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Original Article

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Endoscopic ultrasound-guided injection of carbon nanoparticles suspension to label rectal cancer before neoadjuvant chemoradiotherapy: a retrospective cohort study

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Abstract

Background: Localization of the primary tumor and ensuring safe distal surgical margins (DSMs) following neoadjuvant chemoradiotherapy (nCRT) are challenging in locally advanced rectal cancers (LARCs). This study investigated the effectiveness of carbon nanoparticles suspension (CNS) for labeling the primary tumor and allowing precise tumor resection after nCRT.

Methods: Clinicopathological data of LARC patients who underwent nCRT followed by laparoscopic radical anal preservation surgery at our center between January 2018 and February 2023 were prospectively collected. The patients were divided into the CNS tattooed (CNS) and non-tattooed (control) groups. In the CNS group, CNS was injected in four quadrants on the anal side 1 cm away from the lower tumor margin. DSMs were determined through intraoperative distal rectal examination in the control group and observation of CNS tattoos in the CNS group. DSM lengths and positive DSM rates were compared between the two groups to analyse the feasibility and effectiveness of CNS for labeling LARCs before nCRT.

Results: There was no statistically significant difference in the basic demographic data, effectiveness of nCRT, or post-operative recovery rates between the two groups (all $P > 0.05$). In the CNS group, CNS tattoos were observed on the outside of the rectal wall, with an overall efficiency of 87.1% (27/31). The CNS group had fewer positive DSMs and safer DSM lengths (2.73 ± 0.88 vs 2.12 ± 1.15 cm, $P = 0.012$) than the control group ($P < 0.05$).

Conclusions: Endoscopic ultrasound-guided injection of CNS tattoos before nCRT could effectively label the LARCs, ensuring safe DSMs during anus-preserving surgeries (Chictr.org.cn No.: ChiCTR2300068991).

Keywords: rectal cancer; neoadjuvant chemoradiotherapy; endoscopic ultrasound; carbon nanoparticles suspension; *in situ* resection

Introduction

Neoadjuvant chemoradiotherapy (nCRT) followed by total mesorectal excision remains the standard treatment strategy for middle- and low-level locally advanced rectal cancers (LARCs) [1–3]. In previous studies, ~60% of LARC patients had tumor stage reduction after nCRT [4], with 20%–30% achieving clinical complete response (cCR) and 10%–20% achieving pathological complete response (pCR) [5, 6]. However, the pattern of tumor regression after nCRT was multifocal remission; the residual tumor may become fragmented rather than shrinking in size [7]. This makes intraoperative localization of the primary tumor and ensuring safe distal surgical margins (DSMs) challenging. The ASCRS Manual of Colon and Rectal Surgery proposed that the DSMs after nCRT should follow the original tumor margin prior to nCRT [8]. On the one hand, tumor regression leads to difficulty in identifying the extent of prior tumor infiltration [9]; on the other, rectum wall fibrosis and tissue edema induced by nCRT increase rectal wall thickness [10], making it harder to intraoperatively identify the inferior margin of the mass. These effects may be more pronounced during laparoscopic surgery, as tissue

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edema and fibrosis make it difficult to identify tissue planes, particularly during pelvic dissection, increasing the risk of positive DSMs [11, 12]. This risk can be reduced by labeling the lower margin of the primary tumor prior to nCRT [12, 13].

A novel carbon nanoparticles suspension (CNS) was used as a stain for labeling in our study. Upon injection into the rectal mucosa, the stain could be observed as a distinct black tattoo on the rectal mesenteric fascia. CNS is metabolized slowly and remains visible for long durations. Studies have shown that clear CNS traces can still be seen *in vivo* after 6–12 months [14]. Additionally, there have been no reports of side effects or serious complications with CNS tattooing [15].

Endoscopic ultrasonography (EUS) combines electronic endoscopic technology and ultrasound detection technology to accurately assess submucosal invasion of rectal cancers. EUS is a significant improvement over traditional electronic colonoscopy, which can only be used to observe tumor morphology and boundaries at the mucosal surface [16, 17], and does not facilitate needle penetration into submucosal tumors for labeling.

The present study employed EUS to detect the greatest intramural invasion of tumors and to inject CNS for labeling the lower edge of the tumor before nCRT. The study aimed to investigate the effectiveness of CNS tattooing for long-term labeling of the initial tumor position, thus allowing accurate rectal cancer resections and safe DSMs after nCRT.

Patients and methods

Patient information

This was a retrospective cohort trial (Chictr.org.cn No.: ChiCTR2300068991) conducted in accordance with the Declaration of Helsinki (2013) and approved by the Medical Ethics Committee of the 900th Hospital of the Joint Logistics Support Force, Fuzhou, China (approval number: 2020–004).

We prospectively collected clinicopathological data for LARC patients treated at our center between January 2018 and February 2023. The inclusion criteria of cases were as follows: (i) age of >18 years; (ii) rectal adenocarcinoma confirmed through colonoscopic biopsy; (iii) pelvic magnetic resonance imaging (MRI) confirming that the lower tumor margin was <10 cm from the anal verge and that the tumor was cTNM stage II–III rectal cancer (AJCC 8th edition) [18]; and (iv) successful radical laparoscopic anus-preserving surgery for rectal cancer after nCRT. The exclusion criteria were as follows: (i) other concurrent tumors or distant metastases; (ii) lack of nCRT, disease progression after nCRT, or neoadjuvant chemotherapy alone; (iii) refusal of surgical treatment or treatment involving abdominal perineal resections (APRs), local excision, or the "wait-and-watch" strategy; (iv) emergency surgery for bleeding, perforation, or obstruction; and (v) conversion to open surgery. A total of 86 patients who met the inclusion criteria were divided into two groups: CNS tattooed group (the CNS group; $n = 31$) and non-tattooed group (the control group; $n = 55$) (Figure 1). Clinical data, including sex, age, body mass index (BMI), clinicopathological stage, distance from anal verge, tumor diameter, tumor differentiation, and nCRT regimens, were analysed for the two groups.

CNS labeling

The reagents were CNS (Canaline; 1 mL: 50 mg, Chongqing Lemay Pharmaceutical Joint-Stock Limited Company, China) and sodium hyaluronate (2.5 mL: 25 mg). The equipment included an endoscopic injection needle (nm-200U-0423, Olympus Corp., Japan) and endoscopic ultrasound (EG-3270UK, frequency: 7.5–12 MHz, Pentax Co., Japan).

CNS group patients were placed in the lateral position and examined through the anal route. EUS was used to locate the submucosa and determine the inferior tumor margin (Figure 2A–C). Four points were selected on the anal side at a distance of 1 cm from the lower margin of the tumor. A 1-mL mixture of sodium hyaluronate and saline water (ratio 1:3) was injected using the endoscopic injection needle into each of these four points to elevate the mucosa. This was followed by labeling with 0.1 mL of CNS (Figure 2D–G). Controls were examined through ordinary colonoscopy without any positional staining marks.

nCRT

All patients received preoperative nCRT. Radiotherapy involved a conventional split (long-term) regimen for a total of 5–6 weeks (3D conformal radiotherapy, 45–50.4 Gy [1.8–2.0 Gy per session, 25–28 fractions]), targeting high-risk areas for recurrence and regional lymphatic drainage areas. Radiotherapy was followed by two to five cycles of chemotherapy using a capecitabine single agent, either CAPEOX or mFOLFOX6, before the surgery. Radical laparoscopic surgery was performed 6–8 weeks after completion of nCRT. Repeat colonoscopy prior to surgery showed significant tumor shrinkage, with the CNS tattoo remnants clearly visible (Figure 3A–C).

Surgery and intraoperative measurements

Laparoscopic radical anal preservation surgeries were performed in all patients. Surgeons with >10 years of experience (>40 cases per year) performed the surgeries.

For the CNS group, after the patient was anesthetized, the operation was performed using the conventional five-hole method. The peritoneal cavity was laparoscopically explored to confirm the absence of liver and peritoneal metastases. CNS tattoos were used to determine the location of the primary tumor and the extent of surgical excision (Figure 4A). The lack of observable CNS tattoos indicated that the lower tumor edge was located below the peritoneal reflection. An ultrasound scalpel was used to separate the tissues along rectal propria fascia until the CNS tattoos were observed. The DSM was placed ≥1 cm from the center of CNS tattoos for mid-level rectal cancers. For low-level rectal cancers, the rectum was dissected at the lower edge of CNS tattoos (Figure 4B). Then, the peri-intestinal lymph nodes were thoroughly dissected (Figure 4C). The digestive tract was reconstructed using the double anastomosis technique and the anastomotic tissue was sent for cryopathology. In case of narrow pelvis or hypertrophied rectal mesentery, the dissection and anastomosis were difficult to complete transabdominally, and the DSM position was determined through transanal observation of CNS tattoo remnants on the rectal mucosal surface. Surgical specimens were fully expanded and DSMs were measured immediately after dissection (Figure 4D–F). The abdominal cavity was closed once a negative cryopathology result was obtained. In cases with positive DSMs, rectal tissue excision was carried a further 1 cm downward and the specimen was sent again for cryopathology testing until negative margins were achieved.

For the control group, the location of the primary tumor was determined by using intraoperative distal rectal examination. The remaining surgery was identical to that for the CNS group.

The effectiveness of CNS tattooing was assessed by comparing the operation times, intraoperative bleeding, intestine lengths, positive circumferential resection margin (CRM) rates, DSM lengths, positive DSM rates, and lymph node acquisition between the two groups.

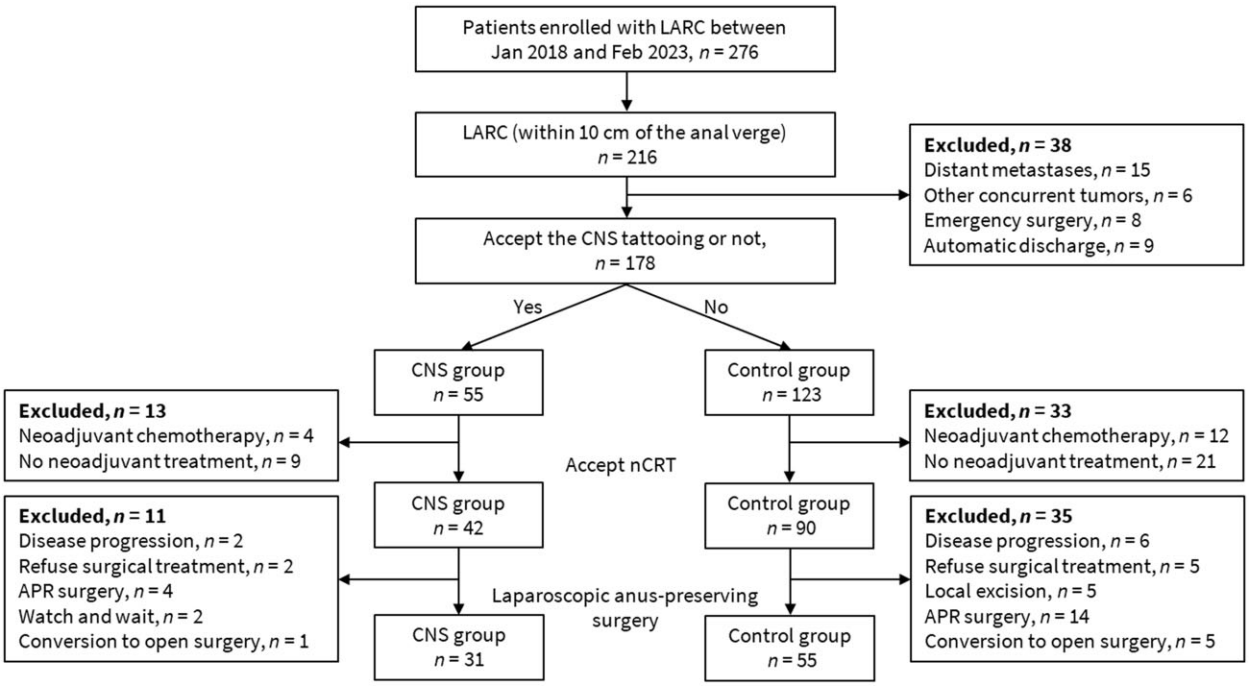

Figure 1. Flow diagram of enrollment. LARC = locally advanced rectal cancer, CNS = carbon nanoparticles suspension, nCRT = neoadjuvant chemoradiotherapy, APR = abdominal perineal resection.

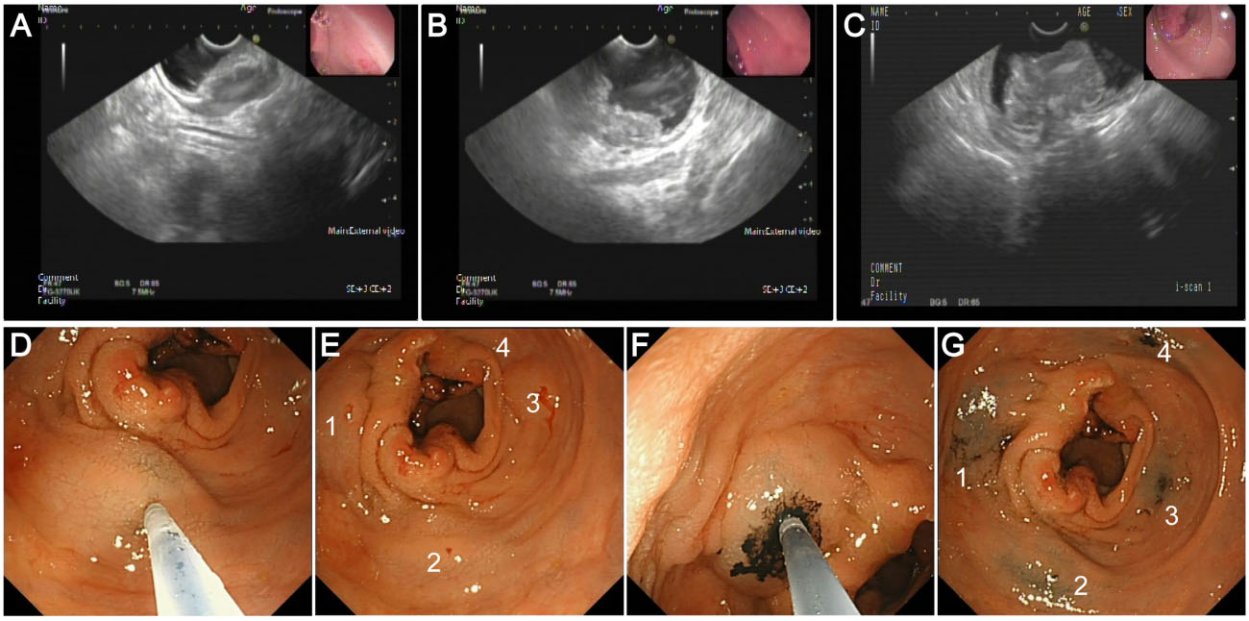

Figure 2. Endoscopic ultrasound-guided CNS tattooing process before nCRT. (A)–(C) Detection boundary of the submucosal layer of the tumor by using endoscopic ultrasound. (D)–(G) A successful labeling process of CNS. (D) 1 mL of mixture of sodium hyaluronate and saline water (ratio 1:3) was injected using an endoscopic injection needle to elevate the mucosa. (E) Four water cushions of sodium hyaluronate around the tumor. (F) About 0.1 mL of CNS was injected into the submucosa. (G) CNS tattooing was completed in four quadrants at the lower margin of the tumor.

Post-operative findings

The post-operative exhaust time, length of hospitalization, and post-operative complications were compared between the two groups to evaluate the safety of CNS labeling.

Statistical analysis

Statistical analysis was performed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA). Normally distributed measurement data are expressed as mean ± standard deviation and were compared using the independent samples *t*-test or t'-test. Non-normally distributed measures are expressed as median (range) and were compared using the Mann–Whitney *U* test. Count data are expressed as numbers and percentages, and were compared using the chi-square (χ^2) test and, if necessary, Fisher's exact probability test. The test level was $\alpha = 0.05$ and $P < 0.05$ was considered statistically significant.

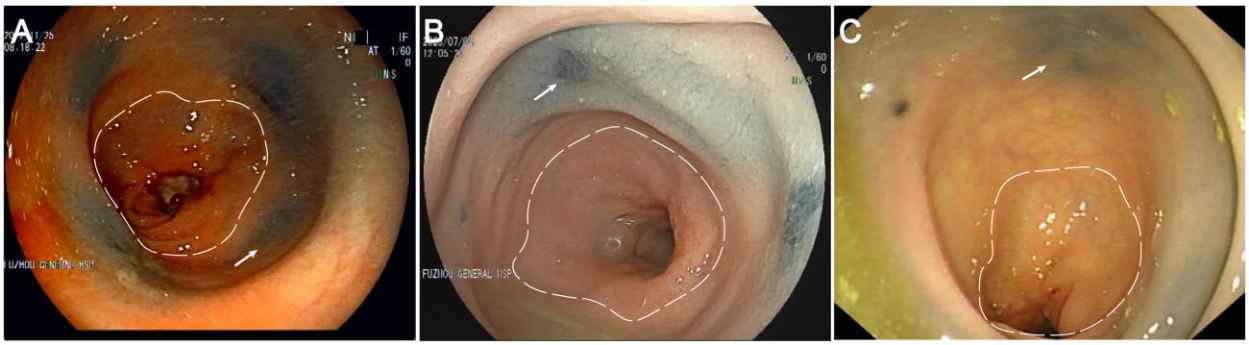

Figure 3. Colonoscopy images after nCRT. (A)–(C) Significant tumor reduction after nCRT. CNS tattoo remnants (white arrows) are clearly visible on the lower edge of the tumors. White dashed boxes indicate the extent of the original tumor.

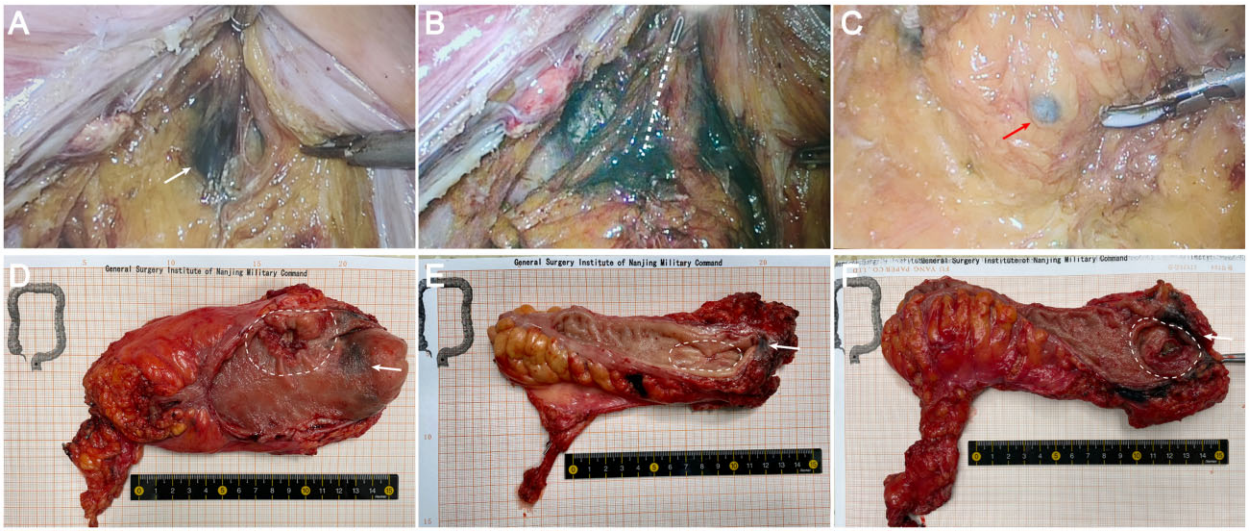

Figure 4. Intraoperative anatomy and post-operative rectal specimens. (A)–(C) Intraoperative dissection of a case of low rectal cancer. (A) Tattoo (white arrow) is visible when dissecting along the propria fascia of the rectum to beneath the peritoneal reflection. (B) Continues to separate 1–2 cm downward along the rectum from the center of the tattoo, followed by intestinal canal naked. (C) Black-stained lymph nodes (red arrow) in the mesentery of the posterior wall of the rectum. (D)–(F) Post-operative specimens of rectal cancer. Tattoos (white arrows) are clearly visible on the inner and outer walls of the rectum, marking the lower edge of the tumor effectively. White dashed boxes indicate the primary tumor area. (D) Rectal cancer 7 cm from the anal verge. (E) Rectal cancer 5 cm from the anal verge. (F) Rectal cancer 3 cm from the anal verge.

Results

A total of 86 patients met the inclusion criteria and were included in the analysis. The participants were divided into two groups: the CNS group ($n = 31$) and the control group ($n = 55$). There were no significant differences in sex, age, BMI, hypertension, diabetes mellitus, or serum tumor marker levels between the two groups (all $P > 0.05$). The two groups were also comparable in terms of tumor differentiation, clinical stage, tumor diameter, distance from anal verge, and chemoradiotherapy regimens (all $P > 0.05$). These results indicate that the two groups had similar baseline conditions (Table 1).

Response to nCRT

We adopted a tumor regression grade (TRG) system (AJCC 8th edition) to assess tumor response to nCRT [18]. Among the 86 patients, 26 (30.23%) achieved cCR after nCRT, including 10 cases in the CNS group and 16 in the control group. Preoperative distal rectal examination was negative and electronic colonoscopy showed significant reduction in the tumor size. Only a white, flat mucosal scar was visible [19] (Figure 3A). MRI showed no obvious tumor or regional lymph node enlargement. Post-operative pathology showed significant tumor regression (TRG score: 0–1) in 37 (43.02%) patients, of whom 16 (18.60%) achieved pCR (TRG score: 0), including 7 cases in the CNS group and 9 cases in the control group. Additionally, 21 patients (24.42%) were close to pCR (TRG score: 1), including 8 cases in the CNS group and 13 in the control group. Eighteen patients (20.93%) showed partial remission (TRG score: 2) after nCRT, with a decrease in tumor size, including 6 patients in the CNS group and 12 in the control group. Furthermore, 31 patients (36.05%) had poor or no tumor regression (TRG score: 3), including 10 cases in the CNS group and 21 cases in the control group. There was no significant difference in nCRT effectiveness between the two groups ($P > 0.05$) (Table 2).

Operative and post-operative specimen management

All patients underwent laparoscopic radical anus-preservation surgery after nCRT. In the CNS group, black-stained tattoos were observed on the outside of the rectal wall in 29 cases, including

Table 1. Basic characteristics of the two groups of patients with rectal cancer

Variable	CNS group ($n = 31$)	Control group ($n = 55$)	P
Median age (range), years	62 (40–82)	62 (33–77)	0.380[d]
Male gender, *n* (%)	18 (58.06)	31 (56.36)	0.878[c]
BMI, kg/m^2 (mean ± SD)	22.17 ± 1.97	21.71 ± 3.13	0.465[b]
Hypertension, *n* (%)	5 (16.13)	11 (20.00)	0.658[c]
Diabetes, *n* (%)	3 (9.68)	8 (14.55)	0.754[c]
Serum CEA (≥5 ng/mL), *n* (%)	12 (38.71)	17 (30.91)	0.463[c]
Serum CA19-9 (≥39 U/mL), *n* (%)	4 (12.90)	6 (10.90)	1.000[c]
Tumor location, *n* (%)			0.393[c]
Med-rectum	18 (58.06)	37 (67.37)	
Low-rectum	13 (41.94)	18 (32.73)	
Distance to the anal verge, cm (median, range)	6.00 (3.60–9.80)	6.70 (3.40–10.00)	0.260[d]
Tumor diameter, cm (median, range)	4.30 (2.20–7.50)	4.50 (2.20–8.80)	0.272[d]
Broder classification, *n* (%)[a]			0.911[c]
Poorly differentiated	3 (9.68)	8 (14.55)	
Moderately differentiated	24 (77.42)	41 (74.55)	
Well differentiated	4 (12.90)	6 (10.91)	
Tumor stage (cTNM), *n* (%)			0.309[c]
II	9 (29.03)	21 (38.18)	
III	22 (70.97)	34 (61.82)	
Receive radiotherapy, *n* (%)	31 (100)	55 (100)	1.000
Chemotherapy regimen, *n* (%)			0.694[c]
Capecitabine	18 (58.06)	37 (67.27)	
CAPEOX	8 (25.81)	11 (20.00)	
mFOLFOX6	5 (16.13)	7 (12.73)	

CNS = carbon nanoparticles suspension, SD = standard deviation, BMI = body mass index, CEA = carcinoembryonic antigen, CA19–9 = glycoantigen 19-9, nCRT = neoadjuvant chemoradiotherapy, cTNM = clinical staging of tumor prior to CRT, CAPEOX = combination of capecitabine and oxaliplatin, mFOLFOX6 = combination of 5-fluorouracil, leucovorin, and oxaliplatin. Mid-rectum cancer was defined as tumors with the lower edge 5–10 cm from the anal verge and low-rectum cancer with the lower edge less than 5 cm from the anal verge.

[a] Mucinous adenocarcinoma was counted as poorly differentiated adenocarcinoma; high-grade intraepithelial neoplasia and high-grade heterogeneous hyperplasia were counted as well-differentiated adenocarcinoma. *P*-values were determined by using [b]independent samples *t*-test or *t*′-test, [c]χ^2 test, or [d]Mann–Whitney *U* test.

Table 2. Comparison of the effectiveness of neoadjuvant chemoradiotherapy between two groups of patients with rectal cancer

Variable	CNS group ($n = 31$)	Control group ($n = 55$)	P
cCR, *n* (%)	12 (32.26)	16 (29.09)	0.759
TRG score, *n* (%)			0.873
0 (pCR)	7 (22.58)	9 (16.36)	
1	8 (25.81)	13 (23.64)	
2	6 (19.35)	12 (21.81)	
3	10 (32.26)	21 (38.18)	
Effective rate, *n* (%)	21 (67.74)	34 (61.82)	0.583

CNS = carbon nanoparticles suspension, cCR = complete clinical remission, TRG = tumor regression grade, pCR = pathological complete remission. *P*-values were determined by using the χ^2 test.

2 cases that showed carbon nanoparticle dispersion leading to ineffective labeling, with an overall efficiency of 27/31 (87.1%). There was no statistical difference between the two groups in terms of operation time (250 [180–420] vs 260 [180–440] min, $P = 0.136$), intraoperative bleeding (125 [50–450] vs 120 [50–500] mL, $P = 0.608$), ileostomy rate, positive CRM rate, and resected intestinal length (16.87 ± 3.65 vs 17.73 ± 4.45 cm, $P = 0.361$). However, the DSMs (mid-rectum, 3.31 ± 0.62 vs 2.51 ± 1.12 cm, $P = 0.006$; low-rectum, 1.92 ± 0.42 vs 1.32 ± 0.73 cm, $P = 0.012$) and the mean number of lymph nodes obtained (23 [16–41] vs 20 [12–34], $P = 0.001$) were greater in the CNS group compared with the control group ($P < 0.05$) (Table 3). In addition, five adverse events occurred in the control group and the DSMs were found to be positive on intraoperative cryopathology, requiring further surgeries. The positive DSM rate in the control group was 5/55 (9.09%). However, the difference was not statistically significant, probably due to the small sample size.

Post-operative recovery and complications

As shown in Table 4, there were no significant differences between the two groups in terms of post-operative exhaust times, peritoneal drainage tube removal times, or post-operative hospitalization times (all $P > 0.05$). Common post-operative complications observed in this study included infections, anastomotic leakage, and intestinal obstructions. There were nine cases of anastomotic leakage: three in the CNS group and six in the control group. There were five cases of intraperitoneal infection: two in the CNS group and three in the control group, with all cases exhibiting anastomotic leakage. There were no cases of intestinal obstruction in the CNS group, but two cases in the control group. In addition, there were five cases of lung infections: two in the CNS group and three in the control group; there were two cases of poor surgical wound healing, one in each group. All these patients were treated conservatively and the complications resolved. The total incidence of complications was 19.35% (6/31) in the CNS group and 21.82% (12/55) in the control group.

Discussion

Sphincter-preserving operations and nCRT have allowed an increasing number of LARC patients to retain the anus. The application of nCRT can reduce the local tumor recurrence rates and prolong the survival rates [20], benefiting a greater number of LARC patients. In recent years, total neoadjuvant therapy has shown additional benefits, including early prevention or eradication of distant tumor micrometastasis, higher pCR rates, longer progression-free survival times, and higher chemotherapy tolerance rates [21, 22]. National Comprehensive Cancer Network (NCCN) guidelines recommend total neoadjuvant therapy as the preferred preoperative treatment for LARC [1]. Advances in neoadjuvant therapy have allowed the shrinkage or even complete

Table 3. Statistics of surgical correlation indicators in two groups of patients with rectal cancer

Variable	CNS group ($n = 31$)	Control group ($n = 55$)	*P*
Operation time, min (median, range)	250 (180–420)	260 (180–440)	0.136[c]
Intraoperative bleeding (median, range)	125 (50–450)	120 (50–500)	0.608[c]
Ileostomy, *n* (%)	27 (87.10)	43 (78.18)	0.464[b]
Excision lengths of intestine, cm (mean ± SD)	16.87 ± 3.65	17.73 ± 4.45	0.361[a]
LN, *n* (median, range)	23 (16–41)	20 (12–34)	**0.001**[c]
CRMs positive, *n* (%)	1 (3.23)	1 (1.82)	1.000[d]
DSMs, cm (mean ± SD)	2.73 ± 0.88	2.12 ± 1.15	**0.012**[a]
Med-rectum	3.31 ± 0.62	2.51 ± 1.12	**0.006**[a]
Low-rectum	1.92 ± 0.42	1.32 ± 0.73	**0.012**[a]
DSMs positive, n (%)	0 (0)	5 (9.09)	0.154[d]

CRMs = circumferential resection margin, SD = standard deviation, DSMs = distal surgical margins, LN = lymph nodes acquisition. *P*-values were determined by using [a]independent samples *t*-test or *t*'-test, [b]χ^2 test, [c]Mann–Whitney *U* test, or [d]Fisher's exact probability test.

Table 4. Post-operative recovery and complications in two groups of patients with rectal cancer

Variable	CNS group ($n = 31$)	Control group ($n = 55$)	*P*
Exhaust time, days (median, range)	2.0 (1.0–4.0)	2.0 (1.0–6.0)	0.945[b]
Peritoneal drainage tube removal, days (median, range)	7.0 (6.0–38.0)	7.0 (6.0–48.0)	0.468[b]
Post-operative hospitalization time, days (median, range)	9.0 (8.0–45.0)	9.0 (8.0–52.0)	0.194[b]
Post-operative complication, *n* (%)	6 (19.35)	12 (21.82)	0.787[a]
Anastomotic leakage	3 (9.68)	6 (10.91)	
Intestinal obstruction	0 (0.00)	2 (3.64)	
Intraperitoneal infection	2 (6.45)	4 (7.27)	
Lung infection	2 (6.45)	3 (5.45)	
Surgical incision infection	1 (3.23)	1 (1.82)	

The removal of the abdominal drainage tube was performed on the sixth day after surgery in all patients without complications. All abdominal infections were in patients with anastomotic leakage. *P*-values were determined by using [a]χ^2 test or [b]Mann–Whitney *U* test.

resolution of primary tumors, making it more difficult to locate the primary tumor intraoperatively. During laparoscopic surgery, the surgeon can only observe the appearance of a part of the rectum and cannot directly observe the tumor location in the rectal cavity. Following tumor shrinkage, it may be impossible to accurately determine the original inferior tumor margin based on intraoperative digital rectal examinations. The tumor may even be confused with an endorectal polyp. Tumor localization based on preoperative colonoscopy and MRIs also showed differences from that observed intraoperatively during tissue dissection. Therefore, it is crucial to label the inferior margin of the primary tumor before nCRT is administered.

Previous studies have reported several methods for preoperative tumor labeling. Commonly used stains include methylene blue, indigo carmine, toluidine blue, lymphatic violet, and hematoxylin and eosin. However, these stains have short durations of staining (<2 days) [15, 23]. Indocyanine green has a short staining time and requires fluorescent imaging equipment for visualization [24]. Although India ink tattooing lasts longer, it may cause local ulcers, inflammation, and even severe peritonitis if it exudes outside the plasma membrane layer [15, 25]. The present study used CNS as a novel tattooing agent that was metabolized slowly after injection into the local intestinal submucosa and sustained for a long period of time (6–12 months) [15], which was enough to complete two to five cycles of nCRT or six to eight cycles of total neoadjuvant therapy before surgery. In our study, the original tattoos could be observed both inside and outside the rectum after nCRT. In addition, although the tumor volume reduced significantly, there was no obvious diffusion of CNS tattoos, allowing the surgeon to accurately determine the primary tumor location and ensure safe DSMs.

CNS tattooing is commonly practiced in gastrointestinal surgery [26], thyroid surgery [27], and breast surgery [28]. It is composed of carbon nanoparticles with an average diameter of ~150 nm, which is larger than the capillary endothelial cell gap (30–50 nm) but smaller than the capillary lymphatic endothelial cell gap (120–500 nm). Therefore, it drains through the lymphatic vessels after local injection, leading to significant lymphatic tropism [25, 29]. Detection rates for perirectal lymph nodes decreased after nCRT [30]. Carbon nanoparticles could partly trace the perirectal lymph nodes, particularly those at the border of the rectal mesenteric fascia, facilitating lymph node dissection and sorting and allowing accurate post-operative pathological staging. Previous studies have shown that CNS tattooing was superior to indocyanine green labeling in lymph node dissection [29]. In addition, carbon nanoparticles do not enter the blood stream, are virtually non-toxic to the human body, and are associated with a lower incidence of allergic reactions. Transient leukocytosis occurred in a few of our patients; no other adverse effects occurred. Therefore, we believe that CNS tattooing is safe and effective for LARC patients who require prolonged primary tumor labeling.

Recent literature suggests a decrease in the safe DSMs for LARC from the original 5 cm to 2 cm or even 1 cm [31–33]. However, international guidelines have not yet specified the safe DSM length after nCRT. Some studies have shown that the distal intramural spread length of rectal cancer after nCRT was <1 cm. Therefore, it is recommended that the DSMs should be >1 cm [34, 35]. It has also been reported that fragmentation of residual tumor cells after nCRT for rectal cancer occurred in ~80% of the patients, resulting in asynchrony between gross and microscopic tumor regression [36]. Fragmented tumor cells may persist in all directions within 3 cm of the edge of the remnant tumor scar after nCRT and are strongly associated with positive margins and a poor prognosis [37]. In addition, MRI was affected by intestinal fibrosis and tumor mucoidosis after nCRT, which decreased the accuracy of rectal cancer restaging and determination of the extent of residual live cancer cells involvement [38]. The 3rd edition

of the ASCRS Manual of Colon and Rectal Surgery guidelines recommends that, based on initial rectal cancer staging prior to nCRT, any area involving the tumor should be resected to avoid the possibility of leaving live cancer cells *in situ* [8]. In this study, three of five positive DSM events in the control group were due to incorrect judgment of the location of the shrinking tumor, which resulted in resection line involvement in the primary tumor, and the other two were due to poor tumor regression. In the CNS group, we labeled the lower rectal cancer margins with CNS before nCRT. Based on its slow metabolism and reduced dispersion, the labeling points persisted regardless of tumor shrinkage, allowing accurate determination of the lower margin of the primary tumor area. This guaranteed the minimum safe DSMs and conformed to the principle of *in situ* resection.

For patients who achieved cCR after nCRT, the study showed no significant differences in overall survival or disease-free survival between the patients treated with a "wait-and-watch" strategy and those treated surgically, but the former group had a higher rate of local recurrences [39]. Recent studies have shown that fluorodeoxyglucose-positron emission tomography, MRI, and computed tomography lacked accuracy for determining pCR [40–42]. The compliance rate between cCR and pCR remains unsatisfactory, with a high risk of residual tumors, including those in the rectal wall beyond the mucosa, intra-mesenteric lymph nodes, and even distant metastases [43]. In our study, only 42.86% (12/28) of patients who achieved cCR also achieved pCR. In the authors' opinion, LARC patients who achieve cCR after nCRT should be actively treated with surgeries. Labeling through EUS-guided CNS injection was routinely recommended for patients undergoing nCRT, facilitating subsequent surgical treatment and follow-up examinations.

There were some limitations in our study. First, this study was not a randomized–controlled trial and selection bias may have existed. Second, the scope of EUS needs to expanded beyond pelvic MRI and colonoscopy to allow CNS injection. Third, a small amount of dispersion was observed after local CNS injection. To prevent CNS dispersion, we preferred to inject a sodium hyaluronate–saline water mixture rather than injecting saline water before CNS injection. Sodium hyaluronate could effectively prevent the dispersion of carbon nanoparticles. The injection dose should be strictly controlled, with four points selected around the same plane of the rectum; 0.1 mL of the solution was injected into each point. CNS injection requires a highly skilled endoscopist in order to prevent spillage out of the needle hole before tissue penetration. The injection needle should be left in place for a few seconds after the injection instead of immediately withdrawing it. In future, we will continue to improve carbon nanoparticles and solvents to reduce the dispersion of carbon nanoparticles, and plan to conduct a multicenter randomized–controlled trial to evaluate the therapeutic effect of CNS tattooing in rectal cancer surgery.

Conclusions

EUS-guided CNS injections allowed effective localization of the primary rectal tumor before nCRT and persisted for a long period of time, allowing precise resection of the shrunken tumor during surgery.

Authors' Contributions

Conception and design: N.L., Y.Z.W., Y.W. Collection and assembly of data: Y.Z.W., C.W.Y., J.Y., W.H.W., Y.C.F. Data analysis and interpretation: W.W.L., J.Y., R.W. Provision of study materials or patients: Y.W., Y.Y.J., R.W. Manuscript writing and revision: Y.Z.W., N.L. All authors read and approved the final manuscript.

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Conflict of Interest

None declared.

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