

Clinicopathological characteristics and treatment of patients with colorectal neuroendocrine carcinoma: A retrospective study of 157 cases

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Abstract. The present study sought to explore the clinicopathological characteristics, and construct a prognostic nomogram predicting disease-free survival (DFS) and overall survival (OS) in patients with colorectal neuroendocrine carcinoma (CRNEC). Data of patients diagnosed with CRNEC from Harbin Medical University Cancer Hospital (Harbin, China) between January 2000 and December 2019 were retrospectively collected. These patients were randomly divided into different groups by histological type, differentiation grade and AE1/AE3 expression. Subsequently, χ^2 test and Fisher's exact test were employed to assess associations between categorical variables and multiple groups, Kaplan-Meier estimates were generated to analyze survival, and univariate and multivariate Cox proportional hazards models were fitted to identify the potential prognostic factors. The multivariate analyses formed the basis for constructing prognostic nomograms. Nomogram validation comprised calculation of the C-index and generation of calibration curves. As a result, three variables were identified as the potential independent predictors for DFS (body mass index, age and differentiation) and OS (sex, age and differentiation). Nomograms were established using these variables based on the multivariate analyses. The C-index for the nomogram prognostic models predicting DFS and OS were 0.843 (95% CI, 0.665-0.936) and 0.819 (95% CI, 0.644-0.919), respectively. The predicted and observed survival probabilities showed concordance across different time points (1, 3 and 5 years), as evidenced by the calibration curves overlapping with the reference lines. Patients with neuroendocrine

tumor-G1/G2 pathological subtypes, low grade differentiation or AE1/AE3(+) expression had longer DFS and OS compared with other groups. Overall, the prognostic nomograms established could effectively predict the prognosis of patients with CRNEC. High-grade CRNEC represents a highly aggressive malignancy associated with an unfavorable clinical outcome. In addition, AE1/AE3 expression may be a helpful biomarker for improving the diagnostic accuracy of patients with CRNEC.

Introduction

Neuroendocrine neoplasms (NENs) originate from peptide-ergic neurons and neuroendocrine cells in the body, with the gastrointestinal tract and pancreas being the most prevalent anatomical sites, accounting for >50% of all neuroendocrine tumors (NETs) (1). A previous study has shown that the incidence of NENs in the United States increased from 1.09 per 100,000 in 1973 to 5.25 per 100,000 in 2004, representing a 3.8-fold rise (2). Another study indicated that among gastrointestinal NENs reported in China from 1957 to 2011, pancreatic NETs were the most common, followed by those in the rectum, appendix, stomach, colorectum, jejunoileum and duodenum, respectively (3). Based on the World Health Organization (WHO) 2010 classification, NETs of the digestive system are mainly divided into three categories: i) NET; ii) neuroendocrine carcinoma (NEC); and iii) mixed adenoendocrine carcinoma (MANEC) (4). According to the European NET Society (ENETS) grading method for NENs of the gastrointestinal tract, well-differentiated tumors are classified as low grade (G1) and intermediate grade (G2), while poorly differentiated tumors are classified as high-grade (G3) (5). Accurate grading requires classifying poorly differentiated NENs, with features such as abnormal cell morphology and active proliferation, as high-grade, whereas well-differentiated tumors are histologically defined by features such as uniform nuclear structures (6).

Colorectal NEC (CRNEC), a relatively uncommon malignancy, includes tumors classified, as per the WHO 2010 criteria, as NET, NEC and MANEC. Currently, the main treatment strategy involves surgical removal followed by chemotherapy.

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Most NEC cases in the colon and rectum are of the large cell type (75%), whereas small cell NEC predominates in the anus (7). NEC cells are positive for at least one neuroendocrine immunohistochemical marker, such as AE1/AE3, chromogranin A, CD56, Ki-67 or synaptophysin (Syn) (8,9). Research shows that CRNEC exhibits high aggressiveness, with hepatic metastases present in 40-50% of cases upon initial detection (10). One study has reported that well-differentiated G3 NETs exhibit a mean Ki-67 index of 30%, significantly lower than the 80% seen in poorly differentiated pancreatic NECs in pancreatic G3 NENs, with a median overall survival (OS) of 99 months versus 17 months, respectively (11).

To the best of our knowledge, studies focusing on CRNEC remain scarce. Factors such as surgical resection and the use of adjuvant chemotherapy have been associated with improved survival in CRNEC. Therefore, the current study conducted a comparative analysis between histopathological factors and survival outcomes for patients with CRNEC, and aimed to provide insights in the clinical management of patients diagnosed with CRNEC.

Materials and methods

Patient selection. The present single-center retrospective study enrolled 157 consecutive patients with CRNEC receiving treatment at Harbin Medical University Cancer Hospital (Harbin, China) from January 2000 to December 2019. There were 102 male and 55 female patients; and the median age was 55 years (age range, 59-81 years). WHO 2019 centralized reclassification standards were used to ensure the diagnostic homogeneity of the patients enrolled over this period (4). Complete clinicopathological data and follow-up information were recorded for the enrolled patients. The study protocol received approval from the Institutional Review Board of Harbin Medical University Cancer Hospital (approval no. 82271845; October 10, 2019) and strictly adhered to the ethical principles of The Declaration of Helsinki (1964) and subsequent amendments. Before treatment, the enrolled patients provided written informed consent for the use of their data in research.

Inclusion criteria and exclusion criteria. The inclusion criteria were as follows: i) Pathologically confirmed diagnosis of CRNEC; ii) age ≥ 18 years with adequate Eastern Cooperative Oncology Group performance status (≥ 2) (12) to tolerate surgical or other treatments (including chemotherapy and radiotherapy); iii) complete clinicopathological records and treatment information; and iv) maintained protocol adherence with follow-up. The exclusion criteria were as follows: i) Patients with idiopathic inflammatory bowel diseases of chronic evolution, notably Crohn's disease and ulcerative colitis; ii) patients with uncontrolled comorbidities or systemic illnesses; iii) patients with poor treatment adherence or unwillingness to comply with therapeutic protocols; and iv) patients with missing follow-up information.

Study variables. According to the WHO 2019 Classification of Digestive System Tumors (5th edition) (4), these neoplasms were categorized into five histological categories based on tumor size, number of lymph nodes affected by cancer and

distant metastasis status: i) NET-G1: Typical carcinoid tumors with well-differentiated morphology and low proliferative activity [Ki-67 index $< 3\%$; mitotic count $< 2/10$ high-power fields (HPFs)]; ii) NET-G2: Moderately-differentiated tumors exhibiting intermediate-grade features (Ki-67 index 3-20%; mitotic count 2-20/10 HPFs); iii) NEC-G3: Poorly-differentiated high-grade malignancies, including small cell carcinoma and large cell NEC subtypes (Ki-67 index $> 20\%$; mitotic count $> 20/10$ HPFs); iv) MANEC: Biphasic tumors containing both gland-forming adenocarcinoma and neuroendocrine components (each constituting $\geq 30\%$ of the lesion); and v) hyperplastic and pre-neoplastic lesions: Non-invasive proliferation with malignant potential.

According to the ENETS 2017 guidelines (13), based on the Ki-67 index and mitotic figures, the neoplasms were assigned three differentiation grades as follows: i) Low-grade (G1): Ki-67 index $\leq 2\%$ or mitotic count $< 2/10$ HPFs; ii) intermediate-grade (G2): Ki-67 index 3-20% or mitotic count 2-20/10 HPFs; and iii) high-grade (G3): Ki-67 index $> 20\%$ or mitotic count $> 20/10$ HPFs.

AE1/AE3 expression. AE1/AE3 expression levels were detected and evaluated by the Department of Pathology (Harbin Medical University Cancer Hospital). Pathological formalin-fixed paraffin-embedded tissues from the enrolled patients were obtained. The manual immunohistochemistry staining assay was followed according to the standard protocols: i) The CRNEC tissues were fixed with 4% paraformaldehyde solution at 4°C for 24 h and embedded in 10% paraffin at room temperature for 48 h; ii) paraffin blocks were sliced at 60-70°C, and dewaxed twice in xylene (10 min each) and in a gradient series of alcohol (100, 95, 85 and 70%) for 5 min; iii) antigen retrieval; iv) endogenous peroxidase activity was blocked by incubating the samples in 0.3% hydrogen peroxide prepared in methanol for 30 min at room temperature; v) tissues were blocked with 10% goat serum albumin (Beyotime Institute of Biotechnology) for 30 min at room temperature; vi) tissues were incubated with an anti-AE1/AE3 primary antibody (1:500; cat. no. 67306S; Cell Signaling Technology, Inc.) overnight at 4°C and with a HRP-conjugated anti-mouse secondary antibody (1:200; cat. no. 8125S; Cell Signaling Technology) for 30 min at room temperature; vii) color rendering using diaminobenzidine (1:20; cat. no. P0202; Beyotime Institute of Biotechnology); viii) recombinant staining of the cell nucleus with hematoxylin (cat. no. 14166S; Cell Signaling Technology, Inc.); ix) dehydration and sealing in an alcohol gradient (95, 95 and 100%) for 3 min and in xylene for 3 min; and x) microscopic examination of three fields using the Aperio Image Scope system (version 12.3.3; Leica Biosystems).

The expression of AE1/AE3 was obtained by immunohistochemistry, authors that evaluated the density and intensity of stained cells were blinded to the clinicopathological data. The density of AE1/AE3-positively stained cells was scored as follows: i) 0: $< 1\%$ stained; ii) 1: 1-10%; iii) 2: 11-50%; iv) 3: 51-75%; and v) 4: 76-100%. The staining intensity of AE1/AE3-positivity was scored as follows: i) 0: No staining; ii) 1: Light yellow staining; iii) 2: Brown-yellow staining; and iv) 3: Yellowish-brown staining. The total AE1/AE3 expression score was calculated by multiplying the density score (0-4) and the intensity score (0-3), yielding a composite score ranging

from 0 to 12. In the present study, patients were separated into two groups based on AE1/AE3 expression: AE1/AE3 negative expression (scores <1 based on stained cell density and intensity) and AE1/AE3 positive expression (scores ≥ 1).

Follow-up. Patients were regularly followed up through medical records (outpatient or inpatient), telephone interviews and scheduled clinical visits. Follow-up assessments included: i) Clinical symptom observation; ii) routine hematological tests (for example, complete blood count, blood biochemistry and tumor markers); and iii) imaging examinations (CT or MRI). Postoperative surveillance was conducted every 3-6 months. If tumor progression, recurrence or metastasis was suspected during follow-up, a repeat biopsy was performed for reassessment and re-grading. Disease free survival (DFS) was defined as the time from initial diagnosis to recurrence or to the last follow-up. OS was defined as the time from initial diagnosis to death or to the last follow-up.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (IBM Corp.) and GraphPad Prism 8.0 (Dotmatics). χ^2 test and Fisher's exact test was used to assess associations between categorical variables and multiple groups. Kaplan-Meier estimates were generated to analyze survival, and differences between survival curves were assessed by log-rank test. Univariate and multivariate Cox proportional hazards models were fitted to identify the potential prognostic factors. Significant variables from the univariate analysis ($P < 0.05$) were included in the multivariate Cox regression model. To address potential confounding from variations in treatment strategies (such as postoperative chemotherapy and radiotherapy), these factors were included as covariates in the multivariate models. The multivariate analyses formed the basis for constructing prognostic nomograms for DFS and OS probabilities. Nomogram validation comprised calculation of the C-index and the generation of calibration curves. A two-tailed $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Study population and characteristics. Overall, 157 patients with CRNEC were enrolled onto the present study, including 102 men (65%) and 55 women (35%). The average age of enrolled patients was 54.89 ± 12.86 years, ranging from 26 to 82 years old. The average body mass index (BMI) was 24.11 ± 3.22 kg/m², ranging from 17.10 to 32.10 kg/m². The primary site of 32 cases (20.4%) was the colon, while the primary site for the other 125 cases (79.6%) was the rectum. During the study period, regarding the tumor-node-metastasis (TNM) stage of patients (14), there were four cases (2.5%) with Tis, 63 cases (40.1%) with stage I, five cases (3.2%) with stage II, 28 cases (17.8%) with stage III, 43 cases (27.4%) with stage IV and 14 cases (8.9%) with an unknown stage. The gross type of CRNECs included elevated type in 72 cases (45.9%), ulcerative type in 22 cases (14.0%) and infiltrative type in 63 cases (40.1%). Lymph node dissection was performed in 46.1% (59/128) of surgically treated patients. All enrolled patients were followed-up, and the last follow-up date was October 2024. There were 39 cases (24.8%) with distant

metastasis and 15 cases (9.6%) with recurrence during the follow-up time. The clinicopathological characteristics of the patients are summarized in Table S1.

Histological types in patients with CRNEC. Based on the WHO 2019 Classification of Digestive System Tumors (5th edition) (4), the pathological types included in the present study were as follows: i) 82 cases (52.2%) with NET-G1; ii) 13 cases (8.3%) with NET-G2; iii) 54 cases (34.4%) with NEC-G3; and iv) eight cases (5.1%) with MANEC.

The clinicopathological characteristics of patients with different histological subtypes of CRNEC are detailed in Table I. In the current study, the total number of lymph nodes includes both metastatic and non-metastatic lymph nodes, whereas the positive number of lymph nodes includes only metastatic lymph nodes. Regarding the clinicopathological characteristics of the patients, the pathological subtypes were significantly associated with age, surgical history, primary site, gross type, tumor size, differentiation, total number of lymph node, positive number of lymph node, TNM stage, perineural invasion, vascular tumor thrombus, AE1/AE3, postoperative chemotherapy and postoperative radiotherapy (all $P < 0.05$).

The mean DFS and OS durations stratified by pathological subtypes were as follows: i) In the NET-G1 group, DFS was 65.63 months (95% CI, 51.70-71.43) and OS was 103.00 months (95% CI, 92.43-109.80); ii) in the NET-G2 group, DFS was 25.20 months (95% CI, 15.20-54.60) and OS was 61.73 months (95% CI, 48.80-91.13); iii) in the NEC-G3 group, DFS was 24.87 months (95% CI, 13.50-34.57) and OS was 50.45 months (95% CI, 25.90-71.10); and iv) in the MANEC group, DFS was 42.32 months (95% CI, 16.53-92.17) and OS was 70.22 months (95% CI, 16.53-128.70). Comparative analysis revealed statistically significant differences in both DFS ($\chi^2 = 40.110$, $P < 0.0001$) and OS ($\chi^2 = 38.290$, $P < 0.0001$) among the four pathological subtypes. The survival curves are presented in Fig. 1.

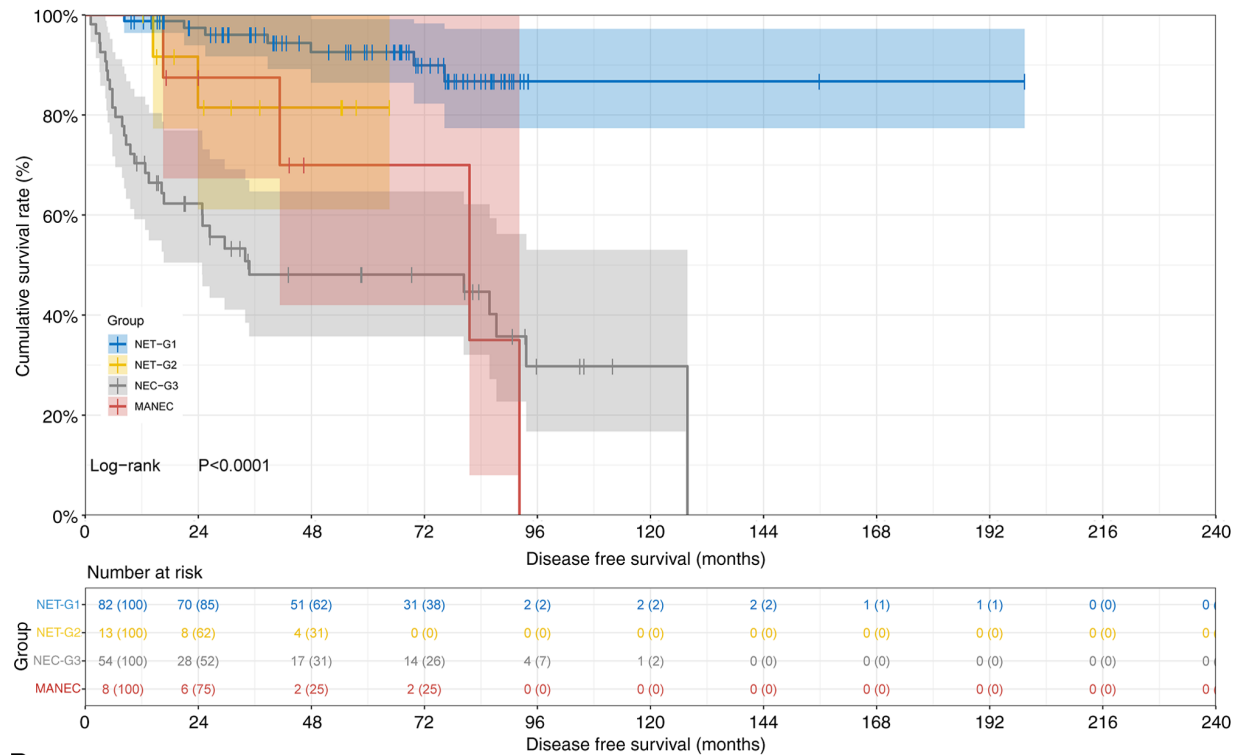
Respectively, the 1-, 3- and 5-year survival rates of DFS and OS stratified by pathological subtypes were as follows: i) In the NET-G1 group, 0.988 (95% CI, 0.964-1.000), 0.960 (95% CI, 0.917-1.000) and 0.926 (95% CI, 0.865-0.991) for DFS, and 0.988 (95% CI, 0.964-1.000), 0.963 (95% CI, 0.924-1.000), 0.951 (95% CI, 0.905-0.999) for OS; ii) in the NET-G2 group, 1.000 (95% CI, 1.000-1.000), 0.815 (95% CI, 0.611-1.000) and 0.815 (95% CI, 0.611-1.000) for DFS, and 1.000 (95% CI, 1.000-1.000), 0.846 (95% CI, 0.671-1.000) and 0.846 (95% CI, 0.671-1.000) for OS; iii) in the NEC-G3 group, 0.704 (95% CI, 0.592-0.837), 0.481 (95% CI, 0.358-0.647) and 0.481 (95% CI, 0.358-0.647) for DFS, and 0.759 (95% CI, 0.653-0.882), 0.574 (95% CI, 0.456-0.722) and 0.537 (95% CI, 0.419-0.688) for OS; and iv) in the MANEC group, 1.000 (95% CI, 1.000-1.000), 0.875 (95% CI, 0.673-1.000) and 0.700 (95% CI, 0.420-1.000) for DFS, and 1.000 (95% CI, 1.000-1.000), 0.875 (95% CI, 0.673-1.000) and 0.750 (95% CI, 0.503-1.000) for OS. The analysis revealed significantly higher 1-, 3- and 5-year DFS and OS rates for NET-G1 and NET-G2 groups compared with the NEC-G3 group, with the MANEC group showing intermediate outcomes. The prognosis was strongly associated with pathological subtype. These findings highlight the prognostic heterogeneity among the CRNEC subtypes, with NET-G1 exhibiting the most favorable outcomes, underscoring the importance of precise histological classification for clinical management.

Table I. Clinicopathological characteristics of patients with colorectal neuroendocrine carcinoma of different pathological subtypes.

Parameters	Group	NET-G1	NET-G2	NEC-G3	MANEC	P-value
Total	-	82	13	54	8	
Sex	Male	53 (64.6)	7 (53.8)	39 (72.2)	3 (37.5)	0.330
	Female	29 (35.4)	6 (46.2)	15 (27.8)	5 (62.5)	
Body mass index, kg/m ²	≤24.49	41 (50.0)	4 (30.8)	27 (50.0)	5 (62.5)	0.669
	>24.49	41 (50.0)	9 (69.2)	27 (50.0)	3 (37.5)	
Age, years	≤55	46 (56.1)	9 (69.2)	17 (31.5)	3 (37.5)	0.029
	>55	36 (43.9)	4 (30.8)	37 (68.5)	5 (62.5)	
Surgical history	No	66 (80.5)	7 (53.8)	47 (87.0)	5 (62.5)	0.005
	Yes	16 (19.5)	6 (46.2)	7 (13.0)	3 (37.5)	
Hypertension	No	64 (78.0)	9 (69.2)	51 (94.4)	7 (87.5)	0.076
	Yes	18 (22.0)	4 (30.8)	3 (5.6)	1 (12.5)	
Diabetes	No	71 (86.6)	12 (92.3)	51 (94.4)	7 (87.5)	0.5675
	Yes	11 (13.4)	1 (7.7)	3 (5.6)	1 (12.5)	
Primary site	Colon	6 (7.3)	3 (23.1)	19 (35.2)	4 (50.0)	<0.001
	Rectum	76 (92.7)	10 (76.9)	35 (64.8)	4 (50.0)	
Gross type	Elevated type	55 (67.1)	4 (30.8)	11 (20.4)	2 (25.0)	<0.001
	Ulcerative type	2 (2.4)	0 (0.0)	18 (33.3)	2 (25.0)	
	Infiltrative type	25 (30.5)	9 (69.2)	25 (46.3)	4 (50.0)	
Tumor size, cm	≤2	66 (80.5)	6 (46.2)	8 (14.8)	1 (12.5)	<0.001
	>2	3 (3.7)	4 (30.8)	28 (51.9)	6 (75.0)	
	Unknown	13 (15.9)	3 (23.1)	18 (33.3)	1 (12.5)	
Differentiation grade	Low-grade (G1)	80 (97.6)	0 (0.0)	1 (1.9)	2 (25.0)	<0.001
	Intermediate-grade (G2)	1 (1.2)	13 (100.0)	1 (1.9)	1 (12.5)	
	High-grade (G3)	1 (1.2)	0 (0.0)	52 (96.3)	5 (62.5)	
Total number of lymph node	≤18	10 (12.2)	6 (46.2)	19 (35.2)	2 (25.0)	<0.001
	>18	4 (4.9)	2 (15.4)	11 (20.4)	5 (62.5)	
	Unknown	68 (82.9)	5 (38.5)	24 (44.4)	1 (12.5)	
Positive number of lymph node	≤5	11 (13.4)	8 (61.5)	17 (31.5)	4 (50.0)	<0.001
	>5	3 (3.7)	0 (0.0)	13 (24.1)	3 (37.5)	
	Unknown	68 (82.9)	5 (38.5)	24 (44.4)	1 (12.5)	
TNM stage	Tis	2 (2.4)	1 (7.7)	1 (1.9)	0 (0.0)	<0.001
	I	57 (69.5)	1 (7.7)	4 (7.4)	1 (12.5)	
	II	1 (1.2)	1 (7.7)	3 (5.6)	0 (0.0)	
	III	6 (7.3)	2 (15.4)	17 (31.5)	3 (37.5)	
	IV	6 (7.3)	5 (38.5)	28 (51.9)	4 (50.0)	
	Unknown	10 (12.2)	3 (23.1)	1 (1.9)	0 (0.0)	
Perineural invasion	No	80 (97.6)	11 (84.6)	49 (90.7)	5 (62.5)	0.006
	Yes	2 (2.4)	2 (15.4)	5 (9.3)	3 (37.5)	
Vascular tumor thrombus	No	81 (98.8)	10 (76.9)	38 (70.4)	5 (62.5)	<0.001
	Yes	1 (1.2)	3 (23.1)	16 (29.6)	3 (37.5)	
AE1/AE3	Negative	29 (35.4)	6 (46.2)	47 (87.0)	4 (50.0)	<0.001
	Positive	53 (64.6)	7 (53.8)	7 (13.0)	4 (50.0)	
Postoperative chemotherapy	No	79 (96.3)	8 (61.5)	27 (50.0)	5 (62.5)	<0.001
	Yes	3 (3.7)	5 (38.5)	27 (50.0)	3 (37.5)	
Postoperative radiotherapy	No	82 (100.0)	13 (100.0)	48 (88.9)	7 (87.5)	0.024
	Yes	0 (0.0)	0 (0.0)	6 (11.1)	1 (12.5)	

Data were analyzed using Fisher's exact test. NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; MANEC, mixed adenoendocrine carcinoma; TNM, tumor-node-metastasis.

A



B

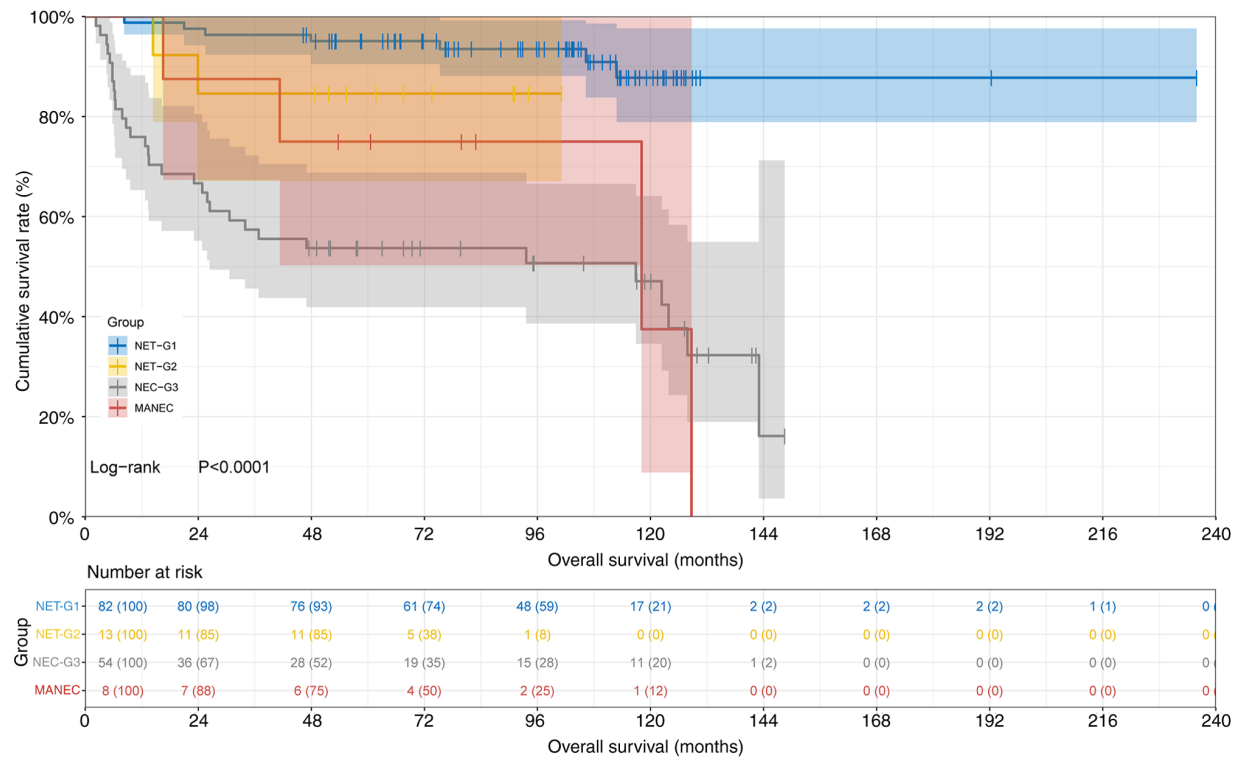


Figure 1. Survival curves of patients with colorectal neuroendocrine carcinoma of different pathological types. (A) Disease free survival and (B) Overall survival curves. MANEC, mixed adenoendocrine carcinoma.

Differentiation in patients with CRNEC. According to ENETS 2017 guidelines, the differentiation types included in the present study were as follows: i) 83 cases (52.9%) with low-grade (G1) CRNEC; ii) 16 cases (10.2%) with intermediate-grade (G2) CRNEC; and iii) 58 cases (36.9%) with high-grade (G3) CRNEC. The clinicopathological

characteristics of patients with CRNEC according to differentiation subtypes are detailed in Table II. Regarding the clinicopathological characteristics of the patients, the differentiation subtypes were associated with age, surgical history, primary site, gross type, tumor size, histological type, total lymph node, positive lymph node, TNM stage, vascular

Table II. Clinicopathological characteristics of patients with colorectal neuroendocrine carcinoma of differentiation types.

Parameters	Group	N	G1	G2	G3	P-value
Total	-	157	83	16	58	
Sex	Male	102 (65.0)	55 (66.3)	8 (50.0)	39 (67.2)	0.413
	Female	55 (35.0)	28 (33.7)	8 (50.0)	19 (32.8)	
Body mass index, kg/m ²	≤24.49	77 (49.0)	40 (48.2)	6 (37.5)	31 (53.4)	0.515
	>24.49	80 (51.0)	43 (51.8)	10 (62.5)	27 (46.6)	
Age, years	≤55	75 (47.8)	46 (55.4)	10 (62.5)	19 (32.8)	0.014
	>55	82 (52.2)	37 (44.6)	6 (37.5)	39 (67.2)	
Surgical history	No	125 (79.6)	68 (81.9)	7 (43.8)	50 (86.2)	0.001
	Yes	32 (20.4)	15 (18.1)	9 (56.2)	8 (13.8)	
Hypertension	No	131 (83.4)	66 (79.5)	12 (75.0)	53 (91.4)	0.095 ^a
	Yes	26 (16.6)	17 (20.5)	4 (25.0)	5 (8.6)	
Diabetes	No	141 (89.8)	73 (88.0)	14 (87.5)	54 (93.1)	0.583 ^a
	Yes	16 (10.2)	10 (12.0)	2 (12.5)	4 (6.9)	
Primary site	Colon	32 (20.4)	7 (8.4)	3 (18.8)	22 (37.9)	<0.001 ^a
	Rectum	125 (79.6)	76 (91.6)	13 (81.2)	36 (62.1)	
Gross type	Elevated type	72 (45.9)	56 (67.5)	5 (31.2)	11 (19.0)	<0.001 ^a
	Ulcerative type	22 (14.0)	2 (2.4)	0 (0.0)	20 (34.5)	
	Infiltrative type	63 (40.1)	25 (30.1)	11 (68.8)	27 (46.6)	
Tumor size, cm	≤2	81 (51.6)	67 (80.7)	7 (43.8)	7 (12.1)	<0.001 ^a
	>2	41 (26.1)	5 (6.0)	5 (31.2)	31 (53.4)	
	Unknown	35 (22.3)	11 (13.3)	4 (25.0)	20 (34.5)	
Histological type	NET-G1	82 (52.2)	80 (96.4)	1 (6.2)	1 (1.7)	<0.001 ^a
	NET-G2	13 (8.3)	0 (0.0)	13 (81.2)	0 (0.0)	
	NEC-G3	54 (34.4)	1 (1.2)	1 (6.2)	52 (89.7)	
	MANEC	8 (5.1)	2 (2.4)	1 (6.2)	5 (8.6)	
Total number of lymph node	≤18	37 (23.6)	11 (13.3)	7 (43.8)	19 (32.8)	<0.001 ^a
	>18	22 (14.0)	6 (7.2)	3 (18.8)	13 (22.4)	
	Unknown	98 (62.4)	66 (79.5)	6 (37.5)	26 (44.8)	
Positive number of lymph node	≤5	40 (25.5)	12 (14.5)	9 (56.2)	19 (32.8)	<0.001 ^a
	>5	19 (12.1)	5 (6.0)	1 (6.2)	13 (22.4)	
	Unknown	98 (62.4)	66 (79.5)	6 (37.5)	26 (44.8)	
TNM stage	Tis	4 (2.5)	2 (2.4)	1 (6.2)	1 (1.7)	<0.001 ^a
	I	63 (40.1)	59 (71.1)	1 (6.2)	3 (5.2)	
	II	5 (3.2)	1 (1.2)	1 (6.2)	3 (5.2)	
	III	28 (17.8)	8 (9.6)	3 (18.8)	17 (29.3)	
	IV	43 (27.4)	4 (4.8)	7 (43.8)	32 (55.2)	
	Unknown	14 (8.9)	9 (10.8)	3 (18.8)	2 (3.4)	
Perineural invasion	No	145 (92.4)	80 (96.4)	14 (87.5)	51 (87.9)	0.085 ^a
	Yes	12 (7.6)	3 (3.6)	2 (12.5)	7 (12.1)	
Vascular tumor thrombus	No	134 (85.4)	79 (95.2)	13 (81.2)	42 (72.4)	0.001 ^a
	Yes	23 (14.6)	4 (4.8)	3 (18.8)	16 (27.6)	
AE1/AE3	Negative	86 (54.8)	30 (36.1)	7 (43.8)	49 (84.5)	<0.001
	Positive	71 (45.2)	53 (63.9)	9 (56.2)	9 (15.5)	
Postoperative chemotherapy	No	119 (75.8)	80 (96.4)	8 (50.0)	31 (53.4)	<0.001 ^a
	Yes	38 (24.2)	3 (3.6)	8 (50.0)	27 (46.6)	
Postoperative radiotherapy	No	150 (95.5)	82 (98.8)	15 (93.8)	53 (91.4)	0.095 ^a
	Yes	7 (4.5)	1 (1.2)	1 (6.2)	5 (8.6)	

^aData were analyzed using Fisher's exact test. NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; MANEC, mixed adenoendocrine carcinoma; TNM, tumor-node-metastasis.

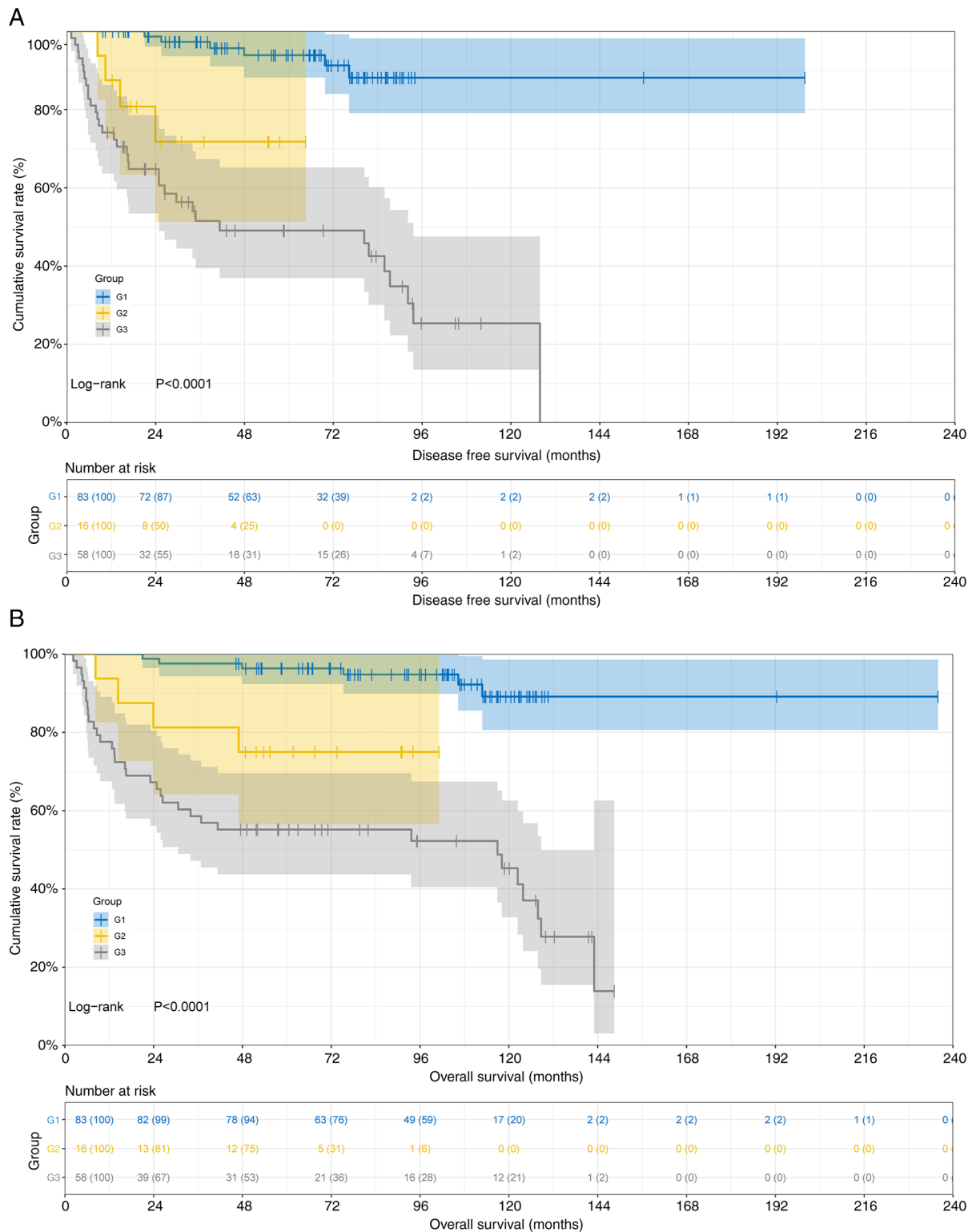


Figure 2. Survival curves of patients with colorectal neuroendocrine carcinoma of different differentiation types. (A) Disease free survival and (B) Overall survival curves.

tumor thrombus, AE1/AE3 and postoperative chemotherapy ($P<0.05$).

The mean DFS and OS durations stratified by differentiation subtypes were as follows: i) In the low-grade (G1) group, DFS was 65.73 months (95% CI, 55.33-71.43) and OS was 103.30 months (95% CI, 92.90-109.80); ii) in the intermediate-grade (G2) group, DFS was 24.57 months (95% CI,

14.40-54.33) and OS was 58.58 months (95% CI, 46.97-90.87); and iii) in the high-grade (G3) group, DFS was 25.67 months (95% CI, 16.23-34.83), OS was 51.90 months (95% CI, 26.43-71.10). Comparative analysis revealed statistically significant differences in both DFS ($\chi^2=42.910$; $P<0.0001$) and OS ($\chi^2=41.220$; $P<0.0001$) among the three differentiation subtypes. The survival curves are presented in Fig. 2.

Respectively, the 1-, 3- and 5-year survival rates of DFS and OS stratified by pathological subtypes were as follows: i) in the low-grade (G1) group, 100.0% (95% CI, 100.0-100.0), 97.3% (95% CI, 93.7-100.0) and 93.9% (95% CI, 88.2-99.9) for DFS, and 100.0% (95% CI, 100.0-100.0), 97.6% (95% CI, 94.3-100.0) and 96.4% (95% CI, 92.4-100.0) for OS; ii) in the intermediate-grade (G2) group, 87.5% (95% CI 72.7-100.0), 71.8% (95% CI, 51.4-100.0) and 71.8% (95% CI, 51.4-100.0) for DFS, and 93.8% (95% CI, 82.6-100.0), 81.2% (95% CI, 64.2-100.0) and 75.0% (95% CI, 56.5-99.5) for OS; and 3) in the high-grade (G3) group, 74.1% (95% CI, 63.7-86.3), 51.6% (95% CI, 39.5-67.3) and 49.1% (95% CI, 37.0-65.2) for DFS, and 77.6% (95% CI, 67.6-89.1), 58.6% (95% CI, 47.2-72.8) and 55.2% (95% CI, 43.7-69.6) for OS. The significant survival differences ($P<0.0001$) among the differentiation grades emphasized that high-grade (G3) tumors require aggressive therapeutic strategies, while low-grade (G1) tumors may benefit from less intensive interventions.

Related prognostic factors identified by Cox regression analysis. According to univariate Cox regression analysis, BMI, age, alcohol drinking, histological type and differentiation were the related factors affecting DFS in patients CRNEC. The multivariate analysis indicated that BMI, age and differentiation were the potential prognostic factors affecting DFS in patients with CRNEC (Table SII). Based on univariate Cox regression analysis, sex, age, diabetes and differentiation were significantly related to OS in patients with CRNEC (Table SIII). The multivariate analysis identified that sex, age and differentiation were the potential prognostic factors affecting OS in patients with CRNEC. The identification of age and differentiation as the potential prognostic factors for DFS and OS provided a foundation for risk stratification and personalized treatment planning in patients with CRNEC.

Establishing a prognostic nomogram. Based on the univariate and multivariate Cox regression analyses, nomograms were developed to predict 1-, 3- and 5-year DFS and OS in patients with CRNEC. The parameters with $P<0.05$, including BMI, age and differentiation were selected via multivariate analyses to construct a nomogram prognostic model of DFS (Fig. 3A). The C-index for this nomogram prognostic model predicting DFS was 0.843 (95% CI, 0.665-0.936). Moreover, the parameters with $P<0.05$, including sex, age and differentiation were selected to comprise the nomogram prognostic model of OS (Fig. 3B). The C-index for this nomogram prognostic model predicting OS was 0.819 (95% CI, 0.644-0.919). Calibration curves with 1,000 bootstrap iterations quantified the precision of the nomograms in estimating DFS and OS probabilities at 1-, 3- and 5-year using the performance of the Cox regression model. The predicted and observed survival probabilities showed strong concordance across different time points (1-, 3- and 5-year), as evidenced by calibration curves closely aligning with the 45° reference line (Fig. 4A-F). The high C-index values and well-calibrated curves demonstrated the robust predictive accuracy of the nomogram, offering clinicians a practical tool for individualized prognostic assessment.

Subgroup analysis for AE1/AE3. According to the results of Tables I and II, AE1/AE3 was significantly associated with histological type and differentiation. There were 71 cases with positive expression of AE1/AE3, and 86 cases with negative expression of AE1/AE3; representative immunohistochemistry images are shown in Fig. S1. The clinicopathological characteristics of patients with CRNEC split according to AE1/AE3 expression are detailed in Table SIV. Regarding the clinicopathological characteristics of the patients, AE1/AE3 expression was associated with gross type, tumor size, histological type, differentiation, TNM stage and postoperative chemotherapy ($P<0.05$).

The mean DFS and OS durations stratified by AE1/AE3 expression were as follows: i) In patients with AE1/AE3(-), DFS was 40.57 months (95% CI, 31.00-56.37) and OS was 79.23 months (95% CI, 67.53-96.33); ii) in patients with AE1/AE3(+), DFS was 53.02 months (95% CI, 29.60-70.23) and OS was 92.25 months (95% CI, 63.03-106.80). Comparative analysis revealed statistically significant differences in both DFS and OS among AE1/AE3 expression groups (DFS: $\chi^2=5.162$, $P=0.023$; OS: $\chi^2=6.681$, $P=0.015$). The survival curves are presented in Fig. 5.

The 1-, 3- and 5-year survival rates of DFS and OS stratified by AE1/AE3 expression were as follows: i) In patients with AE1/AE3(-), 83.7% (95% CI, 76.3-91.9), 69.5% (95% CI, 60.2-80.4) and 68.0% (95% CI, 58.5-79.1) for DFS, and 87.2% (95% CI, 80.4-94.6), 74.4% (95% CI, 65.7-84.2) and 70.9% (95% CI, 62.0-81.2) for OS, respectively; and ii) in patients with AE1/AE3(+), 95.8% (95% CI, 91.2-100.0), 88.9% (95% CI, 81.4-97.1) and 83.8% (95% CI, 74.4-94.6) for DFS, and 95.8% (95% CI, 91.2-100.0), 90.1% (95% CI, 83.5-97.3) and 88.7% (95% CI, 81.6-96.4) for OS, respectively (Fig. 5). The association of AE1/AE3 positivity with prolonged survival suggested its potential role as a favorable biomarker for tumor biology, which could aid in diagnostic and therapeutic decision-making.

Subgroup analysis for age. Based on the univariate and multivariate Cox regression analyses, age was indicated as a potential prognostic factor affecting DFS and OS in patients with CRNEC. The median age of enrolled patients was 55 years; according to the median age, there were 75 patients aged ≤ 55 years and 82 cases patients aged >55 years. Notably, patients >55 years old had a poorer prognosis than those aged ≤ 55 years (DFS: $\chi^2=11.620$; $P=0.0007$; OS: $\chi^2=12.450$; $P=0.0005$) (Fig. S2).

Discussion

CRNEC is a relatively rare malignancy, accounting for 20-30% of all gastroenteropancreatic NENs (GEP-NENs) and $<2\%$ of all colorectal cancers. A majority of CRNECs are non-functional, exhibiting non-specific symptomatology indistinguishable from colorectal adenocarcinoma, including abdominal pain, altered bowel habits and hematochezia (15). CRNECs are typically more aggressive than conventional colorectal carcinoma, with higher rates of metastasis and poorer survival outcomes (16). With advancements in diagnostic technologies, the improvement of living standards and increased health awareness, the detection rate of CRNEC has

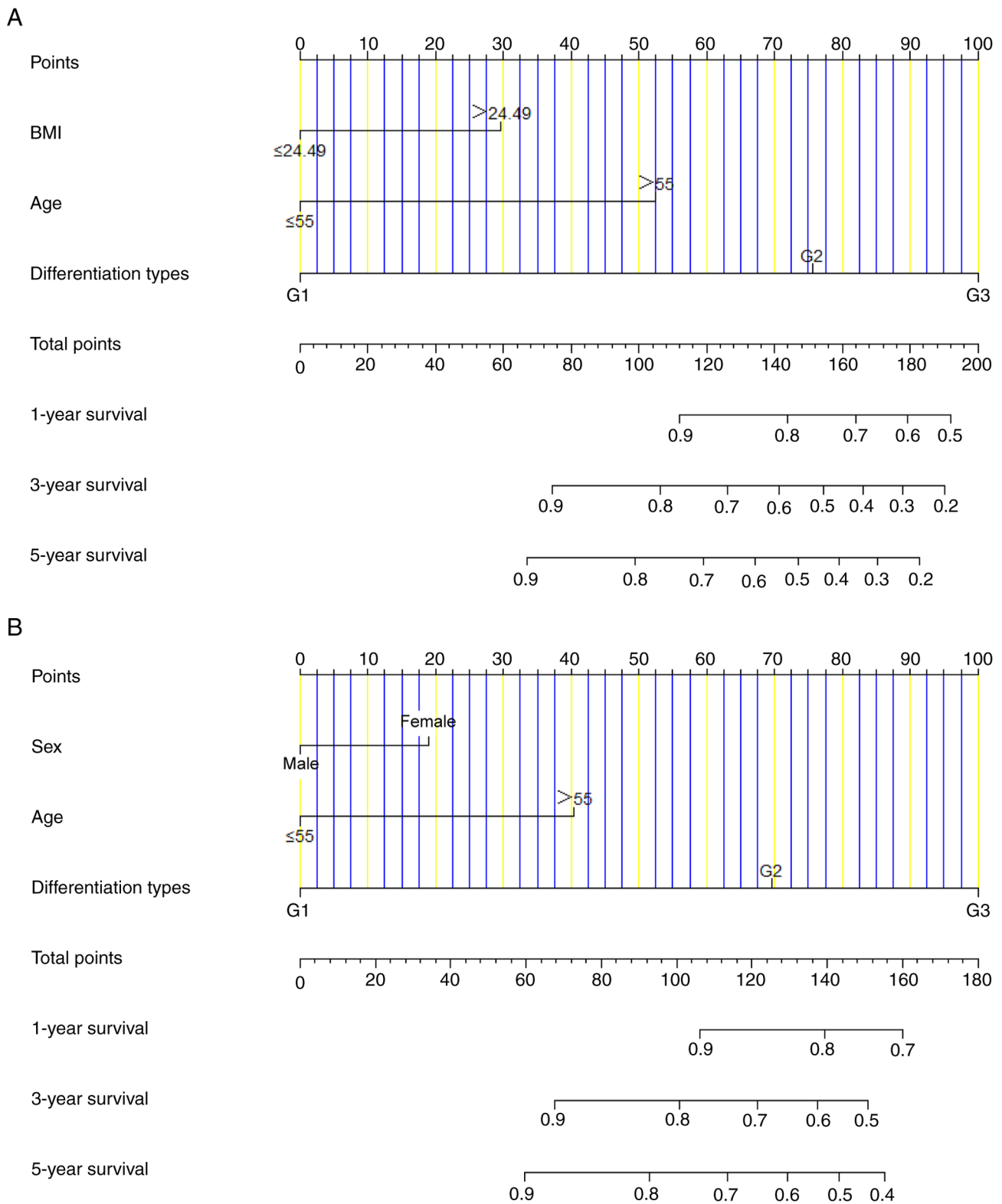


Figure 3. Nomograms for prognosis prediction in patients with colorectal neuroendocrine carcinoma. (A) 1-, 3- and 5-year nomograms for prognosis prediction of Disease free survival; (B) 1-, 3- and 5-year nomograms for prognosis prediction of Overall survival. BMI, body mass index.

notably risen in recent years (17). The diagnosis of CRNEC primarily relies on colonoscopy, biopsy and pathology after surgery, with definitive confirmation requiring immuno-histochemical evidence of neuroendocrine markers, such as Syn, chromogranin A and neuron-specific enolase (18). Surgical resection is the main treatment for CRNEC, while

chemotherapy serves a role as a crucial adjuvant treatment, particularly for tumors with Ki-67 >5%, utilizing regimens including doxorubicin, etoposide and fluorouracil (19). By contrast, conventional colorectal carcinoma is primarily managed with surgery and chemotherapy regimens such as FOLFOX, CAPEOX or FOLFIRI (20).

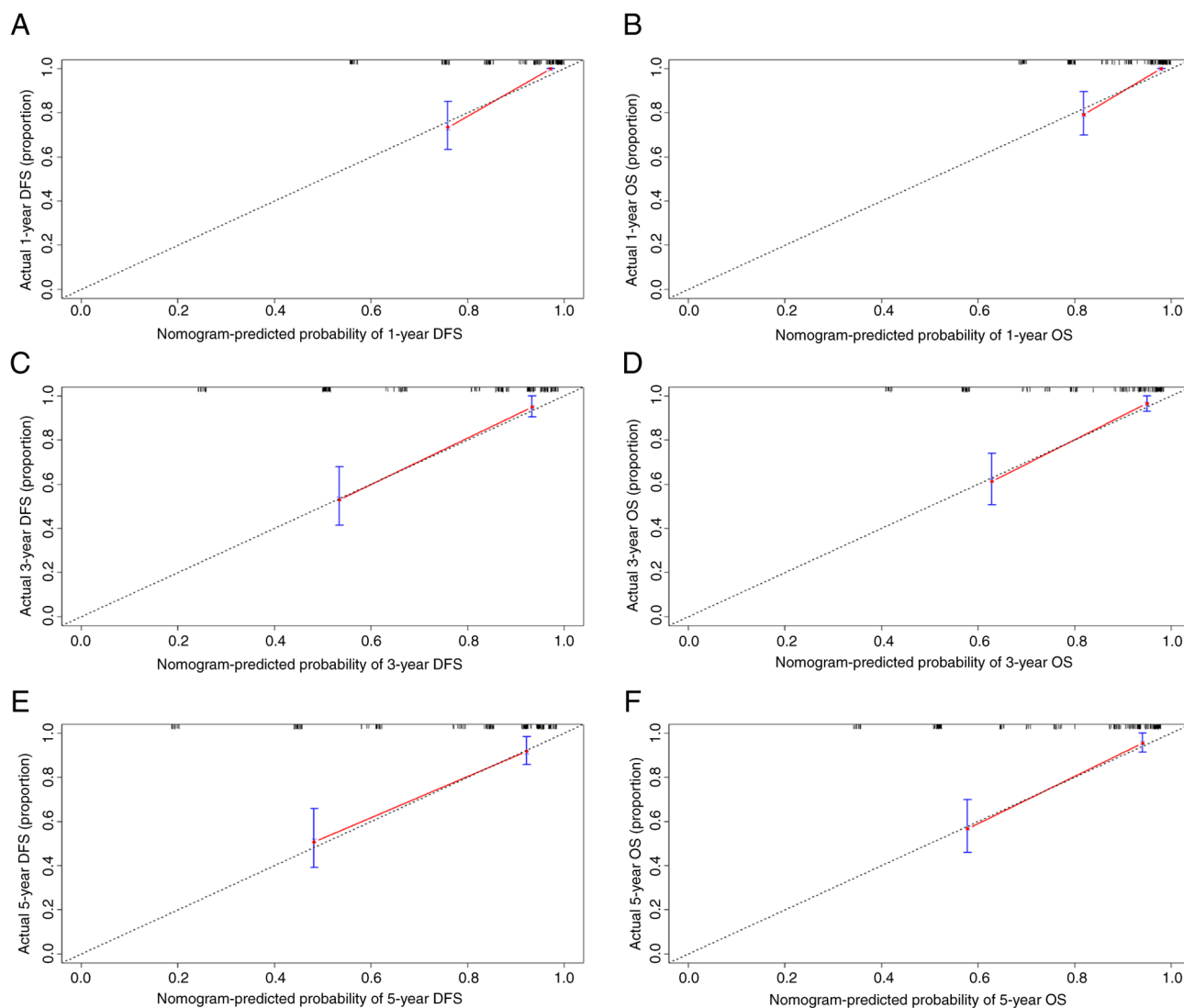


Figure 4. Calibration curves of nomograms in colorectal neuroendocrine carcinoma. (A) 1-, (C) 3- and (E) 5-year calibration curves of nomograms for prognosis prediction of DFS; (B) 1-, (D) 3- and (F) 5-year calibration curves of nomograms for prognosis prediction of OS. DFS, disease-free survival; OS, overall survival.

The present study analyzed the clinicopathological characteristics of 157 cases of CRNEC, including 32 cases of colonic NETs and 125 cases of rectal NETs. According to data from China, rectal NENs are the predominant type of gastrointestinal NETs, and the incidence rate has increased in recent years (21). Studies have also demonstrated that tumor stage and tumor size are the critical prognostic factors for CRNEC (22,23). According to the 5th edition of the WHO Classification of Digestive System Tumors (2019), NETs can be classified as NET-G1, NET-G2, NEC-G3 and MANEC (24). In the present study, of all pathological types included, there were 82 cases graded as NET-G1, 13 cases of NET-G2, 54 cases of NEC-G3 and eight cases of MANEC. The results of the current study demonstrated that the different pathological types were associated with age, gross types, differentiation and AE1/AE3. Additional analysis of prognostic outcomes in patients with CRNEC indicated that the NET-G1 pathological subtype was associated with significantly prolonged DFS and OS, as well as improved prognosis, when compared with the other pathological subtypes. The results of the present study align

with prior evidence, which demonstrated that patients with pancreatic NET-G1/2 had a significantly longer survival time compared with that of patients with poorly differentiated NEC ($P=0.002$) (25). Punekar *et al* (26) revealed that patients with NET-G1/2 had an improved OS rate than those with NEC-G3 and MANEC in colorectal NETs in the SEER database.

The potential mechanisms are that NEC-G3 and MANEC are associated with larger tumors with more aggressive histological features, and more metastatic sites compared with NET-G1/G2. NECs are genomically distinct entities characterized by obligate inactivation of the TP53 and Rb/p16 tumor suppressor pathways (27). Tanaka *et al* (28) observed that MANEC, albeit rare, has a highly aggressive clinical course, and that curative-intent surgery with adjuvant chemotherapy could markedly prolong survival in localized cases.

The present study also analyzed the clinicopathological features among the various differentiation types in patients with CRNEC. Regarding differentiation types, there were 83 low-grade (G1) cases, 16 intermediate-grade (G2) cases and 58 high-grade (G3) cases. The present study revealed that the

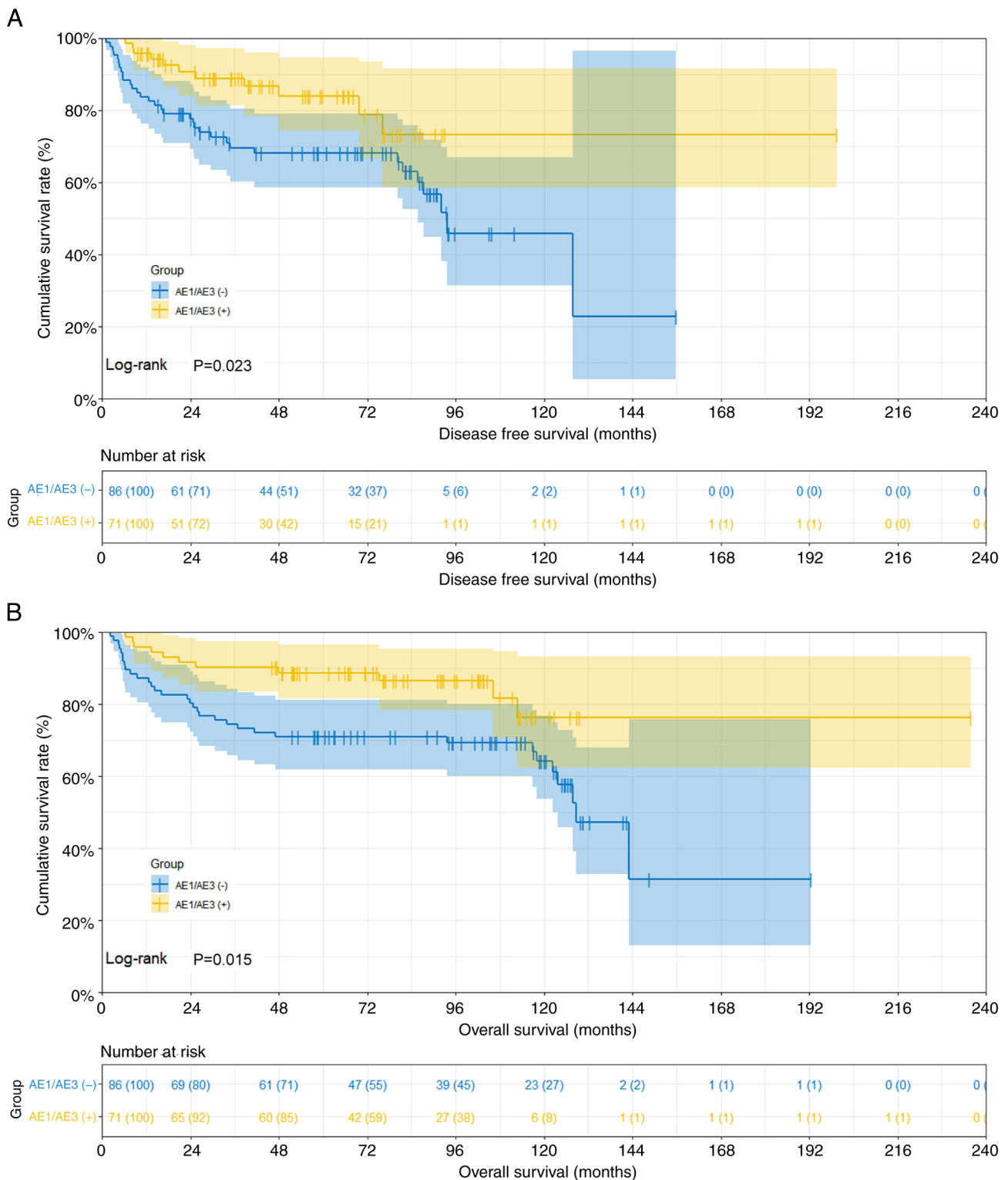


Figure 5. Survival curves of AE1/AE3 expression in patients with colorectal neuroendocrine carcinoma. (A) Disease free survival and (B) overall survival curves.

differentiation types were related to age, primary site, histological type, TNM stage and AE1/AE3 expression. Subsequent analysis of the outcomes of patients with CRNEC revealed that the low-grade (G1) subtype was associated with markedly extended DFS and OS durations and greater prognostic results compared with THE alternative differentiation classifications. Pommergaard *et al* (29) demonstrated that surgical

resection of primary tumors provided favorable long-term survival in locoregional high-grade GEP-NENs and mixed neuroendocrine-non-neuroendocrine neoplasms (MiNENs). Holmager *et al* (30) demonstrated that high-grade MiNENs have a neuroendocrine and a non-neuroendocrine component that is associated with aggressive biological behavior and unfavorable clinical outcomes. Another study indicated that

high-grade CRNECs exhibit rapid disease progression, and are characterized by limited therapeutic responsiveness and low survival rates (31). Moreover, prognostic analysis revealed that patients with high-grade NEC without metastatic disease have an adenocarcinoma component within their tumor, or their response to chemotherapy is associated with modestly improved clinical outcomes. Alese *et al* (32) also demonstrated that patients with high-grade pancreatic NETs exhibited a significantly shorter median OS (6.0 months) than those with other high-grade gastrointestinal NECs (9.9 months). The survival data of the present study indicated that the prognosis of high-grade (G3) tumors was the poorest compared with low-grade (G1) or intermediate-grade (G2) tumors, which was consistent with the aforementioned studies.

Notably, the present study revealed that AE1/AE3 expression was related to histological type and differentiation. According to immunohistochemistry, there were 71 cases with positive expression of AE1/AE3 and 86 cases with negative expression of AE1/AE3 in the current study. Subsequent analysis of the outcomes of patients with CRNEC revealed that AE1/AE3(+) expression was associated with markedly extended DFS and OS durations and improved prognostic results compared with AE1/AE3 (-) expression. AE1/AE3 expression was associated with well-differentiated CRNEC and may indicate favorable outcomes, while loss of expression was frequently observed in poorly differentiated CRNEC. An AE1/AE3 cocktail is the gold standard for detecting cytokeratin expression in immunohistochemistry. AE1 recognizes high (K10, K14-16) and low (K19) molecular weight keratins, while AE3 targets type II high (K1-6) and low (K7-8) molecular weight keratins, and their downregulated expression is a hallmark of high-grade carcinomas with aberrant differentiation (33). Badzio *et al* (34) demonstrated that patients with small cell lung cancer undergoing pulmonary resection with high AE1/AE3 immunoreactivity demonstrated a superior median OS compared with patients with low expression (24.7 months vs. 13.8 months; $P=0.019$). Vasilevska *et al* (35) revealed that AE1/AE3 immunoreactivity was predominantly observed in moderately differentiated endometrial carcinoma, and the clinical implication of AE1/AE3 might aid in the diagnosis of early-stage endocrine carcinoma as well as aiding the detection of micrometastases, leading to improved survival outcomes. In addition, this previous study demonstrated that AE1/AE3 negativity coincided with epithelial-mesenchymal transition activation and aggressive behavior of the carcinoma, such as rapid cell proliferation and metastasis (35). Although this has, to the best of our knowledge, not yet been studied in CRNEC, similar mechanisms may explain the observation in the present study that negative expression of AE1/AE3 in tumors indicated higher rates of vascular invasion and metastasis.

The present study investigated the potential prognostic factors of CRNEC and developed a prognostic nomogram model for DFS and OS. Univariate and multivariate analyses revealed that BMI, age and differentiation were the potential independent predictors of DFS, while sex, age and differentiation emerged as significant determinants of OS. Differentiation grade stratifies survival, with high-grade (G3) tumors predicting adverse outcomes and reduced OS. Moreover, the present study constructed a multivariate-derived

prognostic nomogram that could provide higher accuracy in predicting 1-, 3- and 5-year survival probabilities than single conventional prognostic markers. Calibration analysis demonstrated agreement between predicted and observed 1-, 3- and 5-year survival probabilities in patients with CRNEC, with the model-derived curve closely aligning with the ideal reference line.

Multivariate modeling confirmed age as a potential prognostic factor for DFS and OS in CRNEC. In the present study, patients >55 years old had poorer prognosis than those ≤55 years ($P<0.001$). Lal *et al* (36) demonstrated that individuals with large bowel carcinoids were more likely to be elderly (age >65 years) (OR 2.17; CI, 2.05-2.31; $P<0.0001$), and age was identified as a potential risk factor (35). Another study also indicated that age ≥56 years (HR, 7.434; 95% CI, 1.334-41.443; $P=0.022$) was an independent prognostic factor via multivariate analyses in colorectal NETs (37). Age-dependent therapeutic disparities likely contribute to these findings, as younger patients demonstrated greater utilization of different treatment modalities.

The present study had several limitations that should be acknowledged. Firstly, this investigation employed a single-center retrospective design, and thus has some inherent limitations, including potential selection bias and residual confounding factors despite multivariate adjustments. Secondly, the conducted nomograms were derived from a restricted set of covariates and require external validation in independent cohorts to confirm generalizability. Thirdly, the low incidence of CRNEC inherently limited the cohort size. Therefore, prospective multicenter validation studies with larger samples are warranted to confirm these preliminary findings.

In conclusion, the present study established a nomogram to predict the prognosis of patients with CRNEC with good prediction efficacy. Adverse prognostic factors for predicting DFS and OS time include being aged >55 years and being diagnosed with high-grade differentiation subtypes (including G2 and G3). High-grade CRNECs represent highly aggressive malignancies associated with an unfavorable clinical outcome. In addition, AE1/AE3 may be helpful for improving the diagnostic accuracy of patients with CRNEC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HL conceptualized and supervised the study, and was project administrator. MD and SS acquired, analyzed and interpreted the data. XL designed the methodology. MD

and HL wrote the original draft, and reviewed and edited the manuscript. MD and HL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol received approval from the Institutional Review Board of Harbin Medical University Cancer Hospital (approval no. 82271845) and adhered strictly to the ethical principles of the Declaration of Helsinki (1964) and subsequent amendments. Before treatment, the enrolled patients provided written informed consent for the use of their data in research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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