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Increased nerve density adversely affects outcome in colorectal cancer and denervation suppresses tumor growth

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Abstract

Background The colon and rectum are highly innervated, with neural components within the tumor microenvironment playing a significant role in colorectal cancer (CRC) progression. While perineural invasion (PNI) is associated with poor prognosis in CRC, the impact of nerve density and diameter on tumor behavior remains unclear. This study aims to evaluate the prognostic value of nerve characteristics in CRC and to verify the impact of nerves on tumor growth.

Methods Tissue samples from 129 CRC patients were stained with immunofluorescent markers NF-L and S100B to detect nerves. Nerve diameter and density were measured and normalized. Kaplan-Meier survival analysis and Cox regression models were used to identify prognostic factors. Prognostic models were established using receiver operating characteristic (ROC) curve analysis to assess the predictive value of neural factors. A murine chemical denervation model was employed to disrupt sympathetic nerves using 6-hydroxydopamine, inhibit muscarinic receptor 3 with darifenacin, and ablate sensory neurons with capsaicin.

Results The total nerve density was $0.72 \pm 0.59/\text{mm}^2$, with intratumoral ($0.42 \pm 0.40/\text{mm}^2$) being significantly lower than extratumoral regions ($1.00 \pm 0.75/\text{mm}^2$). The average nerve diameter was $28.14 \pm 6.04 \mu\text{m}$, with no significant difference between intratumoral ($28.2 \pm 7.65 \mu\text{m}$) and extratumoral regions ($27.86 \pm 6.72 \mu\text{m}$). PNI was observed in 65 patients (50.4%). PNI and high normalized nerve density (NND) were associated with shorter overall survival and disease-free survival in CRC patients, with PNI identified as an independent prognostic factor. Patients with PNI exhibit higher NND. Incorporating PNI and NND into ROC curve analysis improved the sensitivity and specificity of survival predictions. In the murine model, chemical denervation of sympathetic, parasympathetic, and sensory nerves significantly reduced rectal tumor volume.

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Conclusions PNI and NND are critical factors influencing CRC patient survival and enhance the accuracy of survival prediction models. Moreover, chemical denervation effectively inhibits rectal tumor growth in vivo, highlighting the potential of neural targeting as a therapeutic strategy in CRC.

Keywords Perineural invasion, Nerve density, Prognosis, Tumor denervation, Neural remodeling

Introduction

Cancer is a major global health challenge, characterized by uncontrolled cell proliferation and the ability to invade surrounding tissues and metastasize to distant organs [1]. Colorectal cancer (CRC) is the third most common cancer worldwide, with millions of newly diagnosed patients each year [2]. In recent years, CRC has become a significant public health burden in China, with increasing incidence and concerning mortality rates [3, 4]. This trend is primarily attributed to the adoption of a Westernized lifestyle, changes in dietary patterns, sedentary behavior, and an aging population [5–8]. The burden of CRC is further compounded by the challenges associated with early detection, limited access to screening programs, and inadequate treatment options. Although chemotherapy remains a cornerstone of cancer treatment, its effectiveness is frequently hampered by resistance, side effects, and recurrence [9]. Thus, understanding the mechanisms driving CRC progression and exploring new therapeutic agents is critical for developing more effective prevention and treatment strategies.

The colon and rectum are innervated by intrinsic enteric neurons and extrinsic efferent and afferent nerves. The enteric nervous system (ENS) includes the myenteric and submucosal ganglia, comprising diverse types of enteric neurons and glial cells [10]. Axons arising from the ENS and from extrinsic neurons provide innervation to regulate intestinal functions [11]. Extrinsic efferent innervation refers to the sympathetic and parasympathetic input from the central nervous system (CNS). Depending on anatomical location, sympathetic innervation of the colorectum arises from the superior, inferior mesenteric, and inferior hypogastric ganglia, while parasympathetic innervation is provided by the vagus or pelvic splanchnic nerves [12, 13]. Sympathetic activation triggers the release of adrenaline, noradrenaline (NA), ATP, and neuropeptide Y, whereas parasympathetic nerves primarily release acetylcholine (ACh), exerting both excitatory and inhibitory control over gastrointestinal tone and motility [14]. Extrinsic afferent nerves refer to sensory neurons that transmit pain, temperature, and other sensory information to the CNS, have been found to interact with cancer cells and influence the tumor immune microenvironment [15]. Mutual interactions between cancer cells and nerves involve the release of neurotransmitters, neuropeptides, and growth factors, which alter tumor behavior and the tumor microenvironment (TME) [16, 17]. Although the specific mechanisms

have not been fully elucidated, nerves and CRC cells are known to communicate with each other, promoting CRC progression [18].

Perineural invasion (PNI) refers to the encasement of tumor cells around nerves, involving over 33% of the nerve circumference or invasion into the nerve sheath [19]. This phenomenon is associated with advanced disease, local recurrence, and poor prognosis in pancreatic ductal adenocarcinoma (PDAC) [20, 21], prostate cancer [22], breast cancer [23], head and neck cancer [24], and CRC [25, 26]. In addition to PNI, nerves undergo alterations during tumor development, such as neurogenesis, nerve hypertrophy and increased density, collectively termed neural remodeling [27, 28]. In CRC, extensive tumor neurogenesis has been shown to be a predictor of decreased overall survival [29]. In gastric cancer, a maximum nerve diameter of $\geq 65 \mu\text{m}$ has been identified as an independent risk factor for recurrence and cancer-related death within 5 years [30]. Additionally, high nerve density can enhance tumor growth and serve as a prognostic factor influencing treatment selection for aggressive oral cavity squamous cell carcinoma (OSCC) [31]. Conversely, low intrapancreatic nerve density has been identified as an independent prognostic factor in PDAC, associated with aggressive tumor behavior [32]. These findings suggest that, neural changes, in addition to PNI, may influence tumor progression and patient prognosis. This study aims to investigate the clinical relevance of neural alterations in CRC and their potential role as prognostic markers. In this study, we investigated the impact of neural alterations on patient survival in 129 CRC tissues. Additionally, we established a chemical denervation mouse model to assess the effect of nerves on tumor growth. Our findings reveal that PNI and normalized nerve density significantly impact CRC patient survival, and chemical denervation effectively inhibited tumor growth.

Materials and methods

Patients and tissues

Primary tumor tissues were obtained from patients who underwent standard colectomy and regional lymphadenectomy at the Gastrointestinal Department of Ren Ji Hospital, Shanghai Jiao Tong University, between April and September 2017. Tumor staging was performed according to the eighth edition of the American Joint Committee on Cancer (AJCC) TNM staging system, based on final histopathological examination. Resected specimens were evaluated for depth of tumor invasion,

differentiation, number of lymph node metastases, and presence of vascular invasion. Patients with synchronous tumors, severe cardiovascular or pulmonary diseases, or those who had received neoadjuvant therapy prior to surgery were excluded from the study. A total of 145 patients were ultimately included in this retrospective review of prospectively collected data. This study was approved by the Ethics Committee of Ren Ji Hospital affiliated to Shanghai Jiao Tong University (approval number: KY2021-120-B), and informed consent was obtained from all participants.

Immunofluorescence

Collected tissue samples were fixed with 4% paraformaldehyde for 1 h and dehydrated overnight at 4 °C. The specificity and validity of the primary antibodies used for immunofluorescence staining, including anti-S100B (Servicebio, GB113884, 1:500) and anti-Neurofilament-light (NF-L, Proteintech, 12998-1-AP, 1:200), were verified (Fig. S1). Fluorescent secondary antibodies CY5-labeled goat anti-rabbit IgG (1:1,000) and FITC-labeled goat anti-rabbit IgG (1:1,000) were incubated for 30 min at room temperature (25 °C). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Images were captured using an upright fluorescence microscope (ECLIPSE C1, NIKON) and scanned with a 3DHISTECH scanner.

Mouse intestinal tissues were stained using the same immunofluorescence protocol. The following antibodies were used: anti-Tyrosine Hydroxylase (TH, Abcam, ab112, 1:100) for sympathetic nerves, anti-Choline Acetyltransferase (VACHT, Abcam, ab317452, 1:100) for parasympathetic nerves, and anti-Calcitonin Gene-Related Peptide (CGRP, Abcam, ab36001, 1:200) for sensory nerves.

Immunohistochemistry

Following chemical denervation, mouse intestinal tissues were fixed in 4% paraformaldehyde for 1 h and dehydrated overnight at 4 °C. For immunohistochemistry (IHC), tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate buffer (pH 6.0) using microwave heating for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Sections were incubated overnight at 4 °C with primary antibodies, followed by secondary antibody incubation and visualization using a DAB substrate kit. Hematoxylin was used for counterstaining. The primary antibodies used included anti-Protein Gene Product 9.5 (PGP9.5, Proteintech, 14730-1-AP, 1:200) as a pan-neuronal marker, anti-TH (Abcam, ab112, 1:200) for sympathetic nerves, anti-Choline Acetyltransferase (VACHT, Abcam, ab317452, 1:100) for parasympathetic nerves, and anti-CGRP (Abcam, ab36001, 1:200) for

sensory nerves. For mouse rectal orthotopic transplantation tumors, 5 µm serial sections of tumor and intestinal tissues were stained with hematoxylin and eosin (H&E).

Quantitative analysis of the nerve diameter and nerve density

Immunofluorescence images for each patient were reviewed independently by two pathologists using SlideViewer software. Tissue regions were divided into two zones: the intratumoral region, comprising the tumor bulk and a 500 µm margin surrounding the tumor-normal boundary, and the extratumoral region, encompassing all other tissue areas (Fig. 1A). In NF-L and S100B-stained sections, all nerves with a transverse diameter exceeding approximately 10 µm were identified. Nerve diameter was measured for each nerve on the slides, and for elongated nerve bundles, the maximum transverse diameter was recorded. PNI was defined as tumor cell encasement of more than 33% of the nerve circumference or invasion into the nerve sheath.

Nerve density was calculated separately for the intratumoral region (intratumoral nerve density, IND) and the extratumoral region (extratumoral nerve density, END) as the number of nerve segments per mm². Contiguous nerve segments were counted as a single nerve trunk. Normalized nerve density (NND) was determined by dividing IND by END [31] (Fig. 1B). Nerve diameters within the intratumoral (intratumoral nerve transverse diameter, INT) and extratumoral (extratumoral nerve transverse diameter, ENT) regions were averaged. The normalized nerve transverse diameter (NNT) was calculated by dividing INT by ENT for each patient. The maximum intratumoral nerve diameter (MND) was also recorded for subsequent analyses.

Clinicopathological analysis and survival analysis

The optimal cut-off values for intratumoral and extratumoral nerve diameters and densities were determined using the 'maxstat' package in R software. Based on these cut-offs, patients were classified into groups with large/small nerve diameters and high/low nerve densities.

Univariate Cox regression analysis was performed using the 'coxph' function in the 'survival' R package to identify prognostic factors for overall and disease-free survival. Multivariate cox regression analysis was subsequently conducted to determine independent prognostic factors. The 'survival' and 'survminer' R packages were used for further analysis and visualization of prognostic differences between groups. Subsequently, the neural parameters were stratified by clinical characteristics, and the differences among the groups were compared to assess significance. The raw data are provided in Table S1.

Receiver operating characteristic (ROC) curve analysis was performed using the 'pROC' R package to calculate

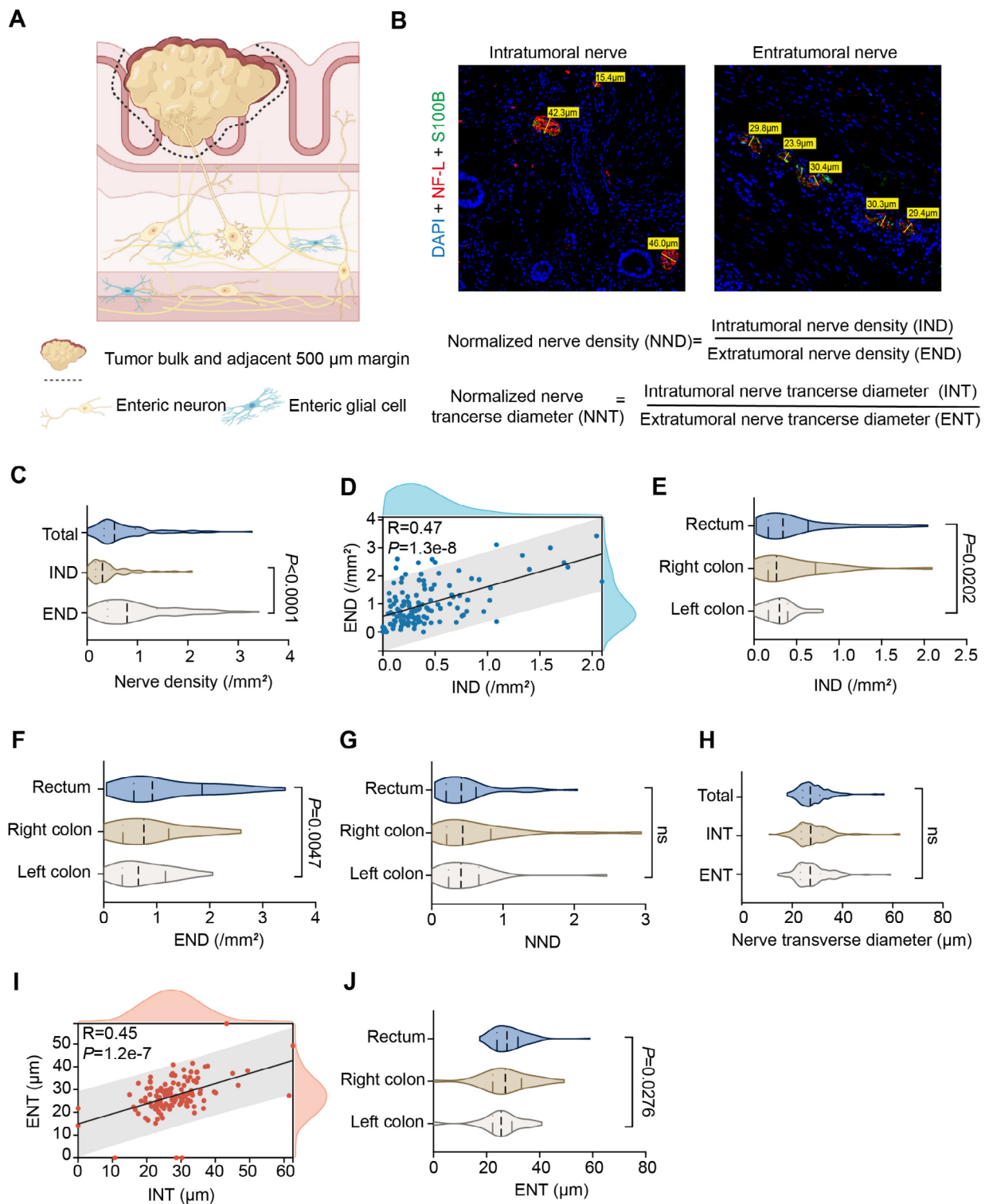


Fig. 1 Acquisition and analysis of neural characteristics in CRC tissues. **(A)** Schematic representation of the zones used for nerve analysis in CRC tissue samples: intratumoral (tumor bulk and adjacent 500 μm margin) and extratumoral (surrounding the tumor). **(B)** Representative immunofluorescence images showing intra- and extratumoral nerve structures: NF-L (red) labels neurons, and S100B (green) labels neuroglia and the myelin sheath. Nerve transverse diameter (μm) is indicated within the figure. **(C)** Comparison of IND and END levels across CRC samples. **(D)** Correlation analysis between IND and END. **(E–G)** Comparison of IND, END, and NND, grouped by tumor location (left colon, right colon, and rectum). **(H)** Comparison of INT and ENT. **(I)** Correlation analysis between ENT and INT. **(J)** Comparison of ENT, grouped by tumor location (left colon, right colon, and rectum). Abbreviations: NF-L, Neurofilament-light; IND, intratumoral nerve density; END, extratumoral nerve density; NND, normalized nerve density; INT, intratumoral nerve transverse diameter; ENT, extratumoral nerve transverse diameter

the area under the curve (AUC). Survival status, follow-up time, and risk scores from the multivariate Cox regression analysis were used for ROC analysis at 12, 36, and 60 months using the 'roc' function in the 'pROC' package. Confidence intervals for the AUC values were calculated using the 'ci' function.

Cell culture

The murine rectal cancer cell line MC38 was obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. MC38 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin, and incubated at 37 °C in a 5% CO₂ humidified atmosphere. All experiments were performed with mycoplasma-free cells. Prior to submucosal injection, MC38 cells were resuspended in PBS at a concentration of 2×10^4 cells/ μ L.

Rectal orthotopic transplantation tumor model

Animal experiments were approved and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Shanghai Jiao Tong University. C57BL/6 male mice (6–8 weeks old) were fasted for one day prior to the experiment. After anesthetizing the mice with isoflurane, they were placed in a supine position. The rectum was carefully exteriorized from the anus using tweezers. Subsequently, 25 μ L (5×10^5 cells per mouse) of tumor cells were injected into the submucosal layer of the rectum using a 1 mL syringe. After needle withdrawal, gentle pressure was applied to prevent cell leakage, and the rectum was repositioned. Following the procedure, the mice were placed on a 37°C heating pad to recover.

14 days after tumor induction, *in vivo* imaging was performed to monitor tumor growth. Mice were injected intraperitoneally with D-luciferin sodium salt (30 mg/mL, 100 μ L) and allowed to move freely for 5 min. The mice were then anesthetized with isoflurane, and imaging was conducted using a small animal *in vivo* imaging system (LINI-T) to confirm successful tumor modeling.

Mouse chemical denervation treatment

14 days after tumor induction, chemical denervation of the sympathetic, parasympathetic, and sensory nerves was performed. Sympathetic nerves were ablated using 6-hydroxydopamine (6-OHDA, MedChemExpress, HY-B1081A), which specifically destroys TH⁺ nerve fibers while sparing parasympathetic VACHT⁺ epithelial nerve fibers, and is non-toxic to tumor cells [33]. The denervation procedure involved two intraperitoneal injections of 6-OHDA: an initial dose of 100 mg/kg followed by 250 mg/kg 24 h later [34]. Notably, some mice exhibited reduced activity and decreased food intake after

the injection of 6-OHDA, but they returned to normal approximately 2 days later, with no significant changes in body weight. For sensory neurons, capsaicin (MedChemExpress, HY-10448A) was administered into the subcutaneous fat tissues of the neck over three consecutive days (50 mg/kg, 80 mg/kg, and 100 mg/kg) [34]. Atropine (5 mg/kg, MedChemExpress, HY-B1205) was injected intraperitoneally immediately before capsaicin injection to prevent acute cardiopulmonary effects due to the excessive release of sensory mediators. Mice exhibited strong struggling behavior during the first capsaicin injection, which diminished with subsequent injections. This dosing regimen of capsaicin has been previously reported to inactivate sensory neurons by reducing neuropeptide levels for 4–6 weeks [35, 36]. No noticeable changes in behavior or feeding were observed after the capsaicin injections. For parasympathetic nerves, the highly selective muscarinic cholinergic receptor 3 (M3R) antagonist darifenacin (DAR, MedChemExpress, HY-A0012), approved by the Food and Drug Administration (FDA), was administered via intraperitoneal injection at a dose of 1 mg/kg/day [37, 38]. No noticeable changes in behavior or feeding were observed following DAR administration.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 26, GraphPad Prism 9, and R 4.0.2. Measurement data are presented as mean $\pm$ standard deviation. Differences between groups were tested using the Chi-square test (or Fisher's exact test where appropriate). Survival rates were calculated using the Kaplan-Meier method, and prognostic factors and survival curves were compared using the log-rank test. Correlations were assessed using Spearman's rank correlation test (weak correlation: $R > 0.1$, moderate: $R > 0.3$, strong: $R > 0.5$). $P < 0.05$ was considered statistically significant.

Results

Clinical baseline characteristics of patients

A total of 145 CRC patients who underwent curative surgery at our institution from April to September 2017 were included. Tumor tissue samples and clinical pathological information were collected, and follow-up was conducted. Of these, 10 patients had primary tumors at other sites, and 6 died from non-tumor-related causes. Consequently, 129 patients were included in the final analysis. The study population consisted of 79 males and 50 females, with a median age of 64.61 ± 10.32 years. The median follow-up was 64 months. At the end of the follow-up period, 45 patients (34.88%) had died. Among the clinicopathologic characteristics, tumor invasion depth (T stage), lymph node involvement (N stage), and AJCC

8th edition staging significantly impacted 5-year survival of patients (Table 1).

Nerve characteristics in CRC

Immunofluorescence staining showed a total nerve density of $0.72 \pm 0.59/\text{mm}^2$, with an IND of $0.42 \pm 0.40/\text{mm}^2$ and an END of $1.00 \pm 0.75/\text{mm}^2$. IND was significantly lower than END (Fig. 1C). A significant positive correlation was observed between IND and END ($R=0.47$, $P=1.3\text{e-}8$, Fig. 1D). According to tumor location, both IND and END were higher in rectal cancer than in left colon cancer (Fig. 1E-F). After normalization, nerve density (NND) showed no significant differences by tumor location, indicating that the site of the tumor did not influence the analysis (Fig. 1G).

The average nerve diameter was $28.14 \pm 6.04 \mu\text{m}$, with an INT of $28.2 \pm 7.65 \mu\text{m}$ and an ENT of $27.86 \pm 6.72 \mu\text{m}$. Nerve diameter did not differ significantly between intra- and extratumoral regions (Fig. 1H). A significant positive correlation was also observed between INT and ENT ($R=0.45$, $P=1.2\text{e-}7$, Fig. 1I). Similar to nerve density, the

ENT in rectal cancer was higher than in left colon cancer ($P=0.0276$, Fig. 1J). However, INT showed no significant differences across different tumor locations (Fig. S2A). After normalization, there was no significant difference in NNT across different tumor locations, confirming that the tumor site did not affect the analysis (Fig. S2B).

PNI was observed in 65 patients (50.4%), higher than previously reported CRC rates [39–41]. The variation in detection rates may be attributed to the reliance on H&E staining in prior studies to detect PNI, whereas our study utilized immunofluorescent labeling to identify all nerves across the entire tissue section, leading to an increased detection rate.

Impact of nerve on patient prognosis in CRC

Kaplan-Meier analysis was conducted to assess the impact of nerve characteristics on patient prognosis. Results showed that patients with PNI had a poorer prognosis ($P=0.041$, Fig. 2A) and showed a trend towards shorter DFS, though not statistically significant ($P=0.071$, Fig. 2B), consistent with previous reports [25, 26]. Furthermore, survival analysis revealed a significant correlation between higher NND and shorter overall survival (OS) ($P=0.044$, Fig. 2C). Higher NND was associated with shorter DFS ($P=0.01$, Fig. 2D). Moreover, patients with higher AJCC stages tended to have higher NND (Fig. S2C, Table S1). However, although patients with a higher AJCC stage also tended to have higher NNT and MND (Fig. S2D-E), higher NNT and MND did not significantly impact OS (Fig. 2E-F).

Univariate Cox regression assessed associations between clinical characteristics and OS/DFS (Table 2). Factors such as Grade, T stage, N stage, M stage, AJCC stage, PNI, and NND were found to influence patient OS and DFS. These characteristics were further analyzed using a nomogram, where the points for each characteristic were considered as indicators of their impact on patient OS. In order of impact, the characteristics were N stage, AJCC stage, T stage, PNI, NND, M stage, and Grade (Fig. S3A). Multivariate Cox analysis showed that N stage and PNI were independent prognostic factors for OS (Fig. 3A).

Furthermore, by analyzing the impact of PNI on nerve density and diameter, we observed that PNI (+) patients had higher NND, NNT, and MND compared to PNI (-) patients (Fig. S3B-D). Combined survival analysis of PNI and NND revealed that PNI (+) patients with high NND had the poorest prognosis, while those PNI (-) with low NND had the most favorable outcome (Fig. 3B-C). The prognostic impact of PNI was independent of NNT and MND (Fig. S3E-F).

We then developed a risk score model incorporating histological grade, T stage, N stage, and M stage to further assess the prognostic impact of PNI and NND. ROC

Table 1 The clinicopathologic characteristics and 5 years survival rate of patients

Characteristics	No. of patients	5-year survival patients (%)	P
Gender			0.87
Female	50	32(64.00%)	
Male	79	53(67.09%)	
Age			0.98
Mean±SD	64.61±10.32	64.60±9.82	
Tumor location			0.08
Rectum	63	43(68.25%)	
Left	38	20(52.63%)	
Right	28	22(78.57%)	
Histologic grade			0.08
Well-Moderate	93	66(70.97%)	
Poor	36	19(52.78%)	
T stage			0.04
T1	6	5(83.33%)	
T2	18	16(88.89%)	
T3	102	63(61.76%)	
T4	3	1(33.33%)	
N stage			8.0e-6
N0	72	60(83.33%)	
N1	34	17(50.00%)	
N2	23	8(34.78%)	
M stage			0.09
M0	119	81(68.07%)	
M1	10	4(40.00%)	
AJCC stage			8.0e-5
I	19	16(84.21%)	
II	52	43(82.69%)	
III	48	22(45.83%)	
IV	10	4(40.00%)	

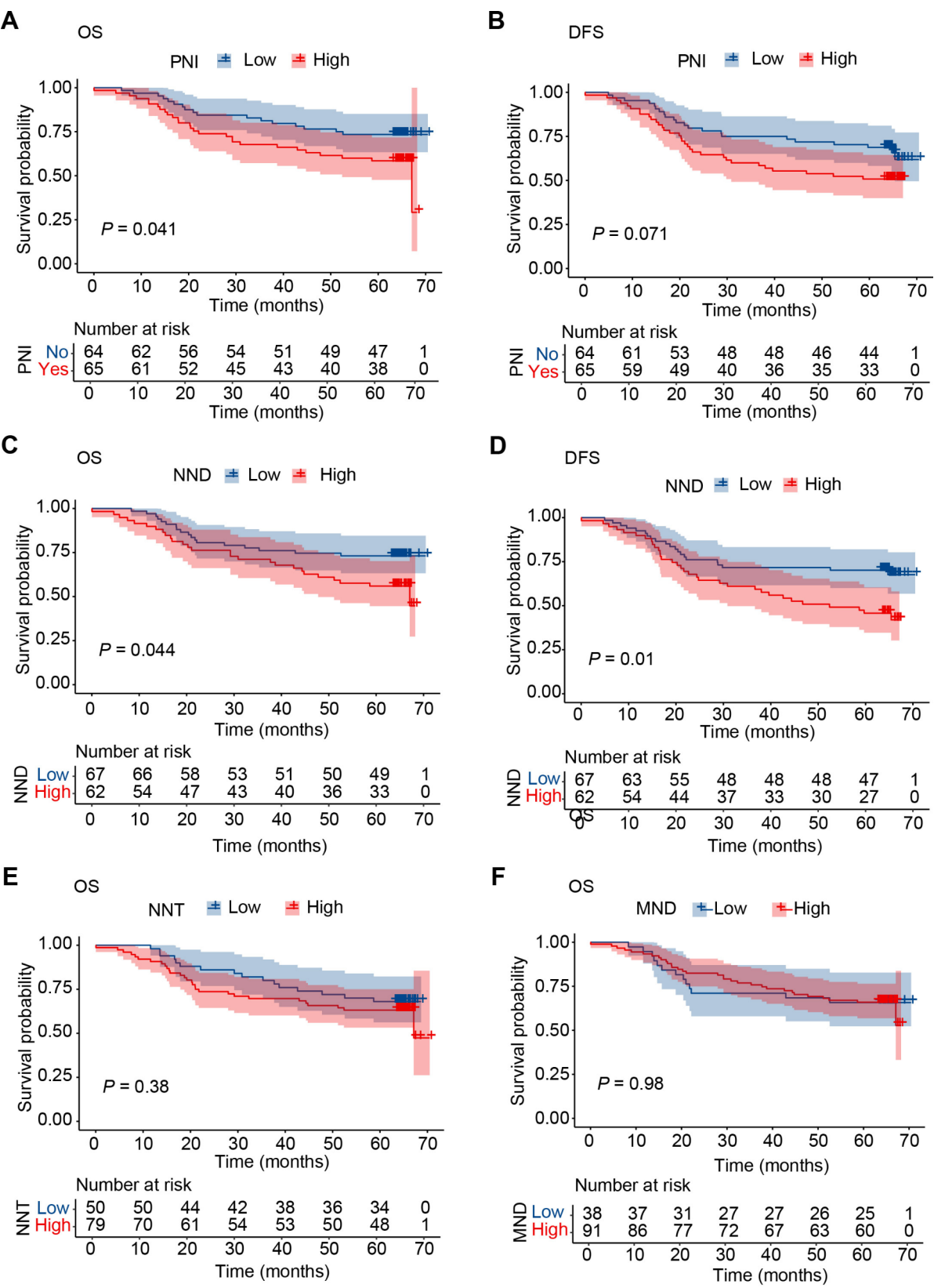


Fig. 2 Impact of PNI and NND on prognosis in CRC patients. **(A–B)** Kaplan-Meier survival analysis assess the impact of PNI on OS and DFS in CRC patients. **(C–D)** Kaplan-Meier survival analysis assess the impact of NND on OS and DFS in CRC patients. **(E)** Kaplan-Meier survival analysis assess the effect of NNT on OS. **(F)** Kaplan-Meier survival analysis evaluating the impact of MND on OS. Abbreviations: PNI, perineural invasion; OS, overall survival; DFS, disease-free survival; NND, normalized nerve density; NNT, normalized nerve transverse diameter; MND, maximum intratumoral nerve diameter

Table 2 Univariate cox analysis of characters for OS and DFS

Characteristics	OS		DFS	
	Hazard ratio (95%CI)	P	Hazard ratio (95%CI)	P
Gender(male vs. female)	0.9028 (0.4972-1.64)	0.737	0.9199 (0.5072-1.668)	0.783
Age (≥ 65 vs. <65 years)	1.165 (0.6472-2.097)	0.611	1.068 (0.6028-1.894)	0.821
Tumor location (rectum vs. colon)	0.7809 (0.4336-1.406)	0.41	0.9405 (0.5514-1.604)	0.822
Grade (III vs. I-II)	2.093 (1.14-3.843)	0.0172	2.001 (1.083-3.698)	0.0269
Tumor diameter (≥ 5 vs. <5 cm)	1.12 (0.6105-2.055)	0.714	1.034 (0.5697-1.875)	0.913
Microsatellite (dMMR vs. pMMR)	0.5661 (0.0779-4.114)	0.574	0.6216 (0.08566-4.51)	0.638
T stage (T3-T4 vs. T1-T2)	3.811 (1.181-12.3)	0.0252	3.019 (1.083-8.416)	0.0347
N stage (N1-N2 vs. N0)	4.398 (2.296-8.424)	7.92e-06	3.431 (1.898-6.202)	4.47e-05
M stage (M1 vs. M0)	2.423 (1.023-5.737)	0.0443	/	/
AJCC stage (III-IV vs. I-II)	4.224 (2.205-8.091)	1.39e-05	3.431 (1.898-6.202)	4.47e-05
PNI (present vs. absent)	1.862 (1.018-3.406)	0.0438	1.654 (0.9228-2.963)	0.091
NND (high vs. low)	1.833 (1.008-3.333)	0.047	2.0252 (1.171-3.503)	0.0116

OS, overall survival; DFS, disease free survival; CI confidence interval

analysis was performed, and the AUC values for 1-year, 3-year, and 5-year outcomes were calculated. The initial model yielded AUC values of 0.77 at 1-year, 0.78 at 3-year, and 0.76 at 5-year (Fig. 3D). After incorporating PNI and NND into the model, the predictive sensitivity and specificity were significantly improved (Fig. 3E). The updated model achieved AUC values of 0.85 at 1-year, 0.80 at 3-year, and 0.79 at 5-year.

Denervation inhibits CRC growth in mice

In normal mouse intestinal tissue, immunofluorescence staining revealed distinct nerve populations, including TH-labeled sympathetic nerves, VAcHT-labeled parasympathetic nerves, and CGRP-labeled sensory neurons (Fig. 4A). An orthotopic rectal cancer model was established by injecting MC38 cells into the sub-mucosa of the rectum. In vivo imaging on day 13 post-injection confirmed successful tumor modeling. On day 14 post-modeling, chemical denervation was performed (Fig. 4B). Intraperitoneal injection of 6-OHDA significantly reduced TH⁺ sympathetic nerves compared to the control group, while subcutaneous injection of capsaicin led to a significant decrease in CGRP⁺ sensory nerves (Fig. 4C). Mice were sacrificed on day 28, and tumors were collected. H&E staining revealed that chemical denervation of sympathetic, parasympathetic, and sensory nerves significantly reduced tumor volume (Fig. 4D-E).

Discussion

This study demonstrates that PNI and NND significantly affect CRC progression and prognosis, offering novel insights into the role of neural components in cancer biology. Results show that higher NND is associated with shorter OS and DFS, suggesting its potential as a valuable prognostic marker. By integrating both PNI and NND into survival models, we improve the accuracy of

risk stratification in CRC patients. Additionally, the use of a chemical denervation model in a murine rectal cancer model reveals that targeting neural components can effectively reduce tumor volume, providing a promising new therapeutic approach that could complement conventional treatments in CRC.

The colon and rectum exhibit region-specific patterns of neural innervation based on their anatomical location. The right sections of the cecum, ascending colon, and transverse colon predominantly receive sympathetic innervation from the superior mesenteric plexus, while the left portions of the transverse colon, descending colon, and sigmoid colon are primarily innervated by sympathetic fibers from the inferior mesenteric plexus. Parasympathetic innervation to the right colon is mediated by the vagus nerve, whereas the left colon and upper rectum are predominantly innervated by the pelvic splanchnic nerves. Sympathetic innervation to the rectum originates from the inferior hypogastric plexus, while parasympathetic innervation arises from the pelvic splanchnic nerves [11, 42]. For afferent nerves, sensory innervation in the colon is primarily mediated by the rectal nerve plexus, pelvic nerve plexus, and lumbar sympathetic trunk, which are responsible for conveying sensory information, such as pain, touch, and pressure [43]. According to our findings, the neural density and diameter in the rectum are higher compared to the left colon. NND was standardized by dividing IND by END, accounting for neural distribution variations. Importantly, NND was shown to better reflect the biological behavior of CRC, with higher NND correlating with lower survival rates. Utilizing NND allows for objective comparisons among CRC patients. To our knowledge, this is the first report to identify NND as a prognostic marker in CRC.

Tumor innervation is frequently discussed in terms of nerve density, with most studies using H&E staining or

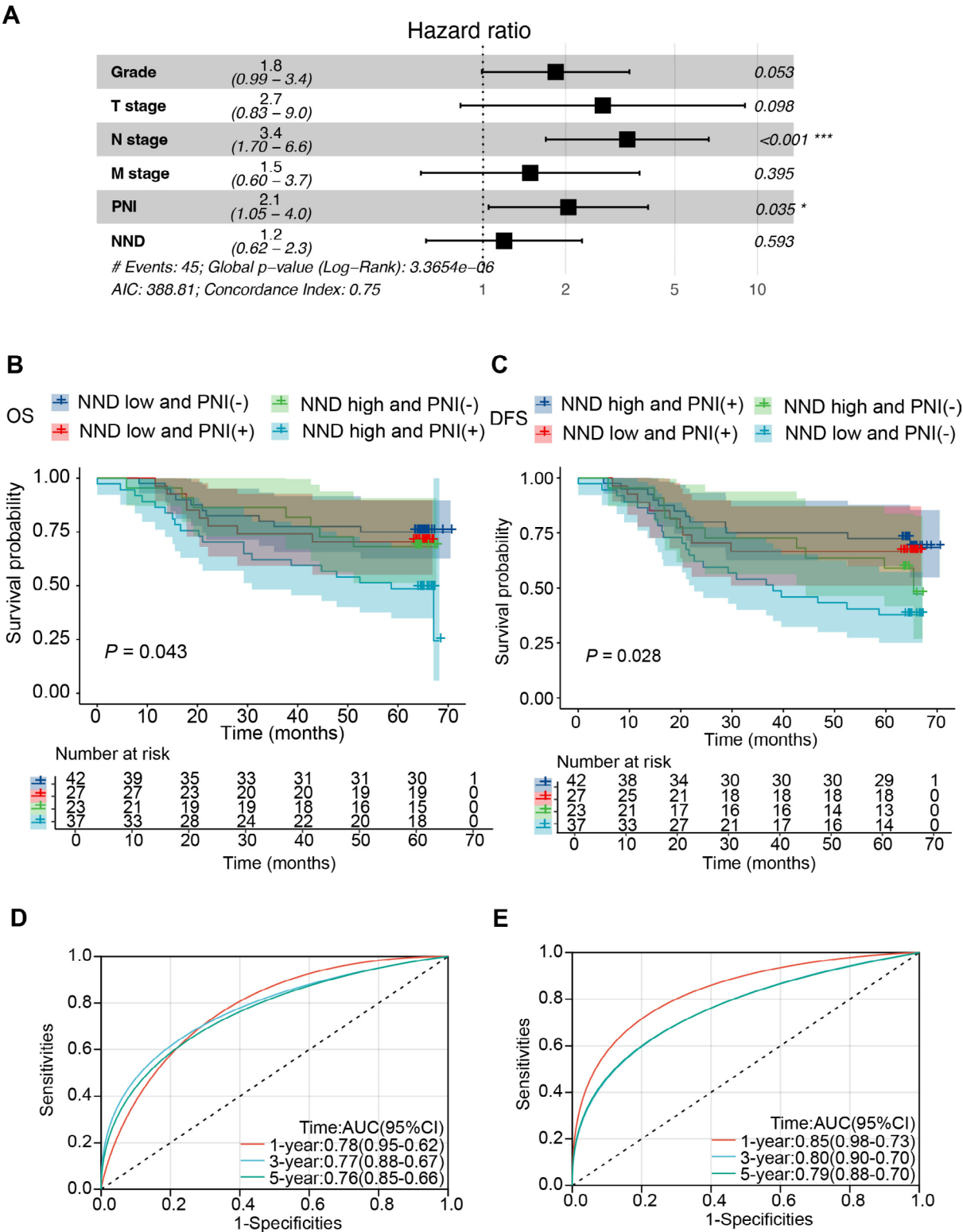


Fig. 3 Combined analysis of PNI and NND on survival in CRC Patients. **(A)** Multivariate Cox regression analysis to identify prognostic factors affecting OS in CRC patients. **(B-C)** Kaplan-Meier survival analysis assessing the combined effect of NND and PNI on OS and DFS in CRC patients. **(D)** ROC curves to predict survival outcomes based on clinicopathological features (histological grade, T stage, N stage, M stage). **(E)** ROC curve combining clinicopathological features, PNI, and NND, demonstrating improved predictive accuracy in survival outcomes. Abbreviations: PNI, perineural invasion; NND, normalized nerve density; OS, overall survival; DFS, disease-free survival; ROC, receiver operating characteristic; AUC, area under the curve

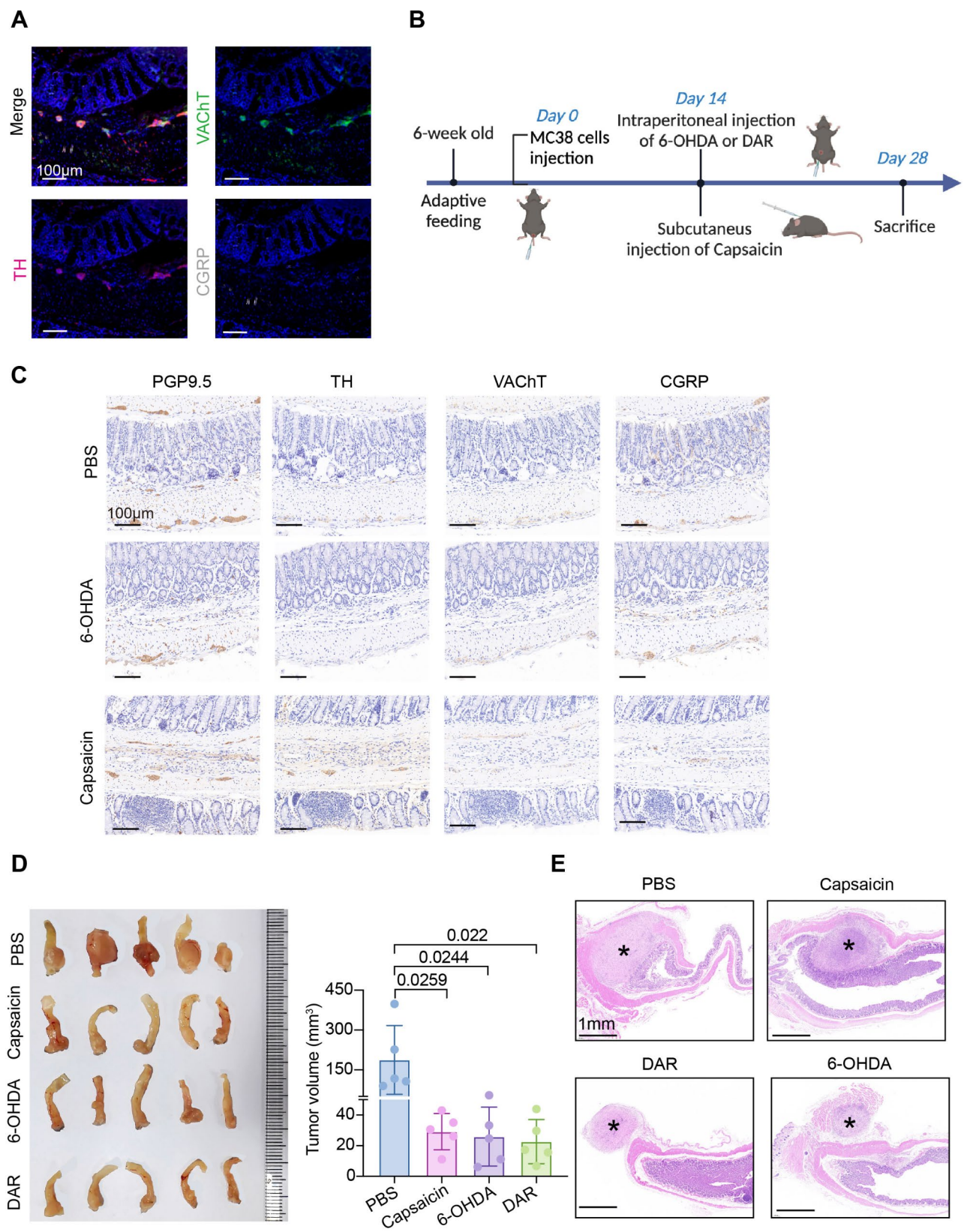


Fig. 4 (See legend on next page.)

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Fig. 4 Effects of denervation on tumor growth in mice. **(A)** Immunofluorescence staining of mouse intestinal tissue: TH (red) labels sympathetic nerves, VACHT (green) labels parasympathetic nerves, and CGRP (gray) labels sensory neurons. Scale bar: 100 μ m. **(B)** Experimental procedure diagram: Mice were injected with MC38 cells to establish rectal orthotopic transplantation tumors, followed by chemical denervation using 6-OHDA, DAR, or capsaicin. **(C)** Immunohistochemistry staining of TH (sympathetic nerves) and CGRP (sensory nerves) shows the reduction in nerve density after chemical denervation. Scale bar: 100 μ m. **(D)** Tumor growth following chemical denervation, showing the effect of neural disruption on tumor volume. **(E)** H&E staining showing tumor size post-denervation. Scale bar: 1 mm. Asterisk (*) indicates tumor area. Abbreviations: TH, tyrosine hydroxylase; VACHT, choline acetyltransferase; DAR, darifenacin; CGRP, calcitonin gene-related peptide; H&E, hematoxylin and eosin

IHC with PGP9.5 to label nerve fibers and calculate nerve density per unit area [29, 37, 44–46]. However, PGP9.5 staining can sometimes result in non-specifically staining of tumor parenchyma, which may lead to inaccuracies in nerve quantification [47, 48]. In this study, we employed NF-L to specifically mark neurons and S100B to label Schwann cells surrounding axons, ensuring accuracy in nerve data collection.

We observed that the IND was lower than the END in CRC tissues. Previous studies have suggested that in CRC, tumor invasion leads to the atrophy of the myenteric and submucosal plexuses of the enteric nervous system (ENS) near the tumor, characterized by a loss of neurons and nerve fibers within these plexuses [49, 50]. This disruption of the ENS can result in the loss of innervation and significant disruption in gut functions, contributing to symptoms such as constipation, diarrhea, and pain [49]. Although the precise mechanisms underlying nerve loss are unclear, they may involve tumor-secreted neuropeptides or local inflammatory processes [50]. A study by Hiraoka et al. in PDAC demonstrated a similar trend, where intrapancreatic nerve size and density declined towards the tumor center, likely due to cancer invasion [32]. Similarly, in CRC samples, nerves in the tumor core were disrupted and replaced by desmoplastic stroma, indicating nerve fiber loss resulting from tumor invasion.

Clinical studies have primarily focused on intratumoral nerves. Despite varying definitions of IND in existing research, high IND has been associated with poor survival outcomes across various cancers. For example, in CRC, neurogenesis characterized by higher nerve density and larger nerve diameters has been associated with decreased cancer-specific OS and DFS [29]. In thyroid cancer, high IND is independently associated with extracapsular extension, a poor prognostic factor [44]. Similarly, in prostate cancer, high IND correlates with extracapsular extension and cancer recurrence [51, 52]. In head and neck cancer, high IND is associated with P53 gene mutation and worse survival outcomes [45]. These findings highlight the potential of IND as a prognostic marker across various tumor types. However, nerve density can vary significantly between different cancers, necessitating tumor-specific analyses to validate its role as a prognostic marker.

To further validate the role of nerves in CRC progression, we used an *in vivo* mouse model. Since it is crucial

to understand which types of nerves are relevant to tumor progression, we separately disrupted sympathetic nerves using 6-OHDA, selectively inhibited M3R receptors in parasympathetic nerves with DAR, and disrupted sensory neurons using capsaicin. The results demonstrated that selective chemical denervation of sympathetic, parasympathetic, or sensory nerves significantly reduced rectal tumor growth, highlighting the crucial role of neural inputs in CRC progression. Several studies have independently highlighted the role of different nerve types in tumor progression. For instance, surgical or pharmacological denervation of the vagus nerve markedly suppresses gastric tumorigenesis [37]. Similarly, surgical or chemical denervation of adrenergic nerves inhibits tumor initiation in prostate cancer [33]. Chemical ablation of sensory neurons in an autochthonous PDAC model slows the initiation and progression of cancer [53]. Mechanistically, sympathetic and parasympathetic nerves influence tumors primarily through the release of neurotransmitters such as NA and Ach. Studies have shown that adrenergic signaling is upregulated in CRC, with β adrenergic receptors (ADRB) being more abundant in tumor sites and correlating with poor prognosis [54]. Adrenergic signaling promotes colon cancer cell proliferation by upregulating key factors such as cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE2), and matrix metalloproteinase-9 (MMP-9), with these effects attenuated by ADRB antagonists or COX-2 inhibitors [55, 56]. Ach and its muscarinic receptors are implicated in CRC progression. Muscarinic Ach receptors, which are predominantly expressed in the gut, can stimulate colon cancer growth by activating the EGFR/ERK signaling pathway, with M3R significantly overexpressed in CRC lesions [57–60]. The role of sensory neurons in CRC is less well understood but has been explored in other cancer types. For example, in PDAC, sensory neurons transmit inflammatory signals to the CNS, promoting neoplasia driven by oncogenic Kras mutations [53]. In melanoma, sensory neurons release neuropeptides that increase CD8⁺ T cell exhaustion, thereby inhibiting anti-tumor immunity [15]. These findings suggest that investigating the roles of different nerves in tumors more thoroughly could lead to novel approaches for tumor inhibition, including the targeted modulation of neural pathways in combination with conventional cancer therapies.

The involvement of nerves in solid malignancies, particularly in CRC, remains a complex and underexplored area of research. Among the various nerve-tumor interactions, PNI is the most extensively studied in CRC. During PNI, tumor cells infiltrate along nerve fibers, using them as conduits for migration. This process facilitates local invasion and distant metastasis, which significantly contributes to poorer patient outcomes, including increased recurrence rates, deeper tissue invasion, and higher rates of lymph node metastasis [26, 61, 62]. Our study further corroborates the association between PNI and adverse prognosis in CRC. We found that patients with PNI (+) and elevated NND exhibited the worst survival outcomes, while patients with PNI (-) and low NND had the best prognosis. Incorporating PNI and NND into survival models improves their predictive accuracy for patient outcomes. Currently, CRC pathological reporting primarily includes the presence or absence of PNI but lacks defined criteria or severity indicators [63]. Our study utilized immunofluorescence-based techniques to assess PNI, resulting in a higher detection rate compared to previous reports [39–41]. This suggests that relying solely on H&E staining may underestimate the prevalence of PNI in CRC. The application of immunohistochemistry (IHC) or immunofluorescence to label nerve-specific markers improves PNI detection rates, and the quantitative analysis of neural parameters could provide more precise prognostic insights [64].

Our study underscores the potential clinical significance of PNI and NND in CRC. Incorporating these factors into tumor staging systems could significantly improve risk stratification, enabling clinicians to identify high-risk patients who may benefit from more intensive treatments or closer monitoring. For instance, patients with significant PNI or elevated NND might be considered for adjuvant therapies, even in cases where traditional staging systems like TNM do not suggest such interventions. This integration would allow for more precise patient classification, considering both the tumor's biological features and its neural interactions, ultimately offering opportunities for more personalized treatment plans. However, further multi-center, longitudinal studies are necessary to refine the criteria for incorporating PNI and NND into clinical practice. These studies would help standardize pathological definitions of PNI and determine the specific cut-off values for high NND, ensuring the clinical applicability of these neural factors.

Based on the current findings, several future research directions are proposed. First, it is critical to delineate the specific roles of sympathetic, parasympathetic, and sensory nerves in CRC progression, as their differential contributions may uniquely influence tumor behavior. Understanding the distinct and collective impacts of these nerve types could provide new insights into how

they drive tumor growth and metastasis. Secondly, the development of nerve receptor-targeted therapeutics represents a promising strategy, offering precise modulation of nerve-tumor interactions. These therapies could enhance efficacy while minimizing off-target effects by selectively targeting neural pathways involved in CRC progression. Thirdly, mechanistic studies are essential to elucidate how nerve inhibition alters tumor behavior, including changes in the TME, immune response, and angiogenesis. Experimental models, such as genetically modified mice with denervation and patient-derived organoid xenograft models, are particularly suitable for studying the impact of nerve innervation on tumor progression. Collaborative efforts between clinical and basic research teams are crucial to accelerate the translation of these findings into clinical practice [65]. Finally, validation in large-scale, multicenter prospective cohort studies, along with longitudinal studies, is crucial to confirm the clinical relevance and therapeutic potential of nerve-targeted therapies [66]. Such studies will provide a comprehensive evaluation of therapeutic efficacy, systemic toxicity, and long-term safety, ensuring that the benefits outweigh associated risks in clinical settings.

In conclusion, this study highlights the critical role of PNI and NND in CRC progression, with PNI identified as an independent prognostic factor. Targeting neural components offers a promising therapeutic strategy for CRC, warranting further exploration in preclinical and clinical settings.

Abbreviations

Ach	Acetylcholine
ADRB	β adrenergic receptors
AUC	Area under the curve
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CRC	Colorectal cancer
DAR	Darifenacin
END	Extratumoral nerve density
ENS	Enteric nervous system
ENT	Extratumoral nerve transverse diameter
IHC	Immunohistochemistry
IND	Intratumoral nerve density
INT	Intratumoral nerve transverse diameter
M3R	Muscarinic cholinergic receptor 3
MND	Maximum intratumoral nerve diameter
NA	Noradrenaline
NF-L	Neurofilament-light
NND	Normalized nerve density
NNT	Normalized nerve transverse diameter
6-OHDA	6-hydroxydopamine
OSCC	Cavity squamous cell carcinoma
PDAC	Pancreatic ductal adenocarcinoma
PGP	Protein gene product
PNI	Perineural invasion
ROC	Receiver operating characteristic
TH	Tyrosine hydroxylase
TME	Tumor microenvironment
VACht	Choline acetyltransferase

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

Hao Wang, Ruixue Huo and Kexin He: Investigation, Writing-original draft; Weihai Li: Software, Visualization; Yuan Gao: Data curation; Wei He: Data curation; Junli Xue, Shu-Heng Jiang and Minhao Yu: Project administration, Writing-reviewing and editing.

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Data availability

Not applicable.

Declarations

Ethics approval and consent to participate

Human Studies: This study was approved by the Ethics Committee of Ren Ji Hospital, affiliated with Shanghai Jiao Tong University (No. KY2021-120-B). Informed consent was obtained from all patients. All procedures were performed in compliance with relevant laws and institutional guidelines and were approved by the Ethics Committee of Ren Ji Hospital. **Animal Studies:** The animal experiments performed in this study were approved by the Ethics Committee of Ren Ji Hospital, affiliated with Shanghai Jiao Tong University (No. RA-2021-387). All animal experiments complied with the ARRIVE guidelines and were conducted in accordance with the U.K. Animals Act, 1986, and associated guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no potential conflicts of interest.

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