 detected genes and those with a high mitochondrial content (>5%). After discarding poor-quality cells, a total of 12,165 cells were retained for downstream analysis. To normalize the library size effect in each cell, we scaled UMI counts using scale.factor = 10,000. Following log transformation of the data, other factors, including "percent.mt", "nCount_RNA" and "nFeature_RNA", were corrected for variation regression using the "ScaleData" function in Seurat (v 4.1.1). The corrected-normalized data metrics were applied to the standard analysis as described in the Seurat R package. The top 1,500 variable genes were extracted for principal component analysis (PCA). The top 30 principal components were kept for Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) visualization and clustering. We performed cell clustering using the "FindClusters" function (resolution = 0.3) implemented in the Seurat R package. Afterwards, the clusters were verified by SingleR package (v1.10.0) and canonical markers (30). Moreover, we utilized"FindAllMarkers" function to identify marker genes between cluster "CRPC_Luminal" and "NEPC_Luminal/NE" with the filter value of absolute log2 fold change (FC) ≥ 0.5 and the minimum cell population fraction in either of the two populations was 0.25 (31).

Pseudotime trajectory analysis

Importantly, after passing quality control, Pseudotime and trajectory analysis of single cells were performed *via* "monocle" R package (v2.24.0) (32–34), genes were placed into the Reversed Graph Embedding algorithm of Monocle to shape the trajectory. Then, Monocle applied a dimensionality reduction to the data and ordered the cells in pseudotime.

TABLE 1 Characteristics of sample cohorts used for the analysis of DFS as well as PFS.

Characteristics	DFS cohort		PFS cohort	
	TCGA (n=276)	GSE21035 (n=138)	TCGA-train (n=292)	TCGA-validation (n=124)
Age (year)				
≤65	121	115	207	85
>65	155	23	85	39
PSA (ng/ml)				
≤10	NA	112	NA	NA
>10	NA	24	NA	NA
Not available	NA	2	NA	NA
Gleason score				
≤6	NA	77	NA	NA
7	NA	48	NA	NA
≥8	NA	13	NA	NA
Disease-free event	248	103	NA	NA
Progression event	NA	NA	63	21
T-stage				
T1/T2	121	86	104	38
T3/T4	155	52	188	86
N-stage				
N0	246	103	239	99
N1	30	12	53	25
Nx	NA	23	NA	NA
Surgery-type				
RP	NA	98	NA	NA
Others	NA	40	NA	NA
Radiation therapy				
Yes	32	18	12	18
No	242	NA	231	92
Not available	2	120	35	14

Ligand–receptor expression and cell interactions

Cell-to-cell communication "CellChat" (v1.4.0) R package was ascertained by evaluating expression of pairs of ligands and receptors within cell populations, thus to reveal the potential interaction between various cells types. Gene expression of 0.2 was set as the valid cutoff point. The specific signaling pathways were selected for further visualization so as to reveal the strength of specific pathways among 16 clusters. In addition, the potential ligand-receptor interaction between luminal/NE cells and other cells was also explored.

Functional analyses and mechanism exploration

Firstly, Gene Set Variation Analysis (GSVA) was performed with the GSVA package (v1.44.0) of R software with default parameters (35). The list of KEGG terms was obtained from the

Gene Set Enrichment Analysis database (https://www.gsea-msigdb.org/gsea/msigdb/genesets.jsp?collection=CP : KEGG).

Furthermore, the DEGs between CRPC-luminal & NEPC-luminal clusters were identified with R package limma (v3.52.0) (36). Then the pathway enrichment analyses, including Gene Ontology (GO) analysis and KEGG analyses were completed to explore distinct pathways (37–39).

Random forest algorithm and artificial neural network model for diagnosis model

A random forest algorithm was applied on 49 samples (Multi-Institute Cohort) from Cbioportal to find the most important genes associated with the phenotype. Briefly, We utilized randomForest R package (v4.7-1.1) to find the most important genes associated with diagnosis status in CRPC and CRPC-NE samples (40). The genes whose "MeanDecreaseGini" > 1 were choose to build the artificial neural network (ANN) model. Based on multilayer perceptron network (MLP), the ANN model consists of input nodes, hidden layers, and an output node (41), In our study, six genes (*HMGN2, MLLT11, SOX4, PCSK1N, RGS16* and *PTMA*) were selected as the input nodes, and one indicator (with or without neuroendocrine differentiation) was used as the output node (42). Consequently, the diagnosis model was validated in samples from Multi-Institute and SU2C/PCF Dream Team (n=216) downloaded from Cbioportal. The sensitivity and specificity of the diagnostic models were evaluated by the receiver operating characteristic (ROC) curves (43).

Construction and validation of prognostic model for DFS and PFS

By comparing CRPC with CRPC-NE *via* "limma" (v 3.52.0) R pacakge, 12 genes highly expressed in both single-cell transcriptome data and Bulk-RNA data were discovered. To begin with, SU2C/PCF Dream Team (n=276) in the Cbioportal dataset were regarded as training cohort. NEPC characteristic genes were analyzed by univariate Cox to obtain candidate prognostic genes (P<0.05), Subsequently, the least absolute shrinkage and selection operator (LASSO) method by "glmnet" (v4.1-4) R package was used to minimize overfitting risk (44), and select the optimal gene combination with the lowest Akaike information criteria (AIC) in a Stepwise Algorithm, Finally, a 2-gene prognostic signature (NE-DFS signature) for DFS was built based on the regression coefficient derived from the multivariate Cox regression model and the optimized genes. The formula are as follows:

$$NE-DFS\ signature\ score = \sum_{i=1}^{n}(\beta_i * \exp i)$$

where n was the number of enrolled genes, βi represented the coefficient of the gene and Exp *i* was the candidate gene's expression level. Then, patients were classified into high- and low- risk groups according to the median, the Kaplan–Meier plot and log-rank test were applied to evaluate differences between the high-risk and low-risk subgroups by the R package "survival" (v3.3-1) (45). The receiver operating characteristic (ROC) curve performed by "timeROC" (v 0.4) R package was used to judge the efficiency of the NE-DFS signature,

Afterwards, we validated the model in the GSE21035 (n=138) cohorts. Data from different platform were modified through "ComBat" function in sva (v3.44.0) package to eliminate batch effects. Similarly, A 3-gene prognosis model for PFS was constructed and validated in TCGA PanCancer cohort (n=416). 416 PCa patients in the dataset were randomly assigned to training (n = 292) and internal validation cohort (n = 124) at a 7:3 ratio, the remaining has been described in detail above.

Immune infiltration and tumor mutational burden exploration

Normalized expression levels (Affymetrix intensity) of gene signatures that distinguish 22 immune cell types from each other and other cell types was downloaded from the Supplementary Table 1 of this article (46), namely LM22 signature. Then we identify the proportions of the 22 immune cells from each sample by "CIBERSORT". The algorithm was run using the LM22 signature and 1000 permutations. For each sample, the final CIBERSORT output estimates were normalized to sum up to one. The Wilcoxon rank-sum test was used to compare the expression differences of 22 types of immune cells between CRPC and CRPC-NE patients. Only cases with a CIBERSORT output of $p < 0.05$ were considered to be eligible for subsequent analysis and visualization. Additionally, waterfall plots were generated to explore the mutation characteristics of the 12 CRPC-NE featured markers by "maftools" (v2.12.0) package (47).

Nomogram construction

Nomogram analysis was constructed in the training group to predict the outcome of the individual. The upper part is the scoring system and the lower part is the prediction system. The 1-, 2-, 3- and 5-year survival rate of PCa patients could exactly be predicted by total points of every factor. Verification of the prediction accuracy of DFS and PFS was performed in patients of the validation group.

Statistical analyses

Besides the Venn diagrams were drawn online (https://bioinformatics.psb.ugent.be/webtools/Venn/). The other statistical analyses and visualization were conducted using the R software (v4.2.0) and Bioconductor (v3.15). Statistical differences between the two groups were assessed using the Wilcoxon test. $P < 0.05$ was considered statistically significant.

Results

Single–cell RNA–seq profiling, clustering and markers

Two Sc-RNA seq datasets (GSE176031 and GSE137829) in the GEO database were used to obtain normal samples (8038 cells), CRPC samples (7119 cells) and NEPC samples (16384 cells). After initial quality control assessment, 12,165 high-quality cell samples isolated from three distinguished types of tissues were screened and illustrated for further analyses (Figure 3A). 1,500 high variable genes and the names of the top 10 genes are marked in Figure 3B. Principal component analysis (PCA) and UMAP was used for preliminary dimension reduction of Sc-RNA seq data (Figure 3C). We subsequently apply t-distributed stochastic neighbor embedding (t-SNE) algorithm on the top 30 principal components to visualize the high dimensional scRNA-seq data, and successfully classified cells into 10 clusters (T cell, Fibroblast, Luminal, NK cell, Monocyte, Endothelial, Basal/Interm, Luminal/NE, B cell, Plasma) by previous canonical cell marker combined with "SingleR" package (v1.10.0), which were later annotated to acknowledged 16 cell types (Figure 3D) according to the sample (Table 2). It can be seen that not all luminal cells in 3 NEPC samples have the characteristics of neuroendocrine differentiation. The cluster "NEPC_Luminal/NE" has neuroendocrine features, while cluster "NEPC_Luminal" does not. Figure 3E illustrates the heatmap of marker gene expression in 16 clusters.

Next, Pseudotime and trajectory analysis were conducted *via* "monocle" package (v 2.24.0) to explore the potential cellular evolution. The predicted pseudotime trajectory began from the upper left and stretched as cells approach the up and bottom right branches (Figure 4A). Intriguingly, cells including fibroblast, luminal, basal/interm as well as Endothelial were mainly localized in the early stages of pseudotime trajectory while immune cells (NK-T cell, B cell, Plasma) with Luminal/NE cells moved towards the termini, implying that T and B cells, as momentous components of tumor microenvironment, may play an indispensable role in the occurrence and development of CRPC and NEPC (Figures 4B, C).

Identification of CRPC-NE featured markers

As set forth in the article, 16 clusters were identified, Figure 5A exhibits the specific markers of basal, Luminal and NE of PCa. A total of 102 genes were identified as DEGs (LogFC>0.5 & pvalue<0.05), which were higher regulated in NEPC_Luminal/NE cluster, namely NEPC cells, than that in CRPC_Luminal and NS_Luminal cluster. Analogously, A Bulk-RNA data consisting of 167 samples (161 CRPC, 6 CRPC-NE) produces 1,529 DEGs (LogFC>0.25 & pvalue<0.05) *via* R package limma (v3.52.0). We selected genes shared between the 102 and 1529 genes (Figure 5B). GO analysis revealed that the 102 DEGs were mainly enriched in the biological processes of the biological oxidation process in mitochondria (Figure 5C). KEGG analysis indicated that the DEGs were mainly enriched in a variety of neurological diseases including Huntington disease, Amyotrophic lateral sclerosis, Pathways of neurodegeneration –multiple diseases and Oxidative phosphorylation (Figure 5D). To further investigate the potential pathway differences between NEPC and CRPC, and thus explain the causes of phenotypic differences between them. GSVA on the scRNA-seq data was conducted (Figure 5E). In contrast with CRPC-luminal, five pathways (KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION, KEGG_PRIMARY_BILE_ACID_BIOSYNTHESIS, KEGG_TAURINE_AND_HYPOTAURINE_METABOLISM, KEGG_LINOLEIC ACID METABOLISM, KEGG_drug_metablism_cytochrome_p450) were obviously down-regulated in NEPC-luminal cells. Nevertheless, distinctively differential KEGG pathways except the above fives were observed in the bulk-RNA data Multi-Institute cohort, which contains 34 CRPC and 15 CRPC-NE samples (Figure 5F).

The exact ligand–receptors among different cell types

It is worthy of exploring the ligand–receptors interactions among 16 clusters, especially the interactions between CRPC and NEPC, we applied CellChat to infer and analyze intercellular communication networks. CellChat revealed a number of crucial ligand–receptor pairs and signaling pathways, including ANGTP, IL16, CSF, LIFR and OSM pathways (Figure 6A), displaying the Luminal/NE cluster regulate CRPC_Endoth and NS_ Endoth clusters through ANGTP signaling pathway, while NS_Fibro cluster displayed vast communication with other cells such as NS_Monocyte, NS_Basal/Interm, CRPC_Endoth, NS_Luminal and NEPC_Luminal clusters (mainly those featured with epithelial and endothelial markers). Intriguingly, NEPC_B

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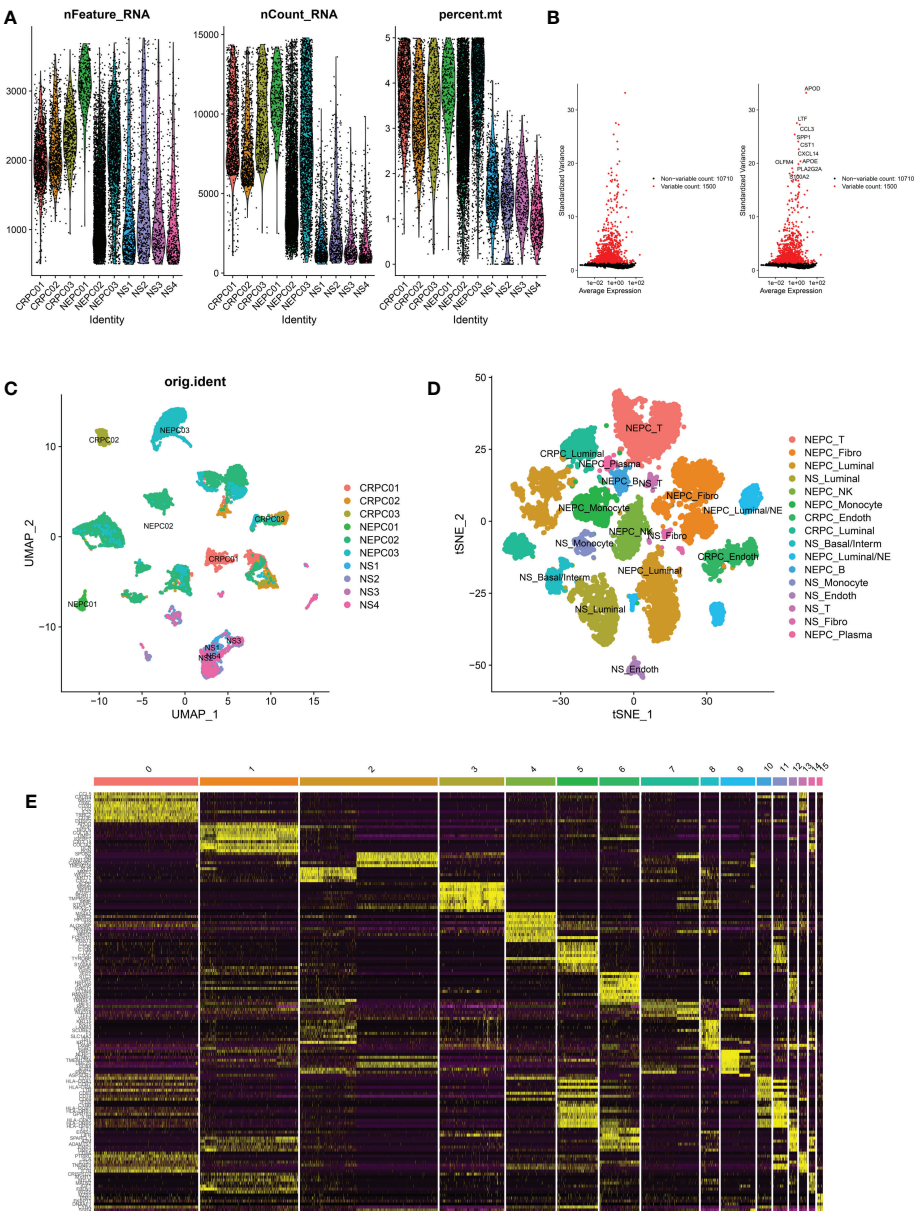

FIGURE 3
Analysis of single-cell RNA seq of 3 CRPC tissues, 3 NEPC tissues and 4 NS tissues. CRPC, castration resistant prostate cancer; NEPC, neuroendocrine prostate cancer; NS, negative samples; Fibro, Fibroblast; Basal/interm, Basal/intermediate; Endoth, endothelial; **(A)**: the number of RNA features (nFeature_RNA) and absolute UMI counts (nCount_RNA) after quality control filtering of each cell. **(B)**: We explored 1,500 high variable genes that exhibit high cell-to-cell variation, and the names of the top 10 genes are marked. **(C)**: Using UMAP dimensionality reduction algorithm, 12165 cells from 10 samples were displayed. **(D)**: Cells were classified into 16 clusters *via* t-SNE dimensionality reduction algorithm based on the source of the cluster, each cluster was marked with the source of the cluster plus the annotated cell types. There may exist 2 same cell types in 16 clusters. **(E)**: Heatmap depicting expressions of top 10 marker genes among 16 clusters.

cluster and NEPC_NK cluster regulate Monocyte cluster through pathways CSF and IL16, respectively, hinting the role of immune intercellular crosstalk is vital. Similarly, cluster NEPC_NK is extensively associated with endothelial and epithelial cells *via* pathways LIFR and OSM. The contribution of each ligand-receptor was showed in (Figure 6B), Notably, the most significant L-R pairs of CSF pathway was CSF1 – CSF1R, previous study has revealed that the CSF1/CSF1R signaling axis has been implicated in prostate cancer oncogenesis and CSF1R blockade lowered (tumor

TABLE 2 Cell cluster distribution and cell marker.

Cell Cluster	Cell marker	Cell Type
0, 13	CD3D, IL7R, TRBC2, CCL5, CCL4, CD8A, CXCR4, ETS1, CD69	T cell
1, 14	DCN, LUM, PTN, APOD, IGFBP5, CCDC80, CFD, LTBP4, COL1A2, FBLN1, MEG3	Fibroblast
2, 3, 7	KRT19, KRT8, KRT18, AR	Luminal
4	NKG7, GNLY, KLRD1, KLRB1, FGFBP2, PRF1, CD8A, CD8B, GZMH, GZMA	NK cell
5, 11	S100A9, EREG, NEAT1, TKT, THBS1, TSPO, CSTA	Monocyte
6, 12	TM4SF1, RNASE1, EGFL7, RAMP3, PLVAP, ECSCR, FKBP1A, EMP1, VWF, EMCN	Endothelial
8	KRT5, KRT19, KRT8, KRT18	Basal/Interm
9	CHGB, ENO2, LMO3, EZH2, SOX2, SIAH2	Luminal/NE
10	CD22, CD79B, LY9, CCR7, IRF8, CD83, BTG1, BANK1	B cell
15	SEC11C, XBP1, PRDX4, SPCS2, SSR3, SDF2L1, MANF, TMEM258, DNAJB9	Plasma

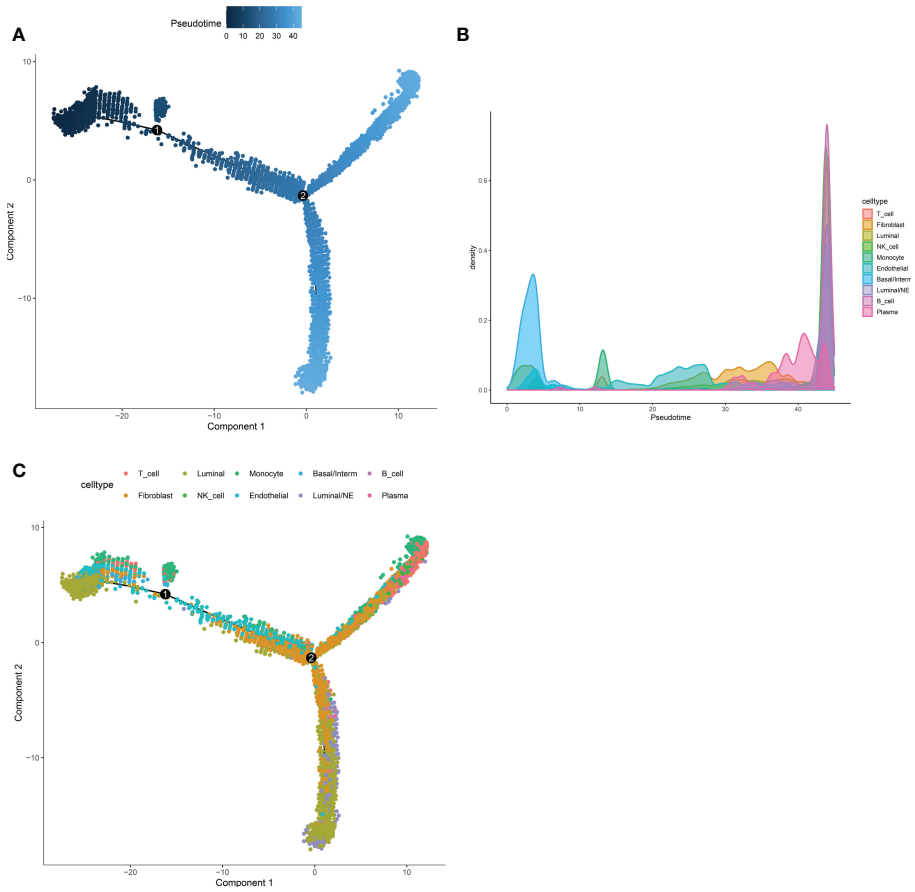

FIGURE 4
Pseudotime and trajectory analysis revealed the tendency curve among various cell types. **(A)** The pseudo time is shown as the depth of the color, the darker the blue, the smaller the pseudo time, which means that the cells appear earlier. The dots above represent cells. **(B, C)** Pseudotime-Density diagram demonstrated cells including immune cells (such as NK-T cell, B cell, Plasma) as well as Luminal/NE cells gather around the destination. X-axis means the value of principal component 1 (the first principal direction of maximum sample change) and Y-axis means the value of principal component 2.

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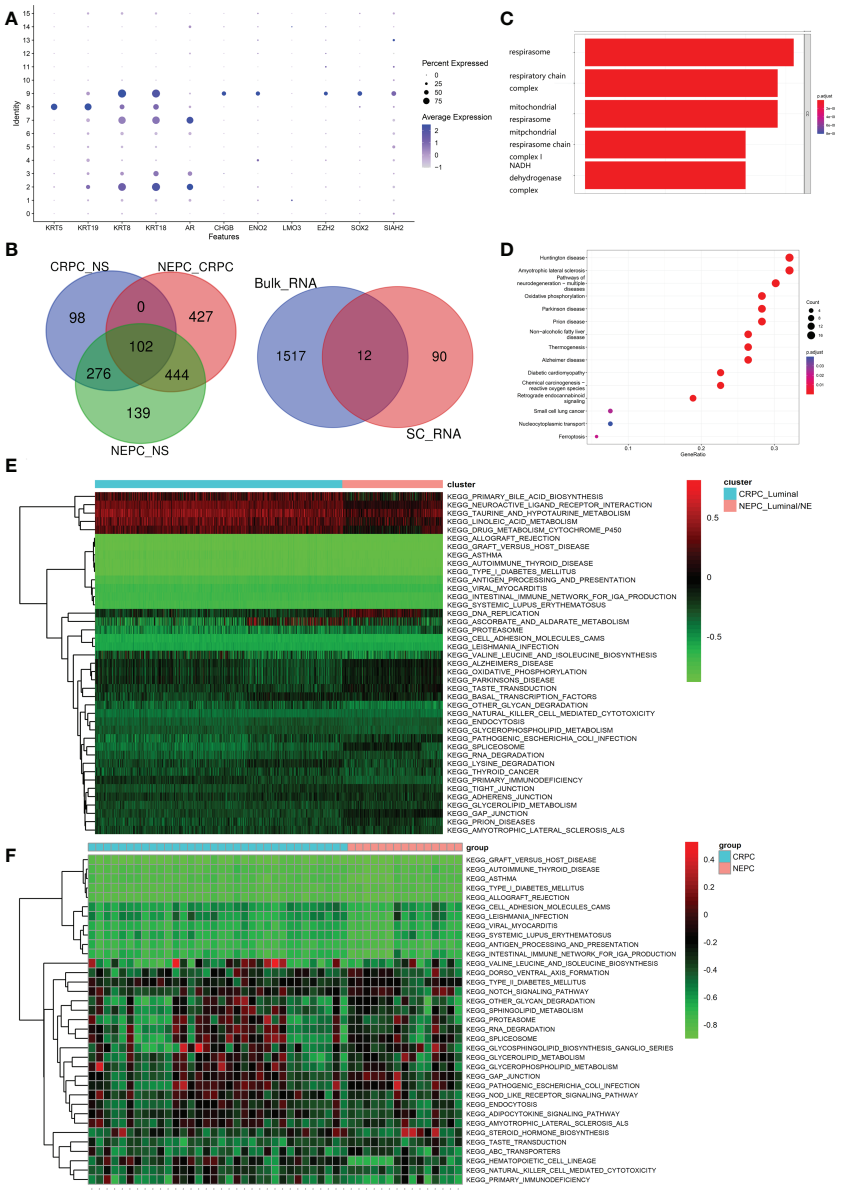

FIGURE 5
Identification and functional analysis of CRPC-NE featured markers. **(A)** Marker gene expression for epithelial cells (KRT5, KRT19, KRT8, KRT18, AR) and neuroendocrine characteristic cells (CHGB, ENO2, LMO3, EZH2, SOX2, SIAH2), in which dot size and color represent percentage of marker gene expression and the averaged scaled expression value, respectively. **(B)** 102 genes with higher expression in NEPC than that in CRPC, and the latter is higher than that in NS were screened out. Then we selected genes shared between the SC-RNA data (102 genes) and Bulk-RNA data (1529 genes). **(C, D)** GO enrichment and KEGG pathway enrichment analysis of differentially expressed 102 genes. **(E, F)** Heatmap illustrating the differential KEGG pathways (upper panel) between CRPC_Luminal cluster and NEPC_Luminal/NE cluster at the single cell RNA-seq level, and discrepant KEGG pathway (lower panel) from the aspect of Bulk-RNA seq. The color indicates the level of pathway expression.

associated macrophage) TAM-induced tumorigenic factors and delayed the emergence of CRPC (48). Besides, tumor-associated macrophage accelerates the survival of CRPC cells upon docetaxel chemotherapy *via* the CSF1/CSF1R-CXCL12/CXCR4 axis (49). We further investigated the specific ligand–receptor interactions among different cell clusters, Particular attention was paid to the interactions of CRPC_Luminal and NEPC_Luminal/NE clusters with other cluster cells (Figure 6C). Distinct cell interactions among luminal/NE, luminal cells as well as other clusters were detected,

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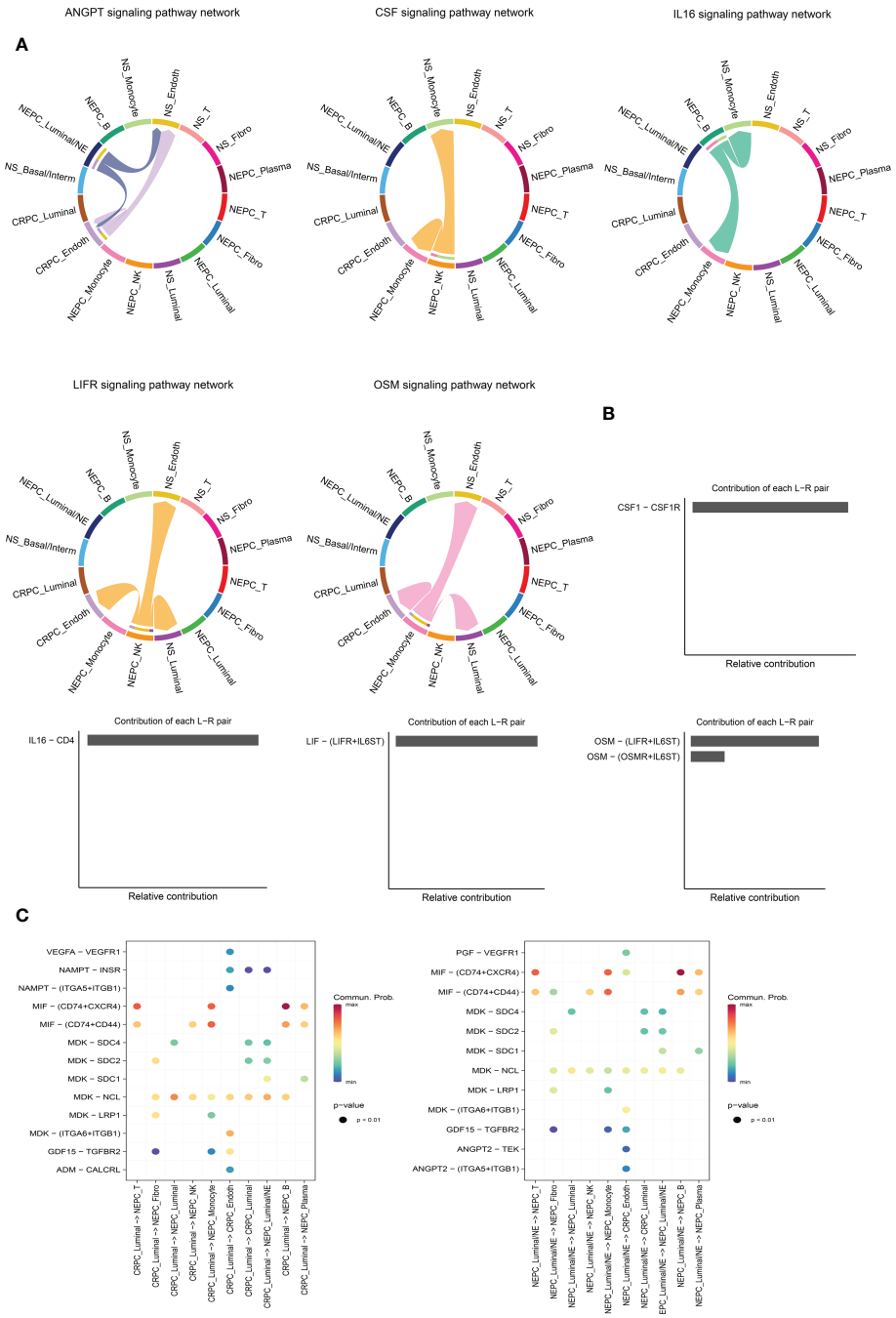

FIGURE 6
Intercellular ligand–receptor prediction among differernt clusters. **(A)** The chord diagram shows the expression of ANGTP, IL16, CSF, LIFR and OSM pathways among different cell clusters. In the peripheral ring, different colors represent different cells, Cells that send the arrow express the ligand, and cells that the arrow points to express the receptor, the more ligand-receptor pairs, the thicker the line. **(B)** Relative contribution of each ligand-receptor pair to the signal pathway, which may affect the overall communication network of the signaling pathway. CSF, IL16, LIFR and OSM pathways are shown in turn. **(C)** The extensive ligand-receptor mediated cellular interaction between different cell clusters of CRPC and NEPC has been further explored and demonstrated. The color gradient indicates the probability of cellular communication.

consisting of MIF – (CD74+CXCR4), MDK – NCL and MDK – LRP1, which might participate in the formation of CRPC or NEPC through relevant channels.

Six–gene diagnostic NEPC signature construction and verification

Firstly, in the training cohort (n=49), we applied the randomForest algorithm to analyze 12 NEPC-featured genes, the number of trees was set as 500 based on the relationship plot between the model error and the number of decision trees, and obtained the most 6 significant genes associated with the phenotype according to the value of "MeanDecreaseGini" (Figure 7A), which reflects the importance of genes. Then k-means unsupervised clustering was utilized to cluster the training cohort with these 6 critical factors (*HMGN2, MLLT11, SOX4, PCSK1N, RGS16* and *PTMA*) (Figure 7B).

In this study, The Multi-Institute cohort was used to build an artificial neural network model using the neural net package. The maximum and lowest data values were normalized before the computation began, and the number of hidden layers was set to 5, the above six genes were selected as the input nodes, and one indicator (with or without neuroendocrine differentiation) was used as the output node Figure 7C. The validation set was utilized to test the model score's classification performance using the expression of genes and gene weight. So far, the diagnosis model was validated in samples from Multi-Institute and SU2C/PCF Dream Team datasets. The sensitivity and specificity of the diagnostic models were evaluated by the receiver operating characteristic (ROC) curves, nearly 0.952 (95% CI: 0.882–0.994) in the train group, indicating that it was robust. The area under the ROC curve (AUC) remains 0.830 (95% CI: 0.692–0.964) in the dataset of SU2C/PCF Dream Team from Cbioportal (Figure 7D).

Immune infiltration and tumor mutational burden analysis

CIBERSORT algorithm was adopted to estimate the abundances of member cell types in a mixed cell population, using gene expression data including 34 CRPC samples and 15 NEPC samples from Multi-Institute cohort (n=49). We used Wilcoxon rank-sum test to explore whether there was a difference in the expression of immune cells between the two groups, The results demonstrated that the infiltration levels of plasma cells, T cells CD4 naive, Eosinophils and Monocytes were significantly different in the two groups (Figure 8A). Particularly, the infiltration levels of plasma cells, T cells CD4 naive, and Eosinophils were significantly higher in cluster CRPC-NE. On the contrary, cluster CRPC appeared higher infiltration levels of Monocytes cells. Combined with the Pseudotime and trajectory of immune cells (Figure 8B), we could conclude that CRPC-NE is closely related to T and plasma cells in the tumor microenvironment, providing a new direction for CRPC-NE immunotherapy. Furthermore, waterfall plot revealed except for genes *CAMTA1*, few mutations were observed of the other 11 CRPC-NE featured genes in CRPC and CRPC-NE samples (Figure 8C).

The prognostic model for DFS and PFS

Univariate analysis was performed to assess associations between 12 DEGs featured CRPC-NE and DFS in the TCGA PanCancer dataset (n=276). According to the selection criteria, 3 DFS associated genes with P<0.05 were screened out for LASSO Cox regression algorithm to ensure the robustness of the prognostic model, afterwards, the lambda.min was determined as the optimal lambda value by tenfold cross-validations, the above 3 prognostic genes with non-zero coefficients were all enrolled (Figure 9A). subsequently, multivariate analysis and Stepwise Algorithm were used to ensure that Akaike information criterion (AIC) is the minimum, thus generating the appropriate gene combination of 2 genes (*STMN1* and *PCSK1N*) with P<0.05, namely NE-DFS signature. On the basis of the coefficients, the risk score was confirmed: NE-DFS signature score = expression level of 0.696 * *STMN1* + expression level of 0.432* *PCSK1N*. According to the median cutoff value of the score, patients were separated into high- and low-risk groups. Kaplan-Meier plots elucidated that the patients with lower scores had better DFS (Figure 9B), $p < 0.05$). Then the potential accuracy of the model was further assessed by the "timeROC" package in the training cohort, with 1-, 2- and 3-year AUCs of 0.784 (95% CI: 0.631–0.938), 0.752 (95% CI: 0.588–0.916) and 0.828 (95% CI: 0.722–0.935) respectively, better than those of Gleason scores and pathological tumor stages (Figure 9C).

External dataset GSE21035 (n=138) were enrolled as validation cohort to evaluate the robustness of the training group. Similarly, the samples were classified into high risk and low risk groups based on median risk score. Kaplan-Meier survival plots revealed that there is a significant difference between the high risk and low risk (p<0.05) (Figure 9B). The AUCs of 1-, 2- and 3- year were 0.899 (95% CI: 0.806–0.992), 0.843 (95% CI: 0.746–0.941) and 0.810 (95% CI: 0.712–0.907) respectively (Figure 9D), demonstrating fabulous predictive potential especially for the DFS within 3 years.

Furthermore, analogous methods were utilized to construct a 3-gene prognostic model for PFS by using TCGA PanCancer (n=416). The Total Cohort were randomly assigned to training (n = 292) and internal validation cohort (n = 124) at a 7:3 ratio. The method to filter the genes is the same as before, firstly, Univariate Cox regression analysis was performed to assess genes significantly associated with PFS ($p < 0.05$).

Subsequently, the LASSO method by glmnet (version 4.0.2) R package for variable selection (Figure 10A). Ultimately, 3 genes,

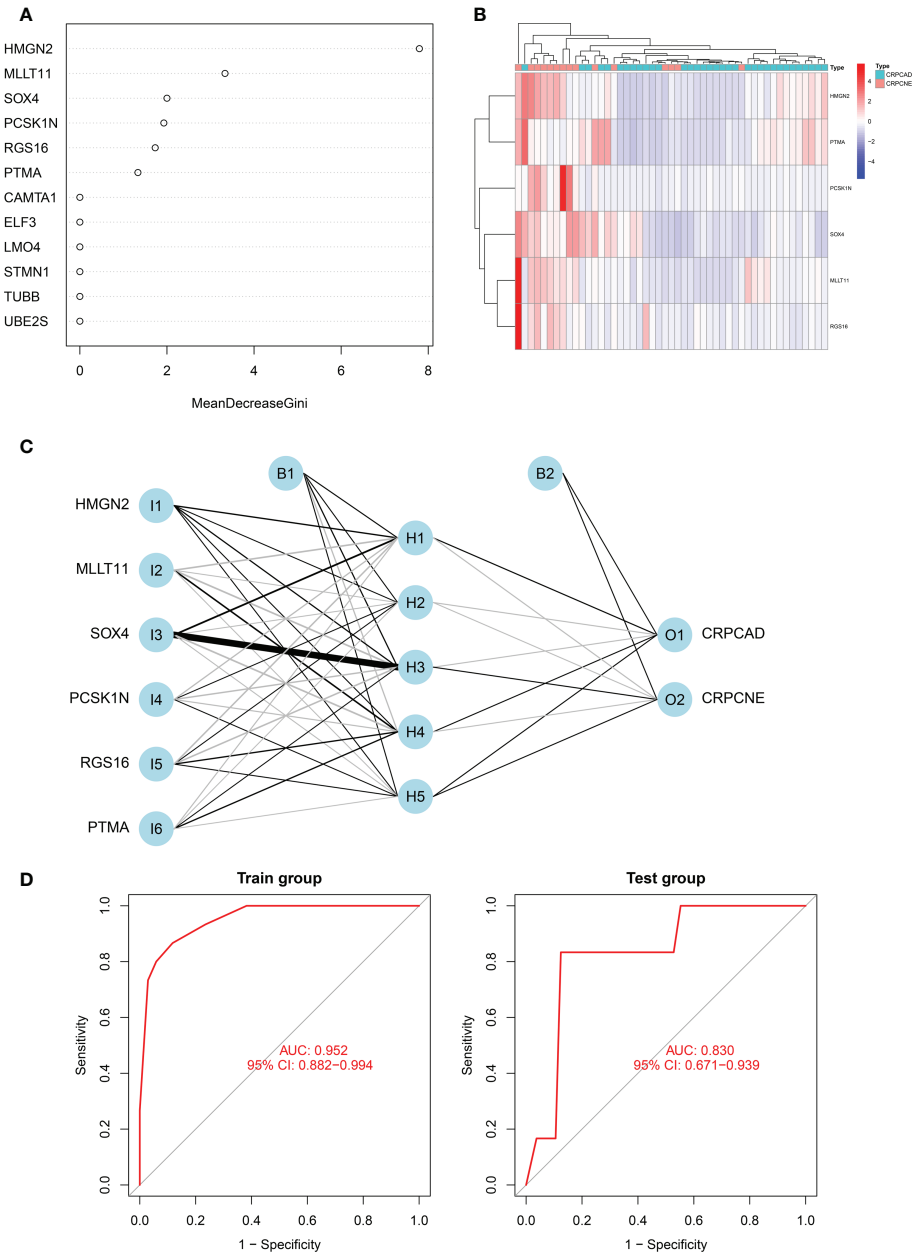

FIGURE 7
Identification of the markers to establish CRPC-NE diagnostic model. **(A)** The figure shows the weight of 12 genes to elucidate the importance of genes to disease classification (CRPC-NE or CRPC). The larger the"MeanDecreaseGini"index, the more likely this gene is to be classified as a characteristic gene. **(B)** Heatmap visualizing the expression levels of the six CRPC-NE diagnostic genes in the Cbioportal training cohort. **(C)** Results of neural network visualization: six CRPC-NE diagnostic genes were selected as the input nodes. Positive weights are connected by black lines, negative weights are connected by gray lines, and the thickness of the lines reflects the weight value. **(D)** The receiver operating characteristic (ROC) curves of 6-gene CRPC-NE diagnostic model in training cohort and validation cohort.

including *STMN1, UBE2S* and *HMGN2* were recognized as NE-PFS signature *via* multivariate Cox and Stepwise Algorithm.

NE-PFS signature score = expression level of 0.302 * *STMN1* + expression level of 0.391 * *UBE2S* + 0.653 * *HMGN2*. The process of building the model has been described in detail above. Compared with the low risk, Kaplan-Meier plots elucidated that the high risk had worse PFS (Figure 10B), $p < 0.05$). The AUC curve presented with decent result in predicting the PFS in training cohort (AUC for 1-, 2-, and 3 years PFS: 0.700 (95% CI: 0.587–0.814), 0.659 (95% CI: 0.566–0.752), and 0.707 (95% CI: 0.622–0.792)) (Figure 10C), then the predictive model was then validated in the internal TCGA PanCancer validation cohort (Figure 10D).

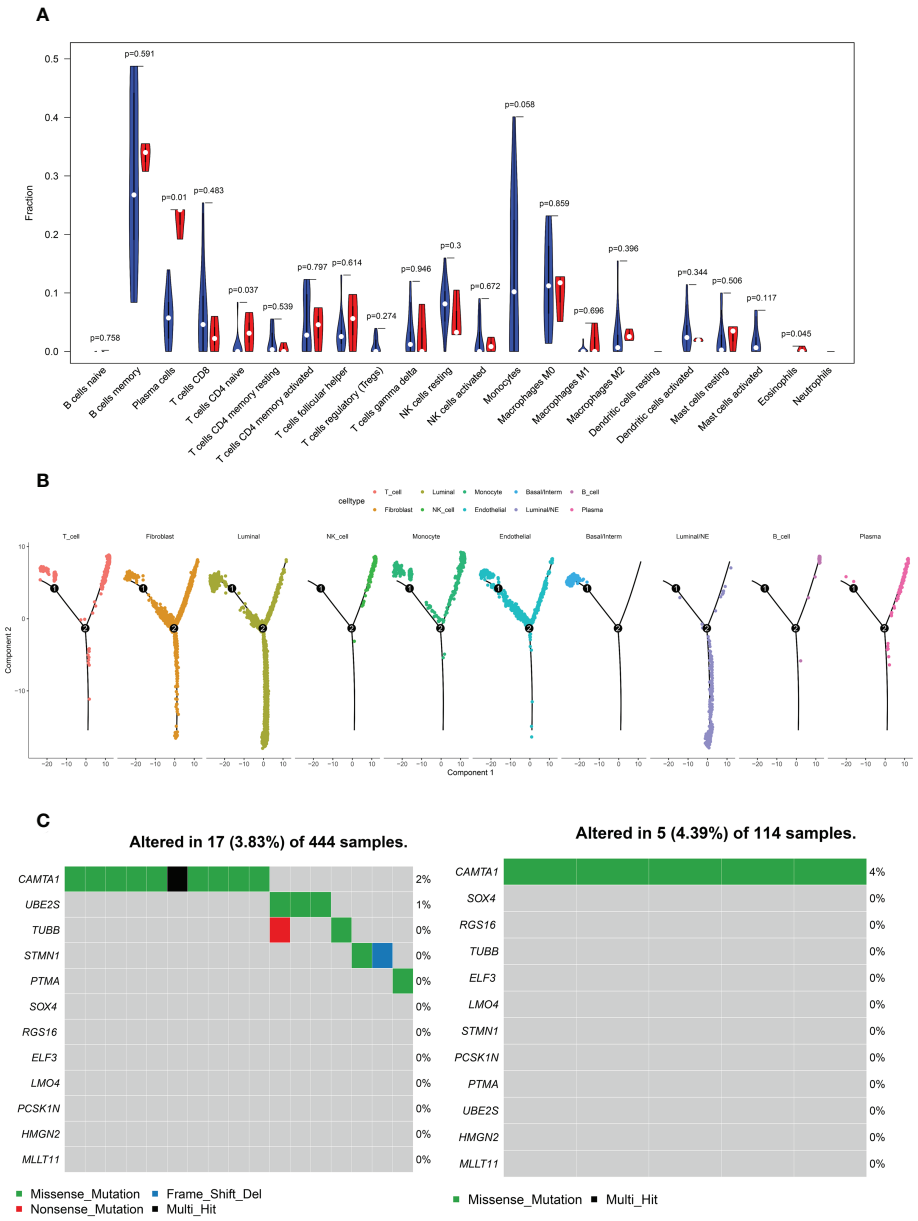

FIGURE 8
The distribution landscape of immune cell, and TMB pattern between CRPC and CRPC-NE **(A)** The difference of 22 immune infiltration between CRPC and CRPC-NE groups, red color indicates the abundance of immune cells in the latter, blue color indicates the abundance in the former. **(B)** Pseudotime trajectory analysis elucidated luminal/NE cluster and immune cells like NK, T, B and Plasma cells moved towards the termini of the trajectory. **(C)** Waterfall plots summarize the mutation landscape of 12 CRPC-NE featured genes in CRPC and CRPC-NE samples, showing that the mutation rate of these genes is low except CAMTA1.

Construction of nomograms

It can be concluded from the above analysis that the NE-DFS signature and NE-PFS signature could independent prognostic indicators for PCa patients. In addition, age, race, tumor stage, gleason scores were also incorporated in the nomogram tool to predict the outcome of individual patients (1, 3 and 5-year DFS and PFS probabilities of PCa in the TCGA PanCancer cohort (Figure 11A). Then, on basis of the total point (the sum score of each variable), the rate of DFS and PFS at 1-, 3- and 5-year can be inferred. In addition, the line-segment in the calibration plots was close to the 45°C line, the model's predictions

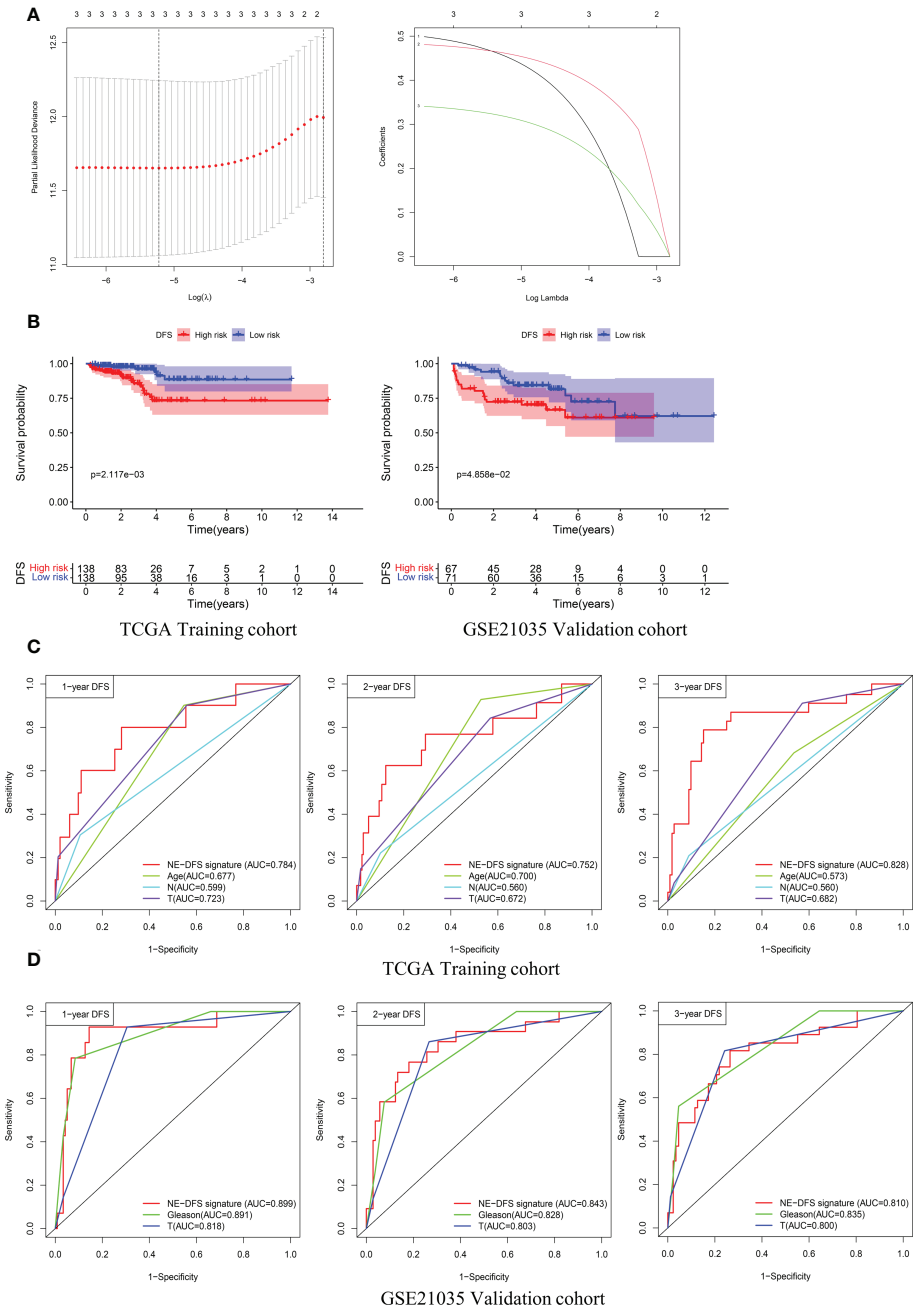

FIGURE 9
A 2-gene prognosis model for DFS (NE-DFS signature) in the TCGA PanCancer training cohort and GSE21035 validation cohort in PCa. **(A)** Three genes significantly correlated with DFS were identified through LASSO regression analysis and ten-fold cross-validations for screening of the optimal parameter lambda **(B)** Kaplan–Meier curves displayed that high-risk group exhibited worse DFS than low risk group in TCGA PanCancer training group (n=276) and GSE21035 group (n=138). **(C, D)** Receiver operating characteristic (ROC) curves of the NE-DFS signature had better Predictive effectiveness than age, tumor stage and lymph node status to evaluate the predictability of DFS at 1-, 2- and 3- year in the TCGA PanCancer training cohort, similar phenomena were observed in the GSE21035 validation group.

of 1-, 3- and 5-year DFS and PFS probabilities were favorably consistent with the ideal predictions (gray line) in both training cohort and validation cohort (Figure 11B), indicating that the nomogram model could be used as reliable indicator to predict DFS and PFS in CRC patients. In addition, we also mapped the calibration curves of the prognosis model. Figure 11C and D showed the calibration curves of recurrence-free survival model and progression-free survival model, respectively.

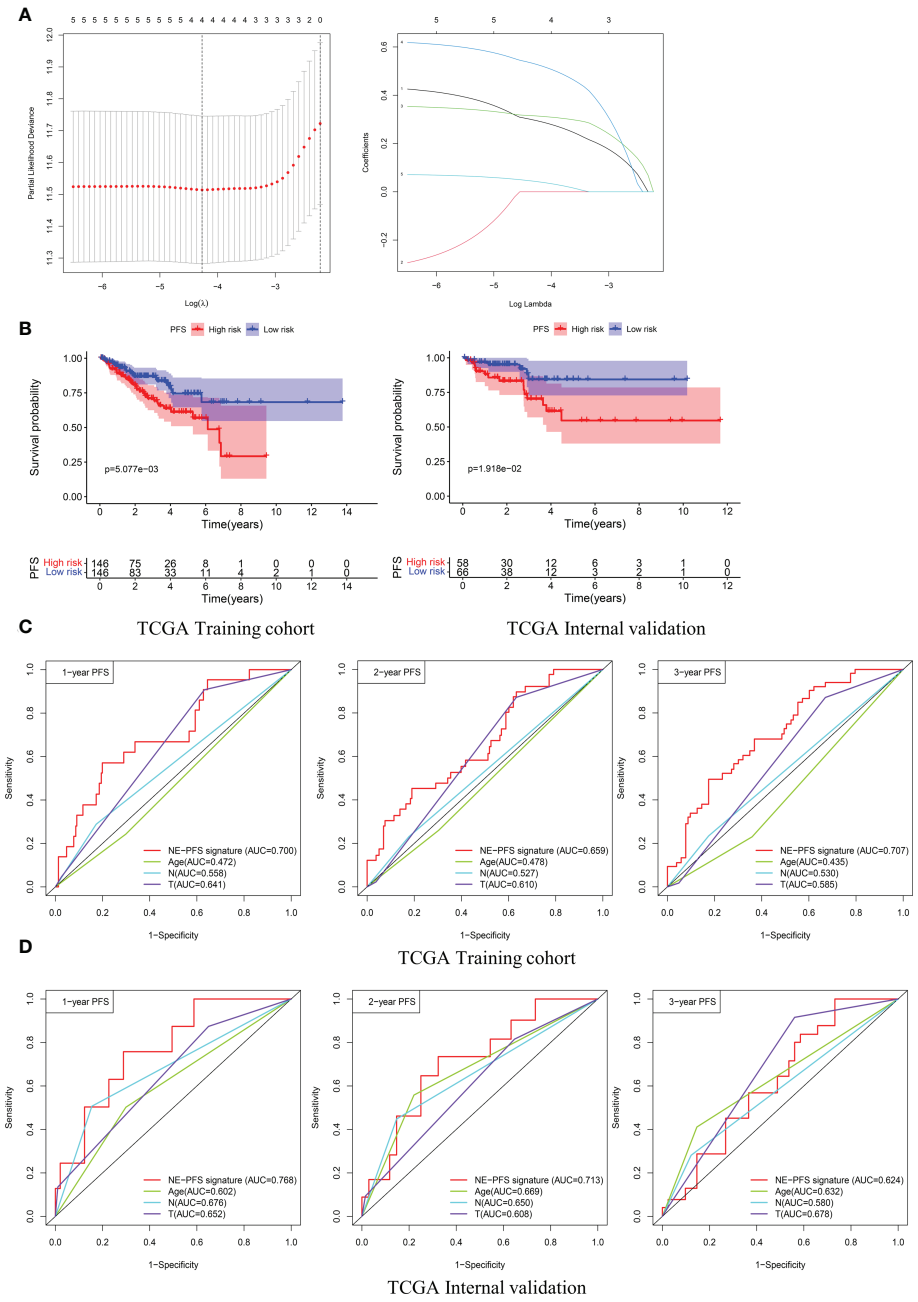

FIGURE 10
Construction and validation of the prognosis model for PFS in the TCGA PanCancer cohort. **(A)** Four genes correlated with PFS were selected for multivariate analysis by LASSO regression analysis. **(B)** Kaplan-Meier plots evaluate the predictive ability of the constructed prognostic model in the TCGA PanCancer training cohort and internal validation cohort, respectively. **(C, D)** NE-PFS signature exhibited better predictive ability than other clinical features as displayed, the 1-, 2- and 3- year AUC for PFS was 0.700 (95% CI: 0.587–0.814), 0.659 (95% CI: 0.566–0.752), and 0.707 (95% CI: 0.622–0.792) in the TCGA PanCancer training cohort.

Discussion

Secondary CRPC and even NEPC emerge as one of the most important killers threatening men's health (50). In present study, Single-cell RNA seq and bulk RNA seq samples were used to discovered 12 differential genes characterized by CRPC-NE, the subsequent result demonstrated that a six–gene diagnostic signature (*HMGN2, MLLT11, SOX4, PCSK1N, RGS16* and *PTMA*) could serve as a reliable predictor to distinguish CRPC-NE from CRPC. Furthermore, we observed that there

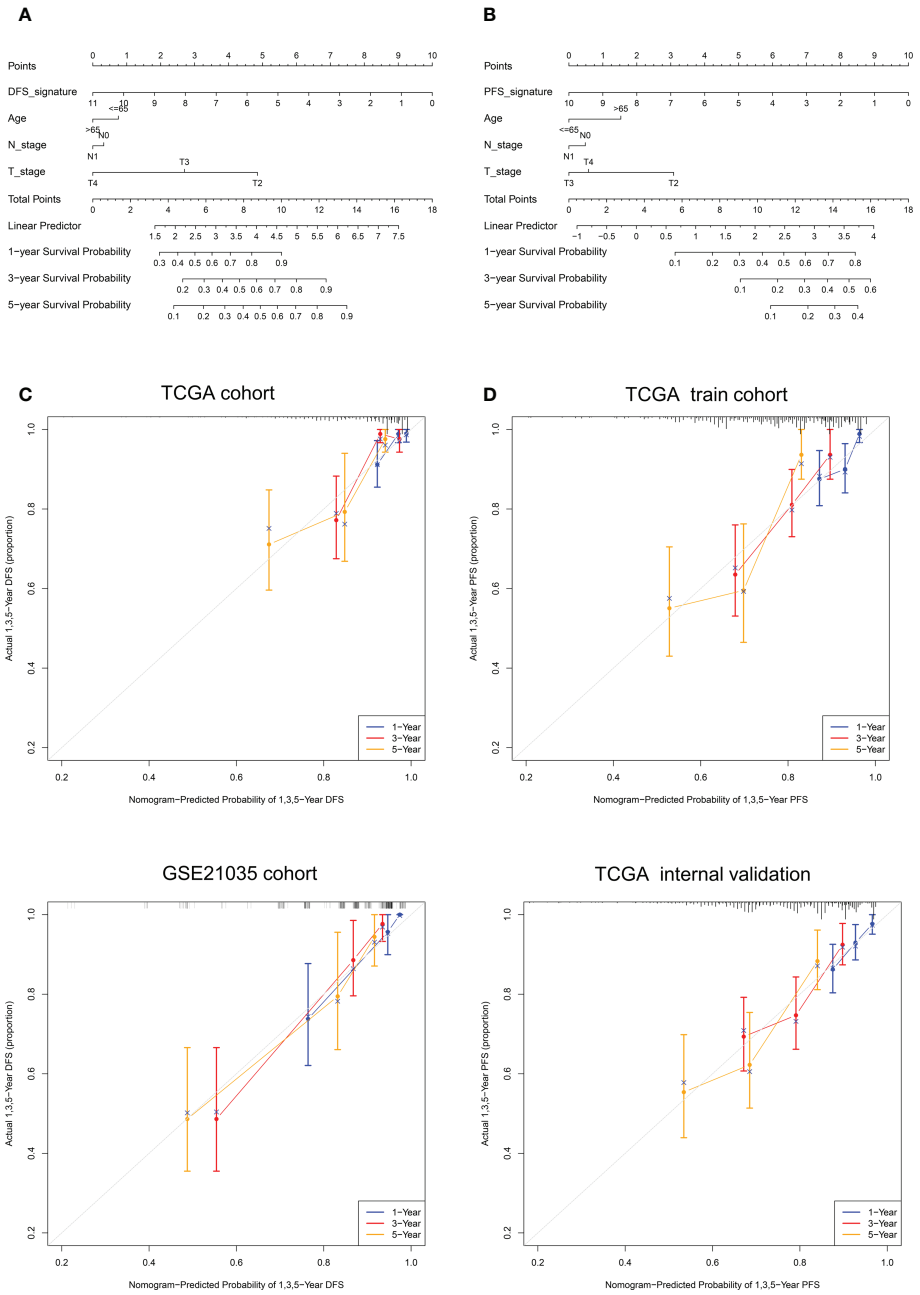

FIGURE 11
Nomogram construction and calibration plot validations for DFS and PFS prediction in PCa. **(A, B)** The composite nomogram consists of the DFS- or PFS- signature and clinical features of the individual patient, by adding the points from variables listed together, the 1-,3- and 5-year survival (DFS or PFS) probability can be inferred by the clinician. **(C, D)** Calibration curves for validation the consistence between 1-, 3- and 5-year (blue, red and orange color, respectively) inferred DFS and actual data in TCGA cohort and GSE21035 cohort. The dashed line represents the best match between the nomogram-predicted probability and the actual data evaluated by Kaplan–Meier analysis.

exists specific ligand–receptors among 16 cell types recognized, including ANGTP, CXCL, IGF, IL16, CSF, LIFR, OSM, and PROS pathways.

As is well-known, macrophage migration inhibitory factor (MIF) is involved in many carcinogenic processes, including cell proliferation, angiogenesis and inhibition of host tumor cell immune surveillance (51, 52). Experiments in LNCaP sublines indicated that during neuroendocrine differentiation, although MIF synthesis decreased, MIF release significantly increased, which may promote cancer progression or recurrence especially

after androgen deprivation (53). It can be seen from Figure 6C that there exists strong intercellular communication between NEPC_luminal/NE cells and T cells, B cells, plasma cells and monocytes *via* MIF (Macrophage migration inhibitory factor) pathway, where the ligand receptor pairs involved are MIF-(CD74 +CD44)and MIF-(CD74+CXCR4). It is worth mentioning that CXCR4 may form a functional MIF receptor complex with CD74, mediating MIF-stimulated, CD74-dependent AKT activation (54), In addition, *in vivo* and *in vitro* experiments showed that the inhibition of CXCR4 reduced the aggressiveness and chemosensitized PCa cells (55, 56), showing that MIF-(CD74 +CXCR4)axis can be used as the target of comprehensive treatment.

Most importantly, immune cell infiltration and GSVA analysis showed that there were also significant differences between CRPC and NEPC in KEGG pathways and immune cell abundance. Drug metablism cytochrome p450 pathway attracts our attention greatly, Cytochrome P450 protein is a monooxygenase involved in the synthesis of cholesterol, steroids and other lipids (57). Drug resistance to ADT such as abiraterone may be caused by overexpression or mutation of *CYP17A1*, increased upstream substrate synthesis, or increased drug metabolism or efflux (58). Studies in LNCaP cells and xenografts have shown that the enzymes required for *de novo* steroidogenesis (including *CYP17A1*) are increased in castration resistance sublines and can produce detectable androgen levels (59–61). Consistently, our study shows that cytochrome P450 pathway is highly expressed in CRPC. In addition, Maayan and Antonio' results showed that the production of dihydrotestosterone by neural-like cells was increased in mice in a *CYP17A1* independent manner under castration conditions (62, 63), accounting for the low expression of cytochrome P450 pathway in CRPC-NE to some extent (Figure 5E). Indeed, there is increasing evidence that prostate cancer cells transdifferentiate into neuroendocrine phenotypes and appear to be strongly induced in an androgen depleted environment (26, 64–66).

In our study, *HMGN2, MLLT11, SOX4, PCSK1N, RGS16* and *PTMA* were newly explored to predict the characteristics of CRPC-NE. Zhang et al. focused only on the bulk-RNA level, which may ignore the differences within the samples. Secondly, the samples with insufficient information are not filtered, resulting in bias consequently, our research overcomes these shortcomings. Previously, as an important developmental transcription factor, sex-determining region Y-box 4 (*SOX4*) proved to be combined with promoters to regulate genes closely related to neuroendocrine prostate cancer, including canonical *EZH2* (67, 68). Our research and previous studies have shown that the expression level of SOX4 increased with the progress of PCa, significantly higher in NEPC compared with CRPC (Figures 5B, 7B) (26, 69, 70). Current experiments also verified that SOX4 knockdown could reduce the proliferation of LNCaP-NEPC cells and inhibit the expression of NEPC markers (71). Prothymosin alpha (PTMA/ProTα) is widely expressed in many tissues and highly conserved in mammalian RNA sequences (Figure 8C) (72). Suzuki et al. demonstrated that the expression level of *PTMA* increased with the progression of normal epithelium, prostatic intraepithelial neoplasia (PIN) to prostate cancer, and was positively correlated with Gleason grade and clinical stage (73), but the relationship with NEPC was unknown.

When it comes to *HMGN2, MLLT11, PCSK1N* and *RGS16*, the diagnostic performance of them for NEPC has not been shown, deacetylation of high mobility group nucleosomal binding domain 2 (*HMGN2*) enhances STAT5A transcriptional activity, thereby regulating prolactin induced gene transcription and breast cancer growth (74, 75). Additionally, AZD1480 inhibits the growth of recurrent castration resistant CWR22Pc xenograft tumors by targeting JAK2-STAT5A/B signal transduction was observed in another study (76). Consequently, it is worth exploring the relationship between *HMGN2* and JAK2-STAT5A/B pathway. Involvement of *MLLT11* promoted the progression of ovarian cancer, bladder cancer and endometrial cancer in previous study (77, 78). Moreover, the granule protein family member *PCSK1N*, also known as ProSAAS, is a protein produced almost entirely by a wide variety of endocrine, neuronal and neuroendocrine cells (79, 80). Recently, the proteolytic neuropeptide PEN derived from the precursor ProSAAS has been identified as a selective, high affinity endogenous ligand for the orphan receptor GPR83. Both of them show regional specific expression in neuroendocrine tissues and may be used as a target for the treatment of neurological and immune diseases (81). Moreover, it is well acknowledged that the abnormal activity of phosphatidylinositol 3-kinase (PI3K) pathway supports the growth of many tumors, including breast, lung and prostate tumors. Studies have shown that G protein signaling 16 (*RGS16*) can act as a tumor suppressor by inhibiting the growth of PI3K dependent breast epithelial cells (82), while inhibiting PI3K/AKT downregulates REST expression and induces NE markers in LNCaP, PC3 and LNCaP95 cells (83). It is known that NEPC has great heterogeneity, integrating these different datasets to deduce 6 markers to predict the characteristics of CRPC-NE may be debatable. Actually, in order to reduce errors, we have eliminated atypical neuroendocrine prostate cancer including Paneth cell neuroendocrine differentiation, large cell neuroendocrine carcinoma, carcinoid, mixed samples and so on to reduce the heterogeneity within NEPC samples in order to produce more reliable biomarkers. What's more, because of the limited sample size in the public database, we are also collecting corresponding data in clinical work. We plan to carry out Bulk-RNA sequencing and SC-RNA sequencing on the same batch of CRPC and NEPC samples, and deduce biomarkers from the SC-RNA and Bulk-RNA sequencing data of the same batch of samples and verify them, so as to better reveal the similarities and differences between CRPC and NEPC.

Regarding the NE-DFS signature and NE-PFS signature, the former can accurately predict DFS in PCa patients, and shows

significant survival differences between low-risk group and high-risk group. It also shows excellent AUC values in GSE20135 (n=138) validation set, with AUC values of 0.899 (95% CI: 0.806 −0.992), 0.843 (95% CI: 0.746–0.941) and 0.810 (95% CI: 0.712 −0.907) for 1-, 2-, and 3-year DFS, respectively, which is significantly higher than the predictive ability of Gleason score and tumor stage. Previous researchers have used multivariable Cox regression analysis to obtain 22 autophagy related genes and build DFS prognosis model, although the AUC value of the prognosis model reached 0.85, there were too many biomarkers, which greatly reduced the clinical practicability (84). On the contrary, although our model only contained two genes (*STMN1* and *PCSK1N*), it still had high accuracy for clinical application. In Wang study, we can observe that the 1- and 3-year prognostic accuracy of AUC is 0.765 and 0.698 in the training cohort, 0.715 and 0.713 in the validation set, respectively (85). As for the NE-PFS signature composed of three markers (*STMN1, UBE2S* and *HMGN2*), the results showed that there was a significant difference in the survival rate between the low- and high-risk groups in the training cohort ($p = 0.005077$) and internal validation cohort ($p = 0.01918$), and the AUC curve of the prediction model at 1-, 2-, 3-year was greater than 0.65. However, due to the limited number of our samples, additional samples are needed to verify the robustness of the above model. We also actively recruit qualified patients and plan to make further verification. Secondly, the molecular mechanism of how the NE-DFS signature and NE-PFS signature affect the prognosis of PCa needs to be clarified through further clinical research.

Conclusion

In the present study, A robust signature composed of six genes for screening CRPC-NE were developed. In addition, we constructed and verified the DFS and PFS prognostic model for prostate cancer patients and the KEGG pathway difference as well as tight intercellular communication between CRPC and CRPC-NE were also further discussed, which is helpful to better guide clinical work.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JL and ZZ conceived and designed the study. JL and YC were responsible for data collection, collation and statistical analysis with bioinformatics methods. ZW, YM and JP carried out data interpretation and chart drawing. JL and YC wrote the manuscript, which was further polished and confirmed by YL and ZZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Comparison of the efficacy of endoscopic submucosal dissection and transanal endoscopic microsurgery in the treatment of rectal neuroendocrine tumors ≤ 2 cm

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Introduction: Currently, complete tumor resection is considered the most effective treatment for rectal neuroendocrine tumors (NETs). Endoscopic submucosal dissection (ESD) and transanal endoscopic microsurgery (TEM) are recommended for rectalNETs ≤2 cm, but it is not clear which method is better. Thus, we evaluated the efficacy of ESD and TEM in the treatment of rectal neuroendocrine tumors (NETs) ≤ 2 cm.

Methods: We conducted a single-centre retrospective cohort study between 2010 and 2021 of rectal NETs ≤ 2 cm in 114 patients with long-term follow-up data who were divided into ESD (n=55) and TEM groups (n=59). Our study assessed differences between groups in the complete resection rate of lesions, recurrence rate, surgical complications, procedure time, and length of hospital stay.

Results: The co-primary outcomes were the complete resection rate of lesions and the recurrence rate. Compared to that in the ESD group, the complete resection rate was significantly higher in the TEM group (91.5% vs. 70.9%, p=0.005). The median follow-up time was 22 months in our study, and the follow-up outcomes suggested that the rates of recurrence were 1.8% (1/55) and 6.8% (4/59) in the ESD and TEM groups, respectively, with no significant difference between the two groups. The secondary outcomes of the evaluation were surgical complications, procedural time, and length of hospital stay. The rate of complications (gastrointestinal bleeding and perforation) was low in both the ESD (7.3%, 4/55) and TEM (5.1%, 3/59) groups. No difference in hospitalization duration was observed between the two groups in our study. However, the procedure time was significantly shorter in the ESD group than in the TEM group (27.5 min vs. 56 min, p<0.001).

Conclusions: Although the rate of complete resection in the TEM group was higher than that in the ESD group, there was no difference in recurrence rates between the two modalities during long-term follow-up. Depending on the qualities of the available hospital resources in the area, one of the two approaches can be adopted.

KEYWORDS

rectal neuroendocrine tumor, endoscopic submucosal dissection, transanal endoscopic microsurgery, treatment, cohort study

Introduction

Neuroendocrine tumors (NETs) are considered to originate from the cells of the diffuse endocrine system. The gastrointestinal (GI) tract is one of the most common sites of NETs, including the stomach, small intestine, appendix, colon, and rectum (1). The small intestine, rectum and colon are the sites with the highest incidence of GI NETs. With a significant increase in morbidities due to rectal NETs, rectal NETs (17.7%) have overtaken small intestinal NETs (17.3%) as the most prevalent GI NETs (2). More than half of patients are diagnosed incidentally, which is attributed to the widespread use of endoscopic screening for colon cancer (3). Rectal NETs are usually small, rarely have symptoms, and are mainly in the anterior or lateral wall of the rectum above the dentate line (4, 5). Most rectal NETs are localized at diagnosis, and distant metastasis is rare (2-8%) (6). The treatment of rectal NETs depends on tumour size. For rectal NET lesions <1 cm, the risk of metastasis is less than 3% (7). The European Neuroendocrine Tumor Society guidelines recommend local resection by an endoscopic or with the transanal technique (7). Tumours between 1-2 cm in size without muscularis or lymphatic invasion can be removed by local resection (7). For rectal NETs ≥2 cm or between 1-2 cm with muscularis or lymphatic invasion or positive margins after local resection, radical surgery is recommended (8). However, a study found no difference in the rate of recurrence between patients with rectal NETs ≤2 cm with or without lymphatic invasion treated by local resection and those treated with radical surgery (9). It is generally accepted that, rectal NETs ≤2 cm with or without lymphatic invasion can be removed by local resection. The available options for local resection include endoscopic polypectomy, endoscopic mucosal resection (EMR), modified EMR, endoscopic submucosal dissection (ESD) and transanal endoscopic microsurgery (TEM) (10, 11). The advantage of ESD and TEM is that the rate of histological complete resection is higher than that of EMR, with a trend towards replacing EMR, especially in Asia (12). Compared to ESD, TEM can achieve full-thickness rectal resection and achieve a higher satisfactory complete resection rate (13). However, the TEM technique also has higher anaesthesia-related adverse events and postoperative morbidity (11). Most critically, TEM is not more effective over the long run than ESD (14). Currently, both ESD and TEM are commonly used techniques for the treatment of rectal NETs ≤2 cm, but there is no consensus on which of the two treatment options is better. Thus, we conducted a single-centre retrospective cohort study with long-term follow-up to compare the efficacy of ESD and TEM in the treatment of rectal NETs ≤2 cm.

Abbreviations: NETs, Neuroendocrine tumors; GI, Gastrointestinal; EMR, Endoscopic mucosal resection; ESD, Endoscopic submucosal dissection; TEM, transanal endoscopic microsurgery; CT, Computed tomography; MRI, Magnetic resonance imaging; WHO, World Health Organization; HPF, High-power fields; LAR, Low anterior resection; HR, Hazard ratio.

Methods

Study design

This study consecutively included 162 patients with rectal NETs ≤2 cm treated with ESD or TEM at Peking Union Medical College Hospital, a tertiary hospital in Beijing, between June 2010 and June 2021. Clinical information, including the patients' baseline data, tumour characteristics, pathological findings, and postoperative status, was collected from each patient through the electronic medical information system. Twelve patients with incomplete pathological findings were excluded. Then, 150 patients were divided into two groups and followed up. Thirty-six patients who were lost to follow-up or had no follow-up were excluded. Finally, a total of 114 patients were eligible for this study and were divided into ESD (55 patients) and TEM groups (59 patients). The study was approved by the Ethics Committee of Peking Union Medical College Hospital (No. K1331R). (The flowchart is shown in Figure 1).

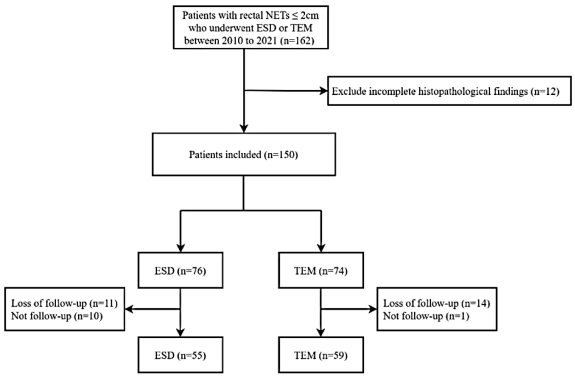

FIGURE 1
Flow chart for patient selection and follow-up.

Treatment procedure

We routinely performed an ultrasound endoscopy or rectal ultrasound to assess tumour size, depth of invasion and pararectal lymph node metastases before ESD or TEM. For the 1-2 cm rectal NETs, computed tomography (CT) or magnetic resonance imaging (MRI) was performed before treatment as a clinical requirement.

All ESD procedures were performed by an experienced endoscopist team, who had completed over 5,000 cases for colonoscopy training and completed more than 300 cases for ESD training. After marking the border with a Dual knife, the submucosa was adequately injected with an injection solution. The mucosa was incised along the anal side, and the lesion was lifted along the submucosa until complete excision was achieved, with electrocoagulation of the wound to stop the bleeding without causing significant muscle damage.

TEM was also required to be completed by an experienced surgeon who had completed over 300 cases for TEM training. After the successful administration of general anaesthesia, the patient was placed in the prone position, the skin in the routine surgical area was sterilized, and sterile towels were laid. After dilation of the anus to approximately two fingers wide, a proctoscope was slowly inserted, the submucosal nodules of the rectum were found, and the proctoscope was fixed on the surgical bed. The back panel of the proctoscope was covered, the mirror tube was inserted, various tubes were connected, CO_2 gas was introduced into the rectum, and the air pressure was regulated to between 12-15 mmHg. The rectum was observed for submucosal nodules with a smooth, yellowish-white surface mucosa. The submucosal nodules were gradually removed along the marker line from right to left, from superficial to deep, along with the entire intestinal wall. Then the rectal wound was sutured and checked for hemorrhage from the wound.

Histopathological assessment

The data of all resected lesions were recorded by one pathologist specializing in gastrointestinal tumours, including tumour size, the status of the cut margins, depth of invasion, lymphovascular invasion, Ki-67 index and mitotic count. The pathological reports were reviewed by another experienced pathologist.

Resection criteria

Lesions with negative lateral and deep margins were considered completely resected (The negative margin defined as no tumor cells contained). Conversely, incomplete resections were defined as lesions with positive lateral or deep margins.

Definition

The procedure time of the ESD was defined to be from the insertion of the endoscope to the removal of the submucosal nodules. The operation time for the TEM was defined to be from the insertion of the rectoscope to the end of the sutured.

The rectal NETs were graded according to 2010 World Health Organization (WHO) classification diagnostic criteria: G1: Ki-67 ≤ 2% and/or mitotic count <2 per 10 high-power fields (HPF); G2: Ki-67 3-20% and/or mitotic count 2-20 per 10 HPF; G3: Ki-67>20% and/or mitotic count >20 per 10 HPF.

Local recurrence was defined as the development of NETs adjacent to previous scars at least 3 months after resection. Distant recurrence was defined as the development of NETs outside the rectal wall. Overall recurrence included local recurrence and distant recurrence.

Follow-up

Outpatient examinations, telephone and email follow-ups were performed. The last assessment colonoscopy combined with CT/MRI was used as the cut-off time for follow-up. Those who did not complete the above examinations and could not be contacted by the researchers were considered lost to follow-up.

Statistical analysis

SPSS 26.0 statistical software (International Business Machines Corporation Inc, New York, USA) was applied to analyse the data. Normally distributed continuous variables are expressed as the mean ± standard deviation, and a two-sample *t* test was used to compare the differences between the two groups. Non-normally distributed continuous variables are expressed as medians, and the Mann–Whitney U test was used to compare the outcomes between the two groups. Categorical variables are expressed as frequencies and percentages, and the $\chi 2$ test or Fisher's exact test was used for comparisons of ESD and TEM. $p<0.05$ was considered statistically significant. Recurrence-free survival for ESD and TEM was *calculated* using the Kaplan–Meier curve, and the analysis software was GraphPad Prism 9 (GraphPad Software Inc, California, USA). Univariate and multivariate analyses were performed using the Cox proportional hazard model, and variables with P<0.05 in the univariate analysis were included in the multivariate analysis.

Results

Patient baseline data and tumour characteristics

In this study, 55 and 59 patients were eventually included in the ESD and TEM groups as determined through doctor–patient communication, respectively. Baseline features (age, sex, history of smoking, alcohol consumption, diabetes mellitus, hyperlipidaemia, personal and family malignancy history, and previous EMR history), as well as tumour characteristics (number, location, size [diameter], depth of infiltration, and lymph node infiltration), are shown in Table 1. There was no difference between ESD and TEM in baseline features and tumor characteristics.

Treatment outcomes of ESD and TEM

Regarding efficacy, the complete resection rate was significantly higher in the TEM group than in the ESD group (91.5% vs. 70.9%, $p=0.005$). There were 16 cases of incomplete resection in the ESD group and 5 cases in the TEM group. The four patients with incomplete resection without lymphovascular invasion in the ESD group were treated with TEM. The two incomplete resection patients with lymphovascular invasion received low anterior resection (LAR) as salvage treatment. Two of five patients with incomplete resection in the TEM group received LAR.

Regarding safety, GI bleeding occurred in three patients in each of the two groups, and GI perforation occurred in one patient in the ESD group. There was no difference in complications between ESD and TEM (7.3% vs. 5.1%, $p = 0.924$). No difference was seen between the two groups in the days of hospitalization. The procedure time of ESD (27.5 min, range 10-60 min) was significantly shorter than that of TEM (56 min, range 20-180 min) ($p<0.001$) (Table 2).

Postoperative pathological assessment and tumor grade

There were 3 cases of lymphovascular invasion in the ESD group and 1 case of lymphovascular invasion in the TEM group. Regarding the Ki-67 index assessment, a Ki-67 index ≤2 was observed in 52 cases in the ESD group and 51 cases in the TEM group, and a Ki-67 index of 3-20 occurred in 3 cases in the ESD group and 7 cases in the TEM group. No cases in the ESD group had a Ki-67 index >20% from pathology, and 1 case in the TEM group had a Ki-67 index >20%. No differences were seen between ESD and TEM in the postoperative pathological assessment, including lymphovascular invasion and Ki-67 index. The grade of rectal NETs was not significantly different between the ESD and TEM groups ($p=0.284$) (Table 3).

Follow-up outcomes of ESD and TEM

The median follow-up time was 22 months (range: 2-117). In the ESD group, the median follow-up time was 19 months (range: 2-75). The median follow-up time was 28 months (range: 2-117) in the TEM group. One patient in the ESD group had local recurrence. No local recurrence was seen in the TEM group. Distant metastases occurred in 4 cases in the TEM group. The rates of overall recurrence were 1.8% and 6.8% in the ESD and TEM groups, respectively, with no significant difference between the two groups (Table 4).

All recurrences were observed in patients with complete lesion excision. There was no recurrences in patients with lesions considered incompletely resected, regardless of whether additional surgical treatment was provided, in the both of two groups.

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TABLE 1 Patients baseline data and characteristics of tumors.

Variable	ESD	TEM	*P* value
N	*55*	59	
Age at diagnosis(y, mean ± SD)	*52.9 ± 11.7*	51.1 ± 12.1	0.429
Sex (F/M)	*35/20*	41/18	0.508
History of smoking (%)	*23 (41.8)*	18 (30.5)	0.209
History of alcohol consumption (%)	*21 (38.2)*	14 (23.7)	0.095
Diabetes mellitus (%)	*5 (9.1)*	7 (11.9)	0.630
Hyperlipidaemia (%)	*4 (7.3)*	7 (11.9)	0.407
Combined malignancy (%)	*6 (10.9)*	8 (13.6)	0.667
History of malignancy in family members (%)	*14 (25.5)*	9 (15.3)	0.175
Previous EMR history	*2 (3.6)*	5 (8.5)	0.493
Number of tumors			0.768
Single lesion	*52 (94.5)*	55 (93.2)	
Multiple lesions	*3 (5.5)*	4 (6.8)	
Distance of the tumor from the anal verge (cm, median, range)	*8 (3-15)*	7 (3-10)	0.106
Tumour size			
Endoscopic evaluation (mm, median, range)	*6 (3-20)*	6 (2-20)	0.476
Histopathological evaluation (mm, median, range)	*7 (2-20)*	6 (2-20)	0.431
Depth of invasion			0.388
Mucosa (%)	*9 (16.4)*	6 (10.2)	
Submucosa (%)	*45 (81.8)*	50 (84.7)	
Muscularis propria (%)	*1 (1.8)*	3 (5.1)	
Plasma (%)	*0 (0.0)*	0 (0.0)	
Lymph node invasion (%)	*1/55 (1.8)*	1/56 (1.7)	0.99

SD, standard deviation; F/M, Female/Male; EMR, endoscopic mucosal resection.

TABLE 2 Treatment outcomes for ESD and TEM group.

Variable	ESD	TEM	*P* value
N	*55*	59	
Complete resection (%)	*39 (70.9)*	54 (91.5)	0.005
Additional salvage treatment	*6 (10.9)*	2 (3.4)	0.229
TEM (%)	*5 (9.1)*	0 (0.0)	0.056
LAR (%)	*1 (1.8)*	2 (3.4)	1.000
Complication (%)	*4 (7.3)*	3 (5.1)	0.924
Bleeding	*3 (5.5)*	3 (5.1)	1.000
Perforation	*1 (1.8)*	0 (0.0)	0.226
Hospitalization (days, median, range)	*4 (2-26)*	4 (1-9)	0.695
Procedure time (min, median, range)	*n=14, 27.5 (10-60)*	n=49, 57 (20-180)	<0.001

TEM, transanal endoscopic microsurgery; LAR, low anterior resection.

TABLE 3 Post-operative pathological assessment and tumor grade.

Variable	ESD	TEM	*P* value
N	*55*	59	
Lymphovascular invasion (%)	*3 (5.5)*	1 (17)	0.561
Ki-67 (%)			0.324
≤2	*52 (94.5)*	51 (86.4)	
3-20	*3 (5.5)*	7 (11.9)	
>20	*0 (0.0)*	1 (1.7)	
Grade of WHO			0.284
G 1	*47 (85.5)*	45 (76.3)	
G 2	*8 (14.5)*	13 (22.0)	
G 3	*0 (0.0)*	1 (1.7)	

WHO, World Health Organization.

Figure 2. showed the recurrence-free survival time of patients with rectal NETs ≤2 cm in both ESD and TEM groups. Univariate Cox analysis revealed that baseline hyperlipidaemia (hazard ratio [HR]: 11.152, 95% confidence interval (CI): 1.721-72.282, p=0.011), depth of invasion (HR: 8.280, 95% CI: 1.027-66.754, p=0.047), and distance of the tumor from the anal verge (HR: 0.327, 95% CI: 0.136-0.778, p=0.013) were associated with recurrence outcomes.

Characteristics of tumors for patients with recurrent rectal NETs

The five patients with recurrent rectal neuroendocrine tumors were all male, and the median age was 44 years, ranging from 26-69 years. The median tumor diameter was 10 mm, ranging from 5-15 mm. Four patients had lesions invading the submucosa, and 1 patient had a lesion invading the muscularis propria. Lymph node invasion was observed in one patient. According to the WHO tumor grade, G1 tumors occurred in 4 cases, and a G2 tumor occurred in 1 case. One patient who underwent ESD was found to have recurrence *in situ* during follow-up. Four patients underwent TEM, and distant metastases were found at follow-up. The information on recurrence in these 5 patients with rectal NETs is summarized in Table 5.

Discussion

In this research, we evaluated the effectiveness of TEM and ESD in the management of rectal NETs under 2 cm. The complete resection rate of lesions and the recurrence rate following treatment during long-term follow-up were the two

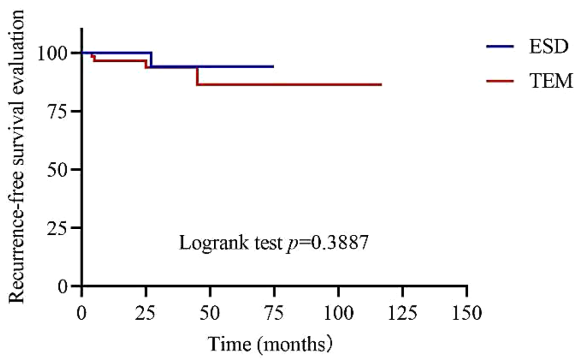

FIGURE 2
Recurrence-free survival time of patients with rectal NETs ≤ 2 cm between ESD and TEM groups.

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TABLE 4 Follow up for ESD and TEM group.

Variable	ESD	TEM	*P* value
N	*55*	59	
Follow-up time (months)	*19 (2-75)*	28 (2-117)	0.012
Recurrence			0.119
No (%)	*54 (98.2)*	55 (93.2)	
Local recurrence (%)	*1 (1.8)*	0 (0/0)	
Distant metastases (%)	*0 (0.0)*	4 (6.8)	

metrics we used to assess efficacy. The full resection rate in the TEM group was much higher than that in the ESD group (91.5% vs. 70.9%). In our investigation, the median follow-up period was 22 months, and the follow-up results indicated that the recurrence rates in the TEM and ESD groups were 6.8% (4/59) and 1.8% (1/55) respectively. Four individuals in the TEM group and one patient in the ESD group among the patients with recurrent rectal NETs both suffered distant recurrence. Differences in surgical complications, procedure time, and length of hospital stay between the ESD and TEM groups were the evaluation's secondary outcomes. Both the ESD (7.3%, 4/55) and TEM (5.1%, 3/59) groups had modest rates of problems. In our investigation, there was no difference in the length of hospitalization between the two groups. However, the ESD group's method took far less time than the TEM group did (27.5 min vs. 56 min).

Traditionally, incomplete resection of the lesion is a factor for poor prognosis, and the goal of local excision is complete resection of the lesion. In our study, the complete resection rate in both the ESD and TEM groups was high, especially in the TEM group. Some studies have also confirmed this result. Sung et al. reported that both ESD and TEM achieved a high complete resection rate in T1 rectal NETs. The study further used propensity score matching and suggested that the rate of complete resection was higher in TEM than in ESD (92.3% vs. 71.2%) (15). Joon et al. found that the complete resection rate of rectal NETs was higher in the TEM group (14/14, 100%) than in the ESD group (19/23, 82.6%). No local recurrence of tumors was seen in any patient, regardless of complete or incomplete resection (16). Unfortunately, the sample size of the study was too small to confirm whether recurrence of rectal NETs was associated with complete resection of the lesion. In our study, no

TABLE 5 Characteristics of tumors for patients with recurrent rectal NETs.

Variable	1	2	3	4	5
Sex	M	M	M	M	M
Age	69	26	62	38	44
Tumor size (mm)	5	14	8	15	10
Depth of invasion	Submucosa	Submucosa	Submucosa	Muscularis propria	Submucosa
Lymph node invasion	No	No	No	No	Yes
Grade of WHO	1	2	1	1	1
Resection type	ESD	TEM	TEM	TEM	TEM
Margin invasion	No	No	No	No	No
Lymphovascular invasion	No	No	No	No	No
Type of recurrence	Local recurrence	Distant metastases	Distant metastases	Distant metastases	Distant metastases
Location of recurrence	Rectum	Liver	Liver	Lymph node	Lymph node
Time of recurrence (month)	27	45	25	5	4
Outcomes after treatment	ESD	Not available	Not available	LAR	Somatostatin analogues

ESD, endoscopic submucosal dissection; TEM, transanal endoscopic microsurgery; LAR, low anterior resection; WHO, World Health Organization.

recurrence was seen in any patients with lesions considered incompletely resected. Therefore, we inferred that whether the resection margin of tumor cells was positive was not associated with tumor recurrence. Chung et al. detected thirteen (3.9%) patients with rectal NETs that presented positive resection margins after treatment with EMR, modified EMR and ESD. Five of thirteen patients accepted additional treatment, but no recurrence was observed in the patients with positive margins, with or without additional treatment (17). Similarly, Pattarajierapan et al. also found that 2.2% of rectal NET patients with positive margins had no recurrence (18). Li et al. reported that 54 patients had incompletely resected lesions out of 428 patients with rectal NETs, and the incomplete resection rate was 12.6%. All patients with rectal NETs underwent treatments including EMR, precutting EMR and ESD. No recurrence of the tumors was observed in the patients with incomplete resection during the follow-up period (19). On the whole, positive lesion margins do not indicate tumor recurrence. The necessity of additional treatment in patients with incomplete lesion excision is debatable. The above studies, including our study, suggest that endoscopic monitoring can be performed for rectal NET patients with incomplete lesion resection rather than additional treatment.

In terms of safety, there was no difference between the ESD and TEM groups in complications, including GI bleeding and perforation, or length of hospitalization. However, the procedure time was significantly shorter in the ESD group than in the TEM group. Compared to ESD, TEM operation needed additional suturing of the intestinal wall, which may extent the procedure time. Moreover, some studies such as Jung et al. and Mao et al. had defined the operation time different, which may cause bias in the procedure time (20, 21).

In previous studies, a number of factors, including tumor size, depth of invasion, lymphatic invasion, presence of central depression, positive resection margin, mitotic rate, and Ki-67 index, were found to predict unfavourable outcomes (22–25). In our study, univariate Cox analysis found that depth of invasion, the distance of the tumor from the anal verge and hyperlipidaemia were correlated with recurrence of the tumor. It has been shown that the depth of infiltration is associated with tumor recurrence, which is consistent with previous studies. Surprisingly, tumor distance from the anus verge and hyperlipidaemia were associated with tumor recurrence. Duan et al. reported that colorectal NET patients with lesions> 5 cm from the anal margin in the rectum have a better prognosis (26). This result may be associated with rectal vascularity and lymphatic distribution. There are few studies on the distance of the lesion from the anal verge affecting tumour recurrence, and this could be further investigated in the future. The relationship between hyperlipidaemia and the recurrence of rectal neuroendocrine tumours is unclear, but a study found that rectal NETs are more likely to occur and persist in areas with high serum cholesterol levels (27).

There was 1 patient and 4 patients in the ESD and TEM groups, respectively. Local recurrence, despite not significantly different, was only seen in the ESD group. All distant recurrence were seen in the TEM group. One of the patients who had distant metastases with a tumor size 15 mm in diameter and muscular involvement received TEM initially. Five months after TEM, lymph node metastasis was found in the rectal mesenteric region and further LAR with lymphadenectomy was performed. No recurrence was observed after 6 months. The choice of local resection or radical resection for rectal NETs between 10 mm to 20 mm remains controversial. The ENETS guidelines recommend local resection for rectal NETs<20 mm with a low mitotic rate and no muscular involvement (7). In addition, Shigeta et al. found that there was no difference in recurrence rate between local resection and radical resection in rectal NETs patients with tumor size>10mm and lymphovascular invasion (9). Therefore, more evidence is needed to clarify whether local or radical resection is more appropriate for rectal NETs between 10-20mm.

There are two limitations in the study. First, there was only one centre included in the study, so the results were limited. Second, the follow-up time was not long enough. The median follow-up times in the ESD and TEM groups were 19 and 28 months, respectively. Patients with incompletely resected lesions were followed up for 28 months. Patients with completely resected lesions were followed up for 27 months. The follow-up period was not long enough to strongly indicate that there would not be any recurrences in the future. In future studies, the follow-up time can be extended to further confirm that the recurrence rates after ESD and TEM are similar.

Conclusion

Despite the fact that the TEM group had a greater percentage of full resection than the ESD group did, there was no difference in the rates of tumor recurrence between the two modalities during long-term follow-up. One of the two ways can be employed depending on the characteristics of the local hospital resources that are accessible.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

RJ, XB and JL designed this study. RJ, XB, TX and QW collected data. RJ completed statistical analysis and wrote the

initial manuscript. XB, XW and JL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Clinical and pathologic characteristics of appendiceal neuroendocrine neoplasms diagnosed during pregnancy

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Introduction: Although appendicitis occurs in approximately 1:1000 pregnancies, appendiceal neuroendocrine neoplasm (ANEN) diagnosis during pregnancy is very rare. Data on presentation, treatment and prognosis is scarce.

Aim: To describe ANEN cases diagnosed during pregnancy.

Materials and methods: A retrospective appraisal of 7 consecutive ANEN patients diagnosed during pregnancy from four Israeli tertiary medical centers and comparison with 17 cases described in the literature from 1965-2021.

Results: Age at ANEN diagnosis was 26.4 ± 3.5 years (range 21-33). Patients were diagnosed between gestational weeks 6-40, most frequently in the third trimester (53%). The most common presenting symptom was abdominal pain. Tumor size was 14.3 ± 8.9mm (range 3-45mm). In patients from our series appendiceal base involvement was reported in 2/7; mesoappendiceal invasion in 5/7; lympho-vascular invasion in 2/7. Ki67 staining was reported in 6/7 cases and ranged from 1-10%. Pathology details were lacking in most of the previously published cases. All 7 pregnancies in our series resulted in term delivery with no complications, whereas in historical cases there were one first trimester abortion, one ectopic pregnancy, and one stillbirth. Right hemicolectomy was performed in 5/7 patients in our series and reported in 2/17 historical cases. All hemicolectomies were performed after delivery, 3-16 months after appendectomy. Local metastases were reported in two cases. Follow-up duration was 7-98 months for our patients and 3-48 months in 5 historical cases. No disease recurrence, distant metastases or mortality were noted.

Conclusions: To the best of our knowledge, this is the largest series describing the extremely rare diagnosis of ANEN during pregnancy. Although pathologic characteristics varied, pregnancy outcomes were usually favorable and long-term prognosis was excellent. This data may suggest that a conservative approach to patients with ANEN diagnosis during pregnancy can be considered.

KEYWORDS

pregnancy, appendix - appendicitis, neuroendocrine tumor, appendectomy, hemicolectomy

1 Introduction

Appendiceal neuroendocrine neoplasm (ANEN) are diagnosed in approximately 0.5% of appendectomies (1–5). These tumors usually harbor excellent prognosis, and rarely require further treatment beyond appendectomy (6, 7). Local lymph node metastases may be present, but distant metastases and disease-related mortality are rare. Right hemicolectomy is suggested for selected patients (8, 9) with large or invasive tumors. Although hormonal hypersecretion syndromes such as carcinoid syndrome and ectopic Cushing's syndrome have been described in ANEN, these are extremely rare (10, 11).

Although appendicitis and appendectomy occur in approximately 1:1000 pregnancies (12, 13), diagnosis of ANEN during pregnancy is extremely rare. Only 12 cases have been reported in the literature since 1965 (14–23), including only one series of four cases (14). Additionally, five cases of incidental ANEN diagnosed during Cesarian section (CS) have been reported (14, 24, 25).The clinical and pathological data in most of these reports is incomplete.

Epidemiologic and clinical characteristics of ANEN in pregnancy have not been systematically described. The effect of the tumor and its resection on pregnancy outcome is unknown. Moreover, the effect of pregnancy and its unique hormonal and immune milieu on tumor progression and spread has not yet been described. As a result, there are no specific guidelines for evaluation and treatment of patients with ANEN diagnosed during pregnancy. Many clinical issues remain to be resolved, such as sensitivity of imaging studies performed during pregnancy prior to appendectomy; the need for further imaging studies after appendectomy during pregnancy and after delivery; and the sensitivity and specificity of biochemical markers such as chromogranin A and 5-hydroxy-indol-acetic acid (5-HIAA) in this context.

The most important clinical dilemma is patient selection for right hemicolectomy. To date, no pregnancy-specific criteria for right hemicolectomy have been suggested. Furthermore, the preferred timing for hemicolectomy, whether during pregnancy (particularly if ANEN is diagnosed in early pregnancy) or in the post-partum period has not been determined.

In this study, retrospective data were collected from 7 Israeli women with ANEN diagnosed during gestation, as well as 17 cases of gestationally-diagnosed ANEN previously described in the literature. Clinical and pathologic characteristics and long-term follow-up data, where available, are described.

2 Materials and methods

Data of 7 ANEN female patients diagnosed during pregnancy were collected from electronic files of four tertiary medical centers in Israel. In addition, data pertaining to another 17 cases described in the literature was retrieved. Data included patient's age, pregnancy week at diagnosis, presenting symptoms and pre-appendectomy imaging studies. Histopathological characteristics of the tumor included size, location in the appendix, depth of invasion, lympho-vascular invasion (LVI), perineural invasion, immunohistochemical staining and proliferation index (Ki-67). Post appendectomy evaluation included imaging, chromogranin A and 5-HIAA testing; right hemicolectomy indication, timing and outcome (if conducted); pregnancy outcome and long-term surveillance data including tumor recurrence and mortality.

2.1 Data analysis

Categorical variables are presented as frequency and percentage. Continuous variables are presented as mean and standard deviation or median and range. Groups were compared using Student t-test. A p-value < 0.005 was considered statistically significant.

2.2 Ethical considerations

The study protocol was approved by the Institutional Ethics Committees of the four Medical Centers (Meir Medical Center, Hadassah Medical Center, Rabin Medical Center and Wolfson Medical Center 0143-21-WOMC). In accordance with Helsinki regulations for clinical studies based on chart review, informed consent was waived.

3 Results

Data of 19 patients with ANEN diagnosed during pregnancy (7 from our series and 12 previously published in the literature) and 5 ANEN cases diagnosed incidentally at CS (all from previous publications) were included in the analysis (Table 1). Historical cases were published between 1965-2019. Patients diagnosed during

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TABLE 1 Clinical characteristics of 24 appendiceal neuroendocrine neoplasms diagnosed during pregnancy.

Case number	Data source	Age et diagnosis (years)	Pregnancy week	Clinical presentation	Pre-operative imaging	Pregnancy outcome	Follow-up duration (months)
1	Current series	27	27	Abdominal pain	US	NA	98
2	Current series	31	6	Abdominal pain	US	Vaginal term delivery	32
3	Current series	21	38	Abdominal pain	US	Vaginal term delivery	11
4	Current series	26	13	Abdominal pain	US-suspected appendicitis	Vaginal term delivery	23
5	Current series	33	30	Abdominal pain	US	Vaginal term delivery	9
6	Current series	23	22	Abdominal pain	US- appendix not seen, MRI-dilated appendix	CS term delivery	15
7	Current series	24	31	Abdominal pain	us- appendix not seen, MRI-dilated appendix	Vaginal term delivery	7
8	Berrios 1965	23	10	Abdominal pain	NA	Vaginal term delivery	48
9	Berrios 1965	26	12	Abdominal pain	NA	NA	NA
10	Jurica 1989	24	21	Abdominal pain	NA	Stillbirth	NA
11	Mclean 1994	30	37	Abdominal pain	NA	CS term delivery	NA
12	Korkontzelos 2005	23	16	Abdominal pain	NA	CS term delivery	NA
13	Pitiakoudis 2008	24	32	Abdominal pain	NA	Vaginal term delivery	NA
14	Gilboa 2008	31	9	Abdominal pain	Trans vaginal US-edematous appendix	1st trimester abortion	NA
15	Thompson 2011	27	NA	Abdominal pain	NA	Ectopic pregnancy	NA
16	Poiana 2012	27	NA	NA	NA	NA	NA
17	panagiotis 2013	22	27	Abdominal pain	MRI-dilated appendix	NA	NA
18	Piatek 2016	28	25	Abdominal pain	US-appendix not seen	Vaginal term delivery	12
19	Vanags 2017	24	35	Abdominal pain	US-appendix not seen	NA	NA
20	Berrios 1965	21	NA	Routine appendectomy during CS	irrelevant	irrelevant	NA
21	Berrios 1965	30	38	Routine appendectomy during CS	irrelevant	irrelevant	NA
22	Syracuse 1979	31	NA	Routine appendectomy during CS	irrelevant	irrelevant	48
23	Gokaslan 2002	30	NA	Routine appendectomy during CS	irrelevant	irrelevant	3
24	Janicki 2019	27	40	Routine appendectomy during CS	irrelevant	irrelevant	36

US, ultrasound; NA, data not available; CS, cesarean section; MRI, magnetic resonance imaging.

gestation presented with abdominal pain, and in most cases were suspected to have appendicitis. Patients diagnosed at CS had appendectomy as a routine procedure or due to abnormal appearance of the appendix. There were no cases of pre-operative tumor diagnosis, and no described signs and symptoms of hormone hyper-secretion syndromes.

Age at ANEN diagnosis was 26.4 ± 3.5 years (range 21-33); there was no significant age difference between cases diagnosed during

pregnancy and those incidentally diagnosed during CS (26 vs. 27.8 years, respectively, p=0.302). As presented at Figure 1, patients were diagnosed at gestational week 6-40, but more frequently in the third trimester. In the 7 cases within our series, pregnancy outcomes were favorable (all resulted in term delivery, 6/7 vaginal delivery and 1/7 CS), whereas in historical cases there were one case of first trimester spontaneous abortion five days after appendectomy (26), one case of ectopic pregnancy implanted on the tip of the appendix that was diagnosed during appendectomy (19) and one stillbirth at 21 weeks' gestation in a patient with concomitant Chlamydia trachomatis infection (15); CS was conducted in 2/5 term deliveries (26). No other post-appendectomy complications were noted.

Data on pre-operative imaging was available for 11 patients. Trans-abdominal ultrasound (US) was performed in in 8/11, magnetic resonance imaging (MRI) in 1/11 and both in 2/11. Trans-abdominal US failed to demonstrate the appendix in 4/11 patients presenting at pregnancy weeks 22-35. MRI demonstrated abnormal findings in the appendix in 3/3 cases. In one additional case, abnormal findings were described in the appendix on vaginal US (26). In all cases the pre-operative imaging results were compatible with the diagnosis of appendicitis but did not reveal the existence of appendiceal tumor.

Tumor histopathological characteristics are presented in Table 2. Tumor size was 14.3 ± 8.9 mm (range 3-45 mm). There was no difference between tumors diagnosed during pregnancy and during CS (13.6 vs. 16.4 mm, respectively, p=0.550) or between our series and historical cases (14.8 vs. 13.5 mm, respectively, p=0.574). Other pathology details were incomplete in most of the previously published cases and in one of our cases. Involvement of the appendiceal base was reported in 3/6 of our series and in no historical cases. Mesoappendiceal invasion was reported in 5/6 of our cases and 3 previously reported cases. LVI was reported in 2/7 of our cases and in 2 of previously reported cases. Ki67 staining was reported in 6/7 of our cases and ranged between 1-10%. Only 5/17 historical cases reported Ki67 staining results, which ranged between 1-2%. For one case in the series of Berrios et al, published in 1965, methenoamine silver and ferrous cyanide staining was reported. Neuroendocrine-specific staining (chromogranin A and synaptophysin) was not reported in cases published before 2005. Positive chromogranin staining was reported in 5/7 of our cases and in 6/17 historical cases. Positive synaptophysin staining was reported in 5/7 of our cases and in 4/17 of historical cases.

Right hemicolectomy was performed in 5/7 patients in our series and reported in 2/17 historical cases. Indications for hemicolectomy were size greater than 2cm in 2 cases, involvement of the appendiceal base in 2 cases, meso-appendiceal invasion in 6 cases, LVI in 3 cases, and Ki67above 2% in 2 cases. All hemicolectomies were performed after delivery, 3-16 months after appendectomy. Hemicolectomy pathology results are presented in Table 2. Local metastases were reported in 2 cases: one had a lymph node metastasis, and the other had a focus of tumor in fat tissue.

Follow-up duration was 7-98 months in our series and 3-48 months in five historical cases. No disease recurrence, distant metastases or mortality were noted. All surveillance imaging studies were negative: abdominal US (1 case), abdominal CT (2 cases), abdominal MRI (4 cases), and Ga68-DOTATATE PET-CT (3 cases). Serum chromogranin A testing (5 cases) and urine 5-HIAA testing (5 cases) during follow-up were within normal range.

4 Discussion

This is the largest series to date of ANEN diagnosed during pregnancy, incorporating 7 new cases together with a review of 17 historical cases. Treatment of neoplastic disease during pregnancy is challenging due to the inherent dilemma between the desire to protect maternal health and the wish to continue the pregnancy and protect the fetus. This challenge is more pronounced in ANEN as data on tumor behavior during pregnancy is limited, and no international guidelines discuss this rare clinical scenario (8, 9, 27). The aim of this study was to gather existing data on ANEN diagnosed during pregnancy in order to assist in clinical decision making.

4.1 Epidemiology

ANEN diagnosis during pregnancy is extremely rare. This is somewhat surprising, because ANEN is more common in women, with a female preponderance of 52-70% of all ANEN patients described in previous reports (2–4, 28, 29). Moreover, ANEN

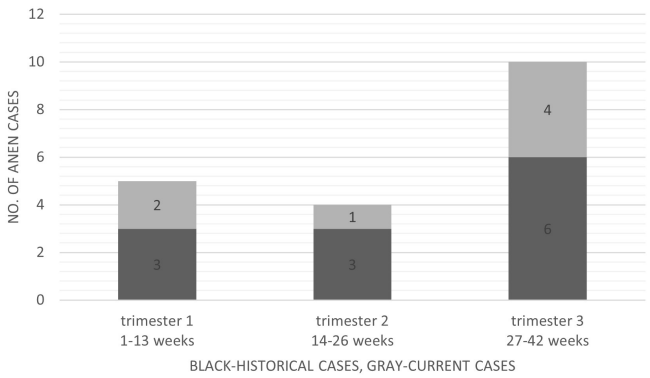

FIGURE 1
Appendiceal neuroendocrine neoplasms diagnosed during pregnancy divided by pregnancy trimester at diagnosis.

TABLE 2 Pathology data of 24 Appendiceal Neuroendocrine Neoplasms diagnosed during pregnancy.

Case number	Data source	Tumor size, mm	Tumor location	Mesoappendix invasion	Lympho-vascular invasion	Ki67 (%)	Mitoses/ HPF	CgA/ synaptophysin staining	inflammation	perforation	Hemicolectomy performed
1	Current series	3	tip	NA	NA	NA	NA	NA/NA	+	–	No
2	Current series	45	base	+	+	1	2	+/+	–	–	Yes, single focus of NEN in fatty tissue
3	Current series	11	tip	+	+	2	NA	+/+	+	–	Yes, no residual tumor
4	Current series	12	base	+	–	4	NA	+/+	+	+	Yes, no residual tumor
5	Current series	13	middle	+	–	10	NA	+/NA	+	–	Yes, no residual tumor
6	Current series	13	NA	+	–	1	0	+/+	–	–	Yes, no residual tumor
7	Current series	14	tip	–	–	2	1	+/+	+	–	No
8	Berrios 1965	3	middle	NA	NA	NA	NA	NA/NA	+	–	NA
9	Berrios 1965	15	middle	NA	NA	NA	NA	NA/NA	NA	NA	NA
10	Jurica 1989	20	tip	–	NA	NA	NA	NA/NA	–	–	NA
11	Mclean 1994	13	NA	+	NA	NA	NA	NA/NA	–	–	No
12	Korkontzelos 2005	22	NA	NA	+	NA	NA	+/NA	–	–	Yes, no residual tumor
13	Pitiakoudis 2008	5	tip	NA	NA	NA	NA	NA/NA	+	–	NA
14	Gilboa 2008	NA	NA	NA	NA	NA	0	+/NA	NA	NA	NA
15	Thompson 2011	NA	NA	NA	NA	NA	NA	NA/NA	NA	NA	NA
16	Poiana 2012	10	Tip	NA	NA	1	NA	+/+	NA	NA	NA
17	panagiotis 2013	4.5	tip	–	NA	2	NA	+/+	+	–	NA
18	Piatek 2016	18	mid	–	NA	1	NA	+/+	+	–	No
19	Vanags 2017	10	NA	NA	NA	1	NA	+/+	NA	NA	NA
20	Berrios 1965	20	NA	NA	NA	NA	NA	NA/NA	NA	NA	NA

(Continued)

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TABLE 2 Continued

Case number	Data source	Tumor size, mm	Tumor location	Mesoappendix invasion	Lympho-vascular invasion	Ki67 (%)	Mitoses/ HPF	CgA/ synaptophysin staining	inflammation	perforation	Hemicolectomy performed
21	Berrios 1965	9	Middle	NA	NA	NA	0	NA/NA	NA	NA	NA
22	Syracuse 1979	15	NA	+	NA	NA	NA	NA/NA	–	–	Yes, 2/67 metastatic lymph nodes
23	Gokaslan 2002	20	tip	NA	+	NA	NA	NA/NA	NA	NA	No
24	Janicki 2019	18	NA	+	NA	1	0	+/+	NA	NA	No

CgA, chromogranin A; NA, data not available; NEN, neuroendocrine neoplasm.
+ means positive, - means negative, +/+ mean both parameters are positive (CgA and synaptophysin).

diagnosis is not uncommon in the reproductive years; in a series published by Rosenblum et al, 31% of reported patients were females between 20-40 years of age (1). Suspected appendicitis is the most common non-obstetric indication for surgical intervention during pregnancy. Appendicitis occurs in approximately 1:1000 births (12, 13). Interestingly, the rate of appendicitis is especially low in the third trimester (30, 31), in contrast to the higher rate of ANEN diagnosis in the 3rd trimester observed in this series.

The reasons for the low rates of both appendicitis in the 3rd trimester and ANEN during pregnancy are not well understood. However, clinicians must be alert to this possibility and know to identify relevant symptoms in order to avoid missed diagnosis of ANEN in the appendix.

4.2 Presentation

All patients diagnosed during pregnancy in the present cohort were admitted with abdominal pain. The majority had suspected acute appendicitis according to clinical and radiologic parameters. Pre-operative imaging with abdominal US or MRI revealed suspicious features for appendicitis but did not demonstrate the intra-appendiceal tumor. This is not surprising as ANEN are frequently not detected radiologically, most probably due to their small dimensions (32, 33). Since the tumors were not suspected pre-operatively, no patients performed pre-operative biochemical specific testing such as chromogranin A or urine 5-HIAA.

4.3 Pathology

Pathology data from previously reported cases was incomplete and did not enable in-depth analysis. Moreover, pathologic processing and diagnosis has changed substantially over the last decades (historical cases were published over seven decades, 1965-2019). Neuroendocrine-specific stainings chromogranin and synaptophysin were not reported in cases published before 2005. Reliable and detailed histopathological data were available in 6/7 of our cohort. Interestingly, a high proportion of tumors in our series had features placing them at 'high risk' for persistence/recurrence according to international guidelines. All six patients with 'high risk' tumors underwent right hemicolectomy, but residual disease was observed in only one (a patient with a 45 mm tumor involving the appendix base, with invasion of the mesoappendix and blood vessels).

These results highlight the controversy over the indication for right hemicolectomy in patients with ANEN. International guidelines suggest hemicolectomy for ANEN >2 cm or ANEN 1-2 cm with worrisome pathologic features (8, 9). However, some authors have questioned these criteria. For example, a retrospective analysis of 263 ANEN patients found that tumor grade, vascular and lymph vessel invasion were associated with lymph node involvement, while tumor size and mesoappendiceal invasion were not (34). A systematic review including 261 patients from 6 studies found that using a cutoff of 2 cm for hemicolectomy, the number needed to treat was very similar to the number needed to harm (35). Interestingly, a SEER database analysis found that right hemicolectomy gave no survival advantage over appendectomy, even after adjusting for tumor stage and grade (36).

Until large-scale studies are available, the decision whether to perform right hemicolectomy should be made by a NEN expert, within the framework of a multidisciplinary team, and taking the patient's will into consideration. Posponement of hemicolectomy to the post-partum period seems to be safe, although this series is to small to draw conclusions.

4.4 Pregnancy and long term outcomes

In the 7 cases of the current series, no post-operative complications were noted, and pregnancy outcomes were favorable. This is in contrast to prior large series, which described high rates of post-appendectomy complications. For example, in a series of over 7,000 cases, there was an almost two-fold increase of post-appendectomy complications in pregnancy such as sepsis, septic shock, transfusion, pneumonia, bowel obstruction and postoperative infection (13). Moreover, approximately 5% of women experience adverse obstetrical outcomes after appendectomy during pregnancy, especially preterm delivery or miscarriage (37). Wei et al. reported adjusted odds ratios of 1.82 for low birth weight, 1.59 for preterm birth, 1.33 for small for gestational age, 1.24 for CS, and 2.07 congenital anomalies in women with acute appendicitis during pregnancy (38). The discrepancy between our data and data from these large series may be influenced by temporal changes in availability of diagnostic tools, anesthetization and surgery methods. The significance of our data is also limited by the small sample size of our cohort.

Follow-up data was available for all 7 cases of our cohort and only 5 historical cases. No cases of tumor recurrence, distal metastases or mortality were reported. The results of imaging and biochemical studies during follow-up were all negative. These results are in concert with previous studies, and allude to excellent long-term prognosis for ANEN diagnosed during pregnancy (1, 2, 6, 29).

4.5 Study limitations

Although this series is the largest reported to date on ANEN diagnosed during pregnancy, its small sample size precludes the formation of definite conclusions.The cases analyzed were treated over a time span of more than 60 years, during which diagnostic and therapeutic approaches have changed substantially. The retrospective nature of the data gives rise to inherent limitations, including potential bias caused by missing or incorrect data.

5 Conclusion

ANEN diagnosis during pregnancy is very rare, occurring most commonly during the third trimester. In this series, all cases were diagnosed post-operatively by the pathologist. In most cases, the post-operative period was unremarkable and pregnancy outcomes were favorable. Local metastases were rare and there were no cases of distant metastases or disease related mortality. This data suggests that a conservative approach to patients with ANEN diagnosis during pregnancy may be considered. However, decision-making needs to be individualized and requires discussion within an experienced multidisciplinary team, including a NEN specialist, gynecologist, pathologist and surgeon. The treatment approach should take into consideration not only the risks related to the tumor itself but also the pregnancy-related psychological burden and relevant outcomes. Larger, multi-center studies are warranted to assess the long-term prognosis of this condition, with emphasis on timing and outcomes of both tumor- and pregnancy- related interventions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Edith Wolfson Medical Center Ethics committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

OT conducted the study and wrote the manuscript. AA, RR, DH, KO, PR-P and SG-G conducted the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Early-onset pancreatic neuroendocrine neoplasms: A distinct disease with improved survival compared with old individuals

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Background: The incidence, clinicopathologic characteristics, treatment patterns, and survival of early-onset pancreatic neuroendocrine neoplasms (EOPanNENs) have not been well explored.

Methods: Patients diagnosed with PanNENs were identified from the SEER database between 2000 and 2018. EOPanNENs were defined as diagnosis in patients aged less than 50 years, while the remaining were defined as later-onset pancreatic neuroendocrine neoplasms (LOPanNENs). Incidence, clinical features, management, and prognosis were analyzed in our study. Multivariable analyses were performed to identify factors associated with overall survival (OS) in EOPanNENs and LOPanNENs, respectively.

Results: A total of 5172 patients with PanNENs were included: 1267 (24.5%) in the EOPanNENs cohort and 3905 (75.5%) in the LOPanNENs cohort. The age-adjusted incidence rate significantly increased among later-onset cases, while it remained relatively stable in early-onset cases. EOPanNENs were more frequently to be female, unmarried, and with better tumor differentiation compared with LOPanNENs. Of note, early-onset patients presented with a higher rate of lymph node involvement, and they were more likely to receive surgical treatment. For local-regional disease at presentation, surgery alone was the most frequently used regimen over the last two decades. With regard to distant stage, a combination of surgery and chemotherapy was more often utilized. Risk factors for PanNENs survival were more correlated with LOPanNENs compared with EOPanNENs. The OS and cancer-specific survival (CSS) were significantly better in the EOPanNENs group. Further analyses showed that EOPanNENs $\leq$ 2cm were associated with more favorable survival outcomes than EOPanNENs>2cm.

Conclusion: EOPanNENs are a clinically rare and distinct entity from LOPanNENs. The advantages in survival for the EOPanNENs cohort over

time were largely driven by the indolent clinical courses including better tumor differentiation and intensified surgical treatment. Further investigations are warranted to better understand the characteristics of this disease subgroup.

KEYWORDS

early-onset pancreatic neuroendocrine neoplasms, later-onset pancreatic neuroendocrine neoplasms, incidence, clinical characteristics, survival

Introduction

Pancreatic neuroendocrine neoplasms (PanNENs), originating from the diffuse endocrine system, are a heterogeneous group of uncommon epithelial tumors with diverse malignant potential (1, 2). Recent years the incidence of PanNENs has risen dramatically, which may primarily be attributed to routine screening and increased detection of asymptomatic disease (3–6). Although PanNENs typically affect elderly individuals, recent data indicate that the number of PanNENs in young adults aged less than 50 years old is steadily increasing. Previous studies focusing on other cancer types demonstrated significantly different epidemiologic characteristics and survival results between early-onset and later-onset cases, such as colorectal cancer (7–9). However, to the best of our knowledge, few large cohort studies have examined the epidemiology, risk factors, treatment patterns, and survival outcomes of patients with early-onset PanNENs (EOPanNENs) given the relative rarity and indolent clinical behaviors in comparison to pancreatic ductal adenocarcinoma (PDAC).

Therefore, the present study sought to systematically analyze and better define the incidence trends, clinical features, management strategies, and prognosis among patients with EOPanNENs over the last two decades using the information derived from a large population-based database in the United States.

Methods

The Surveillance, Epidemiology, and End Results (SEER) program was used to identify patients who were pathologically diagnosed with primary PanNENs on the basis of conventional histology between 2000 and 2018: young age (<50 years), and older counterparts (≥50 years old). EOPanNENs were defined as diagnosis in patients aged less than 50 years of age, while the remaining were defined as later-onset pancreatic neuroendocrine neoplasms (LOPanNENs). The rationale for choosing 50 years old as the age threshold is not solely based on patient numbers, but rather on a combination of statistical considerations and clinical practice experience. Cancer is a complex disease that typically affects individuals aged 50 and above, but the increasing incidence of cancer in young adults under 50 suggests that there are changes in carcinogenic exposures that warrant attention. As mentioned, early-onset cancers typically present distinct pathological and biological features compared with later-onset cases, with these features more commonly observed in patients under 50 years old. Additionally, the age of 50 has been broadly accepted as the threshold for defining early-onset cancers in the medical community, enabling consistent comparisons between different studies and populations. The data on cancer epidemiology, clinicopathologic features, and survival outcomes were retrospectively collected and analyzed. Patients with missing data were not included in our study. The last follow-up time was December 31, 2018. The study was approved by the institutional review board (IRB) of Qingdao municipal hospital, and the informed consent was exempt for the data were obtained from a public database.

Statistical analysis

Continuous data were presented as medians with interquartile ranges (IQR), and were compared using 2-tailed Student t-test. Categorical variables were expressed as number and percentage, and the differences between cohorts were examined by chi-squared test. And the survival outcomes including overall survival (OS) and cancer-specific survival (CSS) were estimated *via* Kaplan-Meier method with log-rank test. Univariate and multivariable Cox proportional hazard models were utilized to identify independent risk factors associated with OS for patients with EOPanNENs. Besides, the treatment distributions stratified by tumor stage (localized, regional, and distant) in the EOPanNENs cohort from 2000 to 2018 were assessed. All analyses were performed by SPSS 22.0 and R software, and a 2-sided $P<0.05$ was deemed to be statistically significant.

Results

Demographics and disease presentation

After inclusion and exclusion criteria were applied, a total of 5172 patients with PanNENs were identified and extracted from the SEER database between 2000 and 2018 for our study: 1267 (24.5%) with histologically confirmed LOPanNENs and 3905 (75.5%) with histologically confirmed LOPanNENs. Using population data from

the SEER program, we calculated the annual number and the age-adjusted incidence of PanNENs cases during the study period, referring to the 2000 US standard population. Among LOPanNENs populations, the incidence rate significantly increased during the period from 2000 to 2018, whereas rate for EOPanNENs remained unchanged, as presented in Figure 1. Demographics, clinical characteristics, and survival outcomes of EOPanNENs vs LOPanNENs were summarized in Table 1.

In the whole study population, a vast majority of patients (90.6%) had a solitary primary tumor while 9.4% had multiple tumors. Non-functional PanNENs patients accounted for approximately 90% of all the enrolled cases. As shown in Table 1, there were significant differences of patients' characteristics among those two cohorts. EOPanNENs patients were more often female (52.4% vs 44.3%) and unmarried (42.9% vs 35.3%). Patients with EOPanNENs were also more frequently to have well to moderately differentiated histologic grade (92.2% vs 89.2%). Of note, compared to patients with LOPanNENs, those in the EOPanNENs cohort had a higher rate of lymph node involvement (29.4% vs 26.1%, P=0.025). Early detection and increasing public attention over the last few decades had led to population stage shift for PanNENs. As presented in Figure 2, the proportion of local-regional disease exhibited an obviously increasing trend among recent years. In terms of the management, patients with EOPanNENs were more likely to undergo surgical intervention (83.3% vs 73.8%). More detailed information on baseline characteristics were given in Table 1.

Treatment distribution

The therapeutic modalities in the EOPanNENs cohort stratified by cancer stage (localized, regional, and distant) were then evaluated, respectively. For local-regional disease at presentation, surgical resection alone was the most frequently used regimen over the last two decades. (Figures 3, 4) With regard to distant stage at presentation, a combination of surgery and chemotherapy was more often utilized among all years between 2000 and 2018 (Figure 5).

Predictors of OS

Cox regression was then performed to select factors that best predicted prognosis of patients with PanNENs. For patients with EOPanNENs, univariate analysis yielded that gender, tumor size, tumor grade, lymph node involvement, tumor stage, surgery, chemotherapy, and radiation were associated with OS. In multivariable Cox proportional hazards model, poor differentiation, advanced tumor stage, and surgical resection were found to be with improved survival outcomes. As for LOPanNENs patients, multivariable survival analysis identified that gender, marital status, tumor size, histologic grade, tumor location, stage, and surgery were independent prognostic factors of OS (Table 2).

Survival disparity between EOPanNENs and LOPanNENs

The overall survival (OS) and cancer-specific survival (CSS) of young adults were significantly better than that of older counterparts (Figure 6). The median OS was 212.0 months for patients with EOPanNENs, while 138.0 months for those with LOPanNENs. In addition, the 3-year OS between EOPanNENs and LOPanNENs in all prespecified subgroups was then assessed in our survival analyses (Figures 7, 8). Cases with EOPanNENs were associated with a significantly better 3-year OS compared with LOPanNENs in all these subgroups except for those with other ethnicity or those who underwent radiation. Surely, patients who received radiation were more likely to have a higher tumor burden and more aggressive tumor biology.

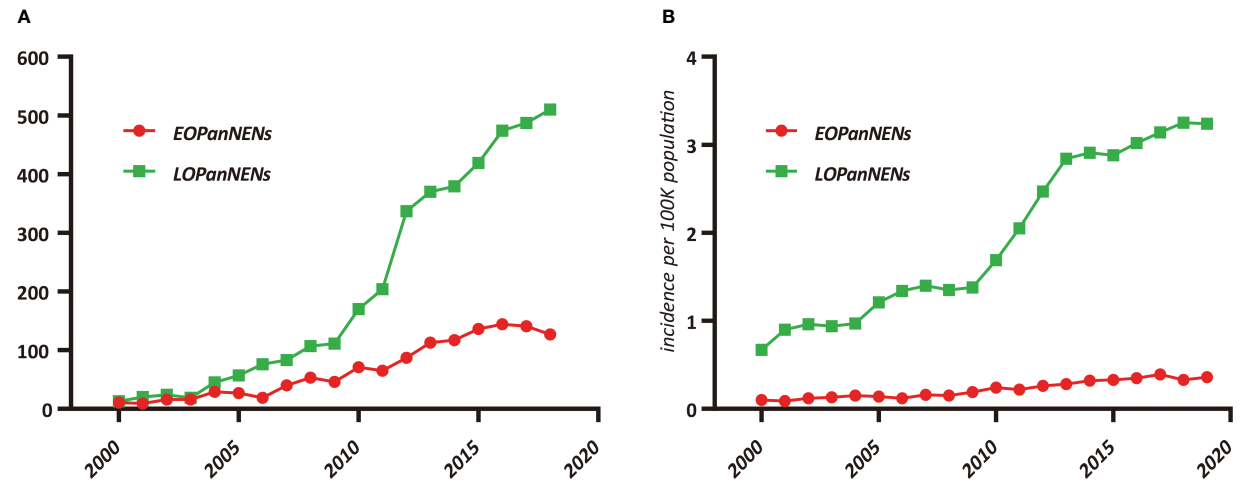

FIGURE 1
Trends in pancreatic neuroendocrine neoplasms (PanNENs) Incidence (2000–2018) in the United States, according to age. **(A)** Incidence of EOPanNENs and LOPanNENs cases per year. **(B)** Age-adjusted incidence of EOPanNENs and LOPanNENs in the overall population.

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TABLE 1 SEER cohort demographics, pathologic characteristics, and survival outcomes.

Variables	Overall (N=5172)	EOPanNENs (N=1267)	LOPanNENs (N=3905)	P value
Age, median (IQR)	60 (50 - 68)	42 (35 - 46)	64 (57 - 71)	
Gender				**<0.001**
Male	2779 (53.7%)	603 (47.6%)	2176 (55.7%)	
Female	2393 (46.3%)	664 (52.4%)	1729 (44.3%)	
Ethnicity				**<0.001**
White	4006 (77.5%)	923 (72.9%)	3083 (79.0%)	
Black	600 (11.6%)	169 (13.3%)	431 (11.0%)	
Other	566 (10.9%)	175 (13.8%)	391 (10.0%)	
Marital status				**<0.001**
Married	3249 (62.8%)	724 (57.1%)	2525 (64.7%)	
Other	1923 (37.2%)	543 (42.9%)	1380 (35.3%)	
Tumor size (cm), median (IQR)	3.0 (1.7 - 5.0)	3.0 (1.8 - 5.0)	3.0 (1.7 - 5.0)	0.846
Tumor grade				**<0.001**
Well differentiated	3575 (69.1%)	901 (71.1%)	2674 (68.5%)	
Moderately differentiated	1090 (21.1%)	280 (22.1%)	810 (20.7%)	
Poorly differentiated	507 (9.8%)	86 (6.8%)	421 (10.8%)	
Tumor number				**<0.001**
Single	4687 (90.6%)	1202 (94.9%)	3485 (89.2%)	
Multiple	485 (9.4%)	65 (5.1%)	420 (10.8%)	
Tumor location				**0.037**
Head	1543 (29.8%)	397 (31.3%)	1146 (29.3%)	
Body/Tail	2710 (52.4%)	625 (49.4%)	2085 (53.4%)	
Other	919 (17.8%)	245 (19.3%)	674 (17.3%)	
Functional status				0.172
Functional	430 (8.3%)	117 (9.2%)	313 (8.0%)	
Nonfunctional	4742 (91.7%)	1150 (90.8%)	3592 (92.0%)	
Lymph node involvement				**0.025**
Yes	1393 (26.9%)	372 (29.4%)	1021 (26.1%)	
No	3779 (73.1%)	895 (70.6%)	2884 (73.9%)	
Liver involvement				**<0.001**
Yes	792 (15.3%)	181 (14.3%)	611 (15.6%)	
No	3991 (77.2%)	950 (75.0%)	3041 (77.9%)	
Unknown	389 (7.5%)	136 (10.7%)	253 (6.5%)	
Tumor stage				0.055
Localized	2655 (51.3%)	619 (48.9%)	2036 (52.2%)	
Regional	1278 (24.7%)	343 (27.1%)	935 (23.9%)	
Distant	1239 (24.0%)	305 (24.0%)	934 (23.9%)	
Surgery				**<0.001**
Yes	3938 (76.1%)	1055 (83.3%)	2883 (73.8%)	

(Continued)

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TABLE 1 Continued

Variables	Overall (N=5172)	EOPanNENs (N=1267)	LOPanNENs (N=3905)	P value
No	1234 (23.9%)	212 (16.7%)	1022 (26.2%)	
Radiation				0.737
Yes	184 (3.6%)	47 (3.7%)	137 (3.5%)	
No	4988 (96.4%)	1220 (96.3%)	3768 (96.5%)	
Chemotherapy				0.664
Yes	736 (14.2%)	185 (14.6%)	551 (14.1%)	
No	4436 (85.8%)	1082 (85.4%)	3354 (85.9%)	
Primary endpoint: OS, months				
Median (95% CI)	151.0 (138.0-164.0)	212.0 (186.7-237.3)	138.0 (125.8-150.2)	**<0.001**†

EOPanNENs, early-onset pancreatic neuroendocrine neoplasms; LOPanNENs, late-onset pancreatic neuroendocrine neoplasms; IQR, interquartile range; SEER, surveillance, epidemiology and end results; OS, overall survival; CI, confidence interval. †Log-rank test, Bold indicates significance.

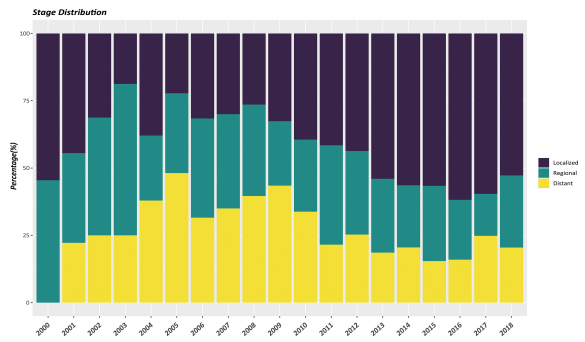

FIGURE 2
Shifts in stage at diagnosis among EOPanNENs patients in the United States, 2000-2018.

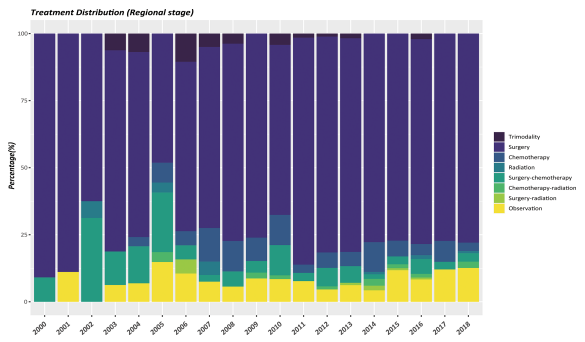

FIGURE 4
Differences in regional stage by type of treatment, 2000-2018, age<50.

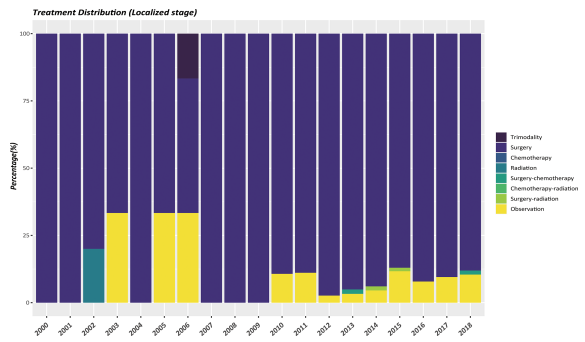

FIGURE 3
Differences in localized stage by type of treatment, 2000-2018, age<50.

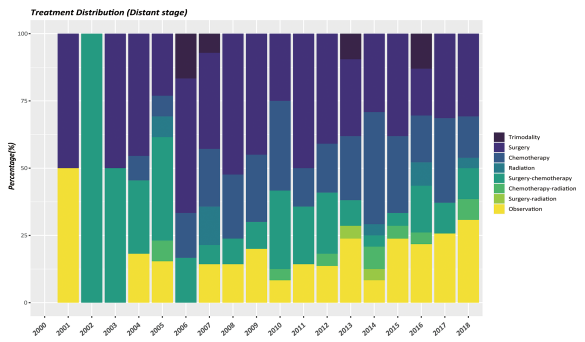

FIGURE 5
Differences in distant stage by type of treatment, 2000-2018, age<50.

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TABLE 2 Univariate and multivariate Cox regression analyses for factors affecting OS in patients with EOPanNENs or LOPanNENs.

Variables	EOPanNENs				LOPanNENs			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Gender								
Male	Ref		Ref		Ref		Ref	
Female	0.66 (0.51, 0.85)	**0.002**	0.79 (0.60, 1.02)	0.073	0.76 (0.67, 0.87)	**<0.001**	0.83 (0.73,0.95)	**0.005**
Ethnicity								
White	Ref				Ref		Ref	
Black	1.24 (0.87, 1.78)	0.230			1.15 (0.95, 1.39)	0.158	0.99 (0.81, 1.20)	0.897
Other	0.79 (0.52, 1.21)	0.279			0.71 (0.55, 0.90)	**0.005**	0.79 (0.61, 1.01)	0.057
Marital status								
Married	Ref				Ref		Ref	
Other	1.19 (1.92, 1.55)	0.188			1.29 (1.14, 1.47)	**<0.001**	1.27 (1.11, 1.46)	**<0.001**
Tumor size								
≤2 cm	Ref		Ref		Ref		Ref	
>2 cm	3.07 (2.06, 4.60)	**<0.001**	1.25 (0.81, 1.93)	0.319	2.96 (2.49, 3.53)	**<0.001**	1.24 (1.01, 1.51)	**0.040**
Tumor grade								
Well differentiated	Ref		Ref		Ref		Ref	
Moderately differentiated	1.74 (1.27, 2.38)	**0.001**	1.35 (0.98, 1.85)	0.066	1.67 (1.42, 1.96)	**<0.001**	1.24 (1.05, 1.46)	**0.012**
Poorly differentiated	7.03 (5.10, 9.69)	**<0.001**	3.55 (2.50, 5.05)	**<0.001**	7.09 (6.13, 8.20)	**<0.001**	3.71 (3.13, 4.39)	**<0.001**
Tumor location								
Head	Ref				Ref		Ref	
Body/Tail	0.92 (0.68, 1.23)	0.565			0.58 (0.50, 0.66)	**<0.001**	0.80 (0.69, 0.93)	**0.003**
Other	1.01 (0.71, 1.44)	0.960			0.86 (0.73, 1.02)	0.084	0.89 (0.75, 1.06)	0.186
Functional status								
Non-Functional	Ref				Ref			
Functional	1.04 (0.64, 1.71)	0.871			0.88 (0.67, 1.16)	0.371		
Lymph node involvement								
No	Ref		Ref		Ref		Ref	
Yes	2.51 (1.94, 3.26)	**0.006**	1.28 (0.95, 1.74)	0.106	1.67 (1.47, 1.90)	**<0.001**	1.13 (0.97, 1.31)	0.119
Tumor stage								
Localized	Ref		Ref		Ref		Ref	
Regional	3.22 (2.08, 4.99)	**<0.001**	1.96 (1.18, 3.23)	**0.009**	2.07 (1.73, 2.48)	**<0.001**	1.48 (1.19, 1.85)	**0.001**
Distant	10.6 (7.11, 15.7)	**<0.001**	4.33 (2.63, 7.12)	**<0.001**	6.47 (5.53, 7.56)	**<0.001**	2.44 (1.97, 3.01)	**<0.001**
Surgery								
No	Ref		Ref		Ref		Ref	
Yes	0.19 (0.15, 0.25)	**<0.001**	0.41 (0.30, 0.58)	**<0.001**	0.17 (0.15, 0.20)	**<0.001**	0.28 (0.24, 0.32)	**<0.001**
Chemotherapy								
No	Ref			Ref		Ref	Ref	

(Continued)

TABLE 2 Continued

Variables	EOPanNENs				LOPanNENs			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Yes	5.38 (4.14, 7.00)	**<0.001**	1.38 (0.99, 1.92)	0.053	4.33 (3.79, 4.94)	**<0.001**	0.95 (0.81,1.12)	0.543
Radiation								
No	Ref		Ref		Ref		Ref	
Yes	4.30 (2.89,6.39)	**<0.001**	1.25 (0.81, 1.94)	0.317	2.85 2.28, 3.56)	**<0.001**	0.99 (0.79, 1.26)	0.953

OS, overall survival; EOPanNENs, early-onset pancreatic neuroendocrine neoplasms; LOPanNENs, late-onset pancreatic neuroendocrine neoplasms; HR, hazard ratio; CI, confidence index; Ref, reference. Bold indicates significance.

Exploratory analyses

In order to better define the impact of tumor size on survival outcomes in EOPanNENs, we analyzed the clinical characteristics and survival between patients with EOPanNENs ≤ 2cm and those with EOPanNENs>2cm. As shown in the Table 3, the baseline characteristics were significantly different among these two cohorts. Kaplan-Meier curves indicated that the OS and CSS were more favorable in patients with EOPanNENs ≤ 2cm, as compared with that in patients with EOPanNENs >2cm (Figure 9).

Discussion

In this comprehensive study of pancreatic neuroendocrine neoplasms (PanNENs), our population-based analysis found a steady rise of the incidence of LOPanNENs in the United States over last two decades, whereas the incidence of EOPanNENs remained relatively stable. And we further investigated the differences between EOPanNENs and LOPanNENs using the largest cohort of PanNENs cases reported as yet, with a focus on epidemiology, clinicopathologic characteristics, and survival outcomes. The findings of our study suggested that patients with EOPanNENs were associated with distinct clinical features and prognosis in comparison to those with LOPanNENs. Results from subgroup analyses further indicated that PanNENs survival was generally better for patients diagnosed before 50 years old. Additionally, our analysis showed that a high percentage of patients with EOPanNENs were diagnosed as having lymph node involvement. Surgery remained the most frequently utilized therapy in cases with local-regional disease, while those with distant disease were more likely to be treated with a combination of surgery and chemotherapy.

The incidence of PanNENs is projected to steadily increase, likely attributable to the high-resolution imaging and increased utility of diagnostic techniques (10, 11). A large proportion of neoplasms were diagnosed incidentally during imaging conducted for an unrelated diagnosis (12). Our results showed that the number of LOPanNENs patients increased more pronounced than the EOPanNENs cases. With respect to the annual incidence, LOPanNENs patients experienced a faster increase than EOPanNENs patients. As shown in our study, the annual age-adjusted incidence of EOPanNENs remained unchanged, while a marked increase of LOPanNENs occurred in United States during

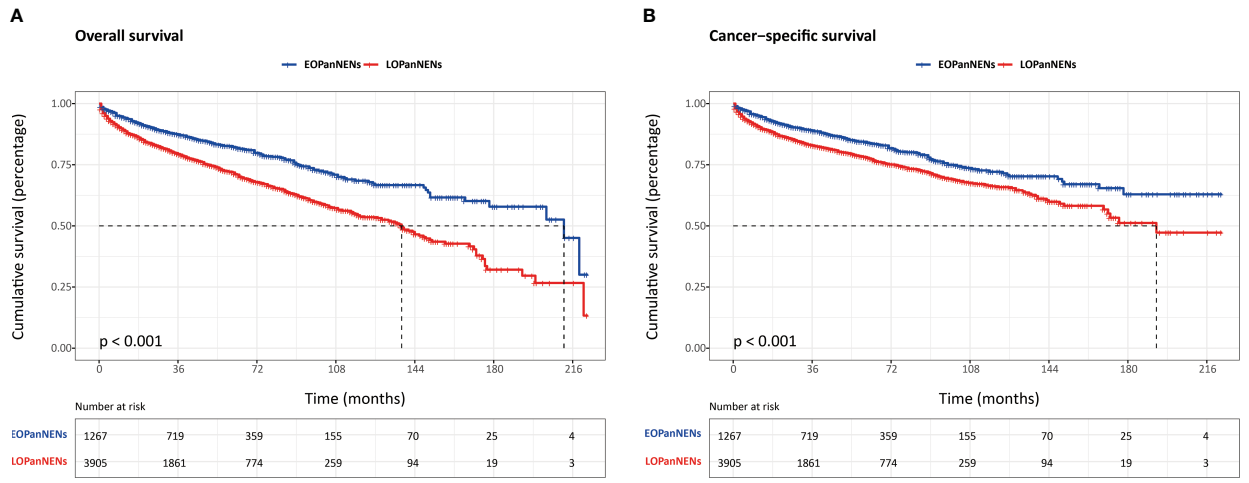

FIGURE 6
The overall survival **(A)** and cancer-specific survival **(B)** between EOPanNENs and LOPanNENs.

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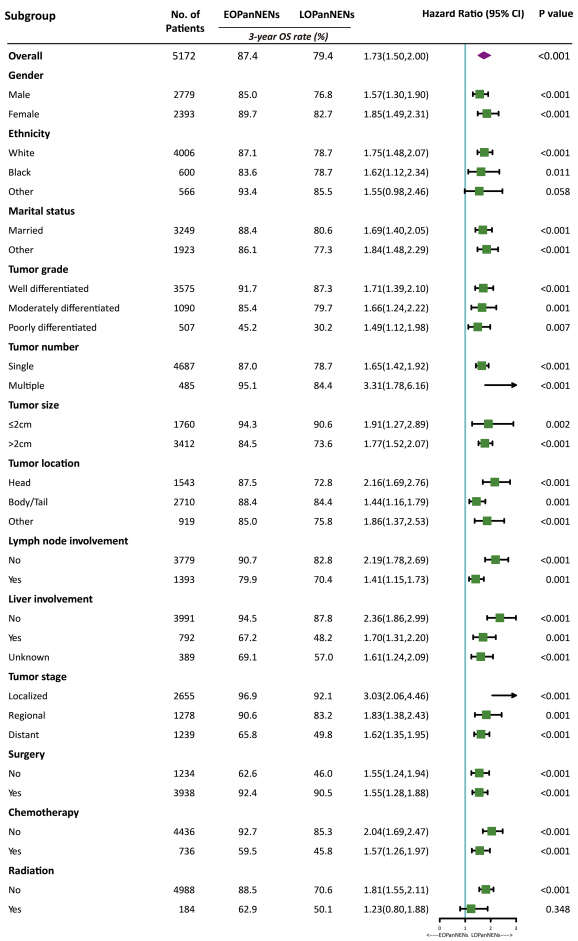

FIGURE 7
3-year overall survival of EOPanNENs compared with LOPanNENs in subgroups of patients with different tumor characteristics and treatment types.

the last two decades. The explanation for this phenomenon might in part be the enhanced availability of routine monitoring in elderly adults. Among stage groups, local-regional disease accounted for the majority of PanNENs, which might also mainly be caused by the rise in early detection capability (13). By the way, both of the EOPanNENs and LOPanNENs cohorts shared the similar stage distribution according to our study.

It is reported that PanNENs exhibited a slight male predominance (14). However, in our study, early-onset patients showed a female preponderance (52.4%) compared with men (47.6%). The exact cause of gender differences between EOPanNENs and LOPanNENs was not well-learned. Previous studies argued that the distribution of risk factors might play a role in the sex disparity (15, 16).

Patients with EOPanNENs in our study seemed to be associated with lower tumor burden and less aggressive behaviors compared to those with LOPanNENs, except for the higher rate of lymph node involvement. Delayed diagnosis in younger patients and presentation with more metastatic lymph nodes highlighted the necessity for the great awareness of the disease on general public, as well as the enhancement of detection ability. Younger patients were more frequently to receive surgical treatment, making it convenient to evaluate the lymph node status. And our study also demonstrated that early-onset patients had significantly better survival outcomes compared to later-onset cases despite more lymph node involvement of EOPanNENs.

Even though there existed numerous treatment options for PanNENs, surgery remained the cornerstone of treatment, which has been proved to be with survival benefits (17–20). Some studies even concluded that cancer-directed surgery can also provide improved survival outcomes in patients with distant diseases (21–25). Consistent with prior findings, we found that patients with EOPanNENs were more likely to complete surgical resections for the primary tumor, and the OS and CSS were significantly better in these patient population compared to LOPanNENs. As for metastatic patients, surgery and chemotherapy were more commonly be proposed as an adequate management as it conferred survival advantages in selected patients (26).

A significant difference in risk factors existed between EOPanNENs and EOPanNENs cohorts. Similar to other studies focusing on the whole PanNENs population, survival analyses using the SEER database confirmed previous results of the prognostic significance of gender, tumor diameter, tumor differentiation, location, stage at presentation, and surgery for LOPanNENs (27, 28). While only poor differentiated tumors, advanced stage, and surgical intervention were significantly associated with OS in the patients with EOPanNENs, which again confirmed EOPanNENs as a unique clinical entity.

Our study has several limitations. First, given the retrospective nature, it is unlikely to avoid the selection biases. And the SEER database does not record novel medications and treatments that have been adopted to improve survival in patients with PanNENs. Second, information regarding to treatment regimens, perioperative complications, and disease recurrence were not available in the public data source, which may limit the generalization of the conclusion (29). However, such drawbacks are inevitable and inherent to any retrospective, population-based analysis. Furthermore, the dichotomy at 50 years of age has its limitations. While there are differences in epidemiology, clinicopathological, and molecular characteristics between early-onset and later-onset tumors, these features are less likely to change dramatically at precisely 50 years of age. We recognize the constraints of using a dichotomy at 50 years of age, but we chose this cutoff point to ensure consistent collection and interpretation of existing evidence on early-onset cancers. In reality, the heterogeneity within this group should also be taken into account. Considering the varying age distribution of cancer diagnosis by different organs, the optimal screening and treatment strategies for specific age groups should be tailored based on the specific organ site affected. The strength of our study compared to previous studies is the largest sample size of PanNENs patients utilized to characterize the clinicopathologic features and survival outcomes for the first time.

In conclusion, unlike the rapid increase in incidence rate of LOPanNENs patients, the age-adjusted incidence of EOPanNENs remained stable according to the analysis of SEER database between 2000 and 2018. Diagnoses of better tumor differentiation represented a larger proportion of the EOPanNENs cohort over the last two decades, together with the higher rate of surgical treatment, resulting in the more favorable survival outcomes compared to LOPanNENs.

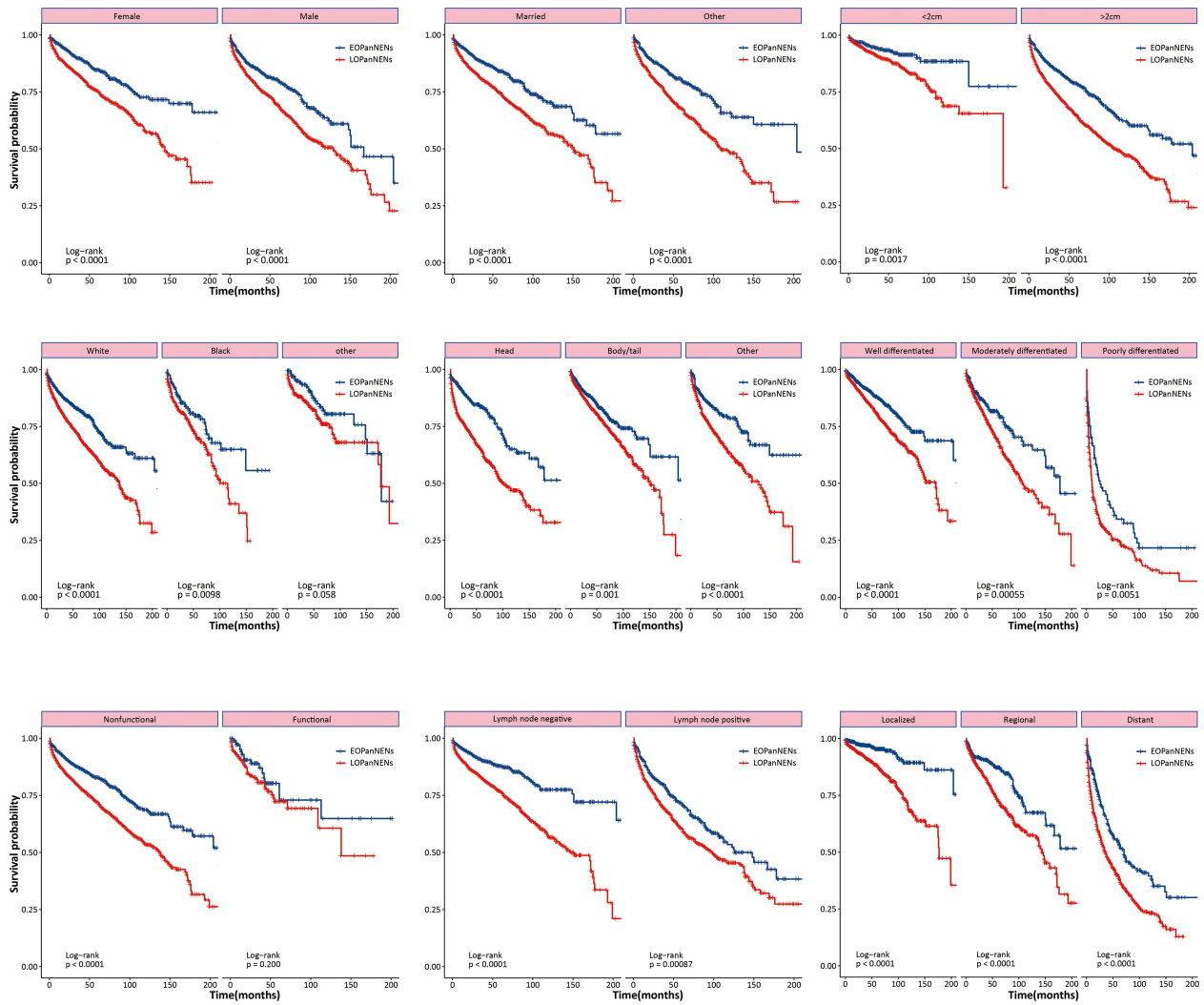

FIGURE 8
Subgroup analyses of overall survival between patients with EOPanNENs and LOPanNENs.

TABLE 3 Patient characteristics of EOPanNENs ≤2 cm versus >2 cm in the SEER database.

Variables	EOPanNENs ≤2 cm (N=399)	LOPanNENs >2 cm (N=868)	P value
Gender			**0.001**
Male	162 (40.6%)	441 (50.8%)	
Female	237 (59.4%)	427 (49.2%)	
Ethnicity			0.658
White	284 (71.2%)	639 (73.6%)	
Black	56 (14.0%)	113 (13.0%)	
Other	59 (14.8%)	116 (13.4%)	
Marital status			0.903
Married	229 (57.4%)	495 (57.0%)	
Other	170 (42.6%)	373 (43.0%)	
Tumor grade			**<0.001**
Well differentiated	322 (80.7%)	579 (66.7%)	

(Continued)

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TABLE 3 Continued

Variables	EOPanNENs ≤2 cm (N=399)	LOPanNENs >2 cm (N=868)	P value
Moderately differentiated	60 (15.0%)	220 (25.3%)	
Poorly differentiated	17 (4.3%)	69 (7.9%)	
Tumor number			0.687
Single	380 (95.2%)	822 (94.7%)	
Multiple	19 (4.8%)	46 (5.3%)	
Tumor location			0.871
Head	121 (30.3%)	276 (31.8%)	
Body/Tail	200 (50.1%)	425 (49.0%)	
Other	78 (19.5%)	167 (19.2%)	
Functional status			0.153
Functional	30 (7.5%)	87 (10.0%)	
Nonfunctional	369 (92.5%)	781 (90.0%)	
Lymph node involvement			**<0.001**
Yes	47 (11.8%)	325 (37.4%)	
No	352 (88.2%)	543 (62.6%)	
Liver involvement			**<0.001**
Yes	20 (5.0%)	161 (18.5%)	
No	364 (91.2%)	586 (67.6%)	
Unknown	15 (3.8%)	121 (13.9%)	
Tumor stage			**<0.001**
Localized	315 (78.9%)	304 (35.0%)	
Regional	52 (13.0%)	291 (33.5%)	
Distant	32 (8.0%)	273 (31.5%)	

EOPanNENs, early-onset pancreatic neuroendocrine neoplasms; SEER, surveillance; epidemiology and end results; CI, confidence interval. Bold indicates significance.

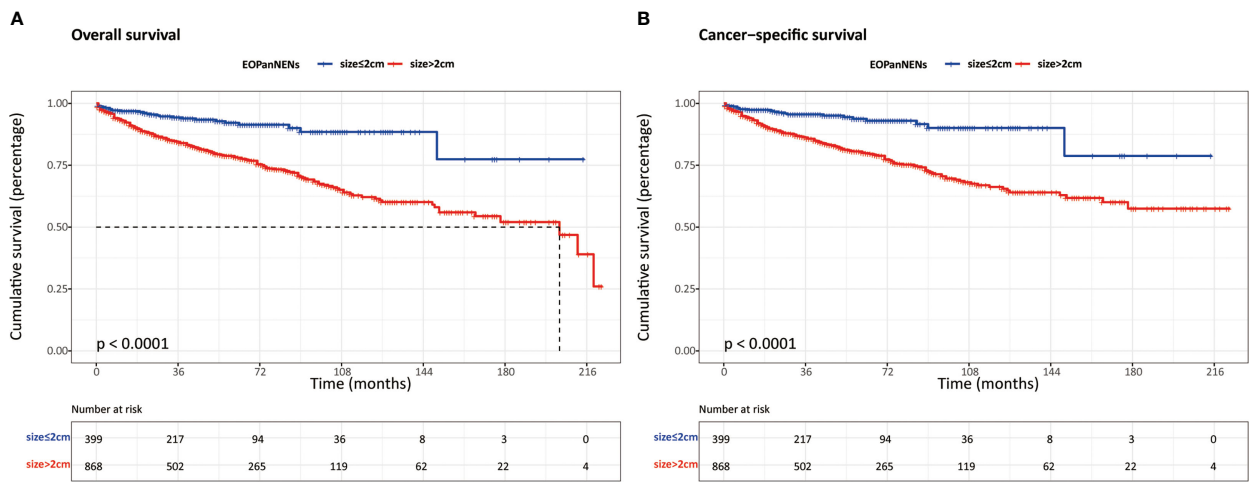

FIGURE 9
The overall survival **(A)** and cancer-specific survival **(B)** between EOPanNENs ≤2cm and EOPanNENs >2cm.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://seer.cancer.gov/.

Author contributions

GS and LL contributed to the conception. CL and KL collected data. ZY and LL designed the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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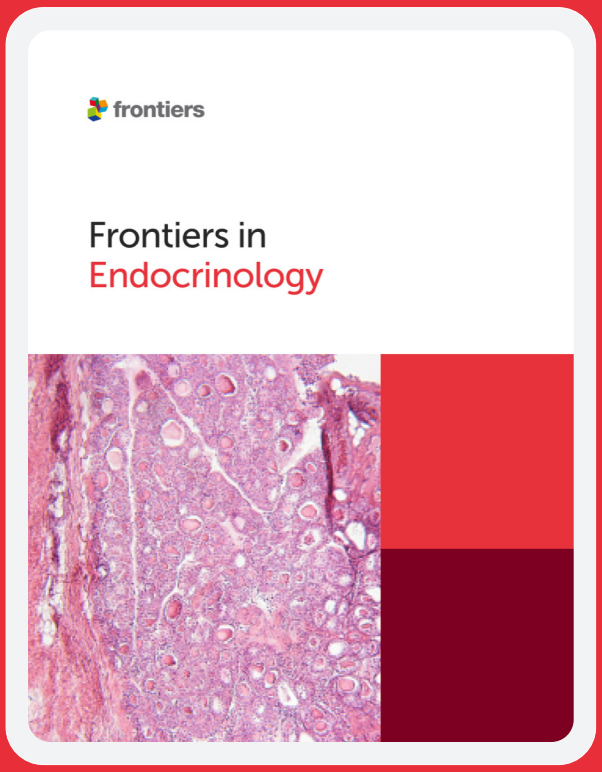

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