



OPEN Histopathological evaluation of the efficacy of carbon nanoparticle staining in detecting lymph node metastasis in colorectal cancer surgery

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The aim of this study is to conduct a retrospective analysis on the correlation between lymph node staining and lymph node metastasis following laparoscopic radical resection of colorectal cancer using a carbon nanoparticles tracer. Additionally, the study seeks to investigate the potential clinical implications of carbon nanoparticles (CNs) in the context of colorectal cancer surgery. Clinical data was collected from patients who underwent laparoscopic radical resection of colorectal cancer at a ward of the Department of General Surgery, Nanjing Drum Tower Hospital between January 2022 and December 2023. Patients were pathologically confirmed to have primary colorectal cancer with lymph node metastasis. The patients were divided into two groups based on whether they received carbon nanoparticle suspension injection under colonoscopy prior to the operation: the carbon nanoparticles group (CNs group, $n=80$) and the control group (non-CN group, $n=39$). The histological sections of all lymph nodes were reviewed, assessing lymph node staining and tumor metastasis. There were no statistically significant differences in sex, age, body mass index, operation time, blood loss, tumor location, and postoperative pathological stage between the CNs group and the control group. However, the average number of lymph nodes differed significantly between the two groups ($P=0.001$). A total of 1626 lymph nodes were obtained from the CNs group. Among these, 207 were identified as metastatic lymph nodes, with 27 showing staining with carbon nanoparticles (1.66%) and 182 showing no staining (11.19%). Of the remaining 1417 normal lymph nodes, 787 were found to be stained with carbon nanoparticle (48.4%) while 630 were not. Out of the 80 patients in the CNs group, none exhibited staining solely in metastatic lymph nodes, 56 patients (70%) did not exhibit staining in either metastatic or normal lymph nodes, 19 patients (23.75%) exhibited staining in both metastatic and normal lymph nodes, and 5 patients (6.25%) did not exhibit staining in either type of lymph node. The utilization of carbon nanoparticle staining prior to laparoscopic colorectal cancer surgery has been shown to enhance the detection of lymph nodes, providing valuable assistance to surgeons and pathologists. However, the efficacy of carbon nanoparticle staining in identifying metastatic lymph nodes is limited, as non-metastatic lymph nodes may also be stained. Consequently, reliance solely on lymph node staining for precise dissection during surgery is not advisable. There is a critical need for the development of more accurate active targeting tracers.

Keywords Colorectal cancer, Carbon nanoparticles, Laparoscopy, Lymphadenectomy

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Colorectal cancer ranks among the most prevalent malignancies globally, as indicated by recent epidemiological findings identifying it as the third most common cancer and a leading cause of cancer-related mortality¹. Radical surgery is currently considered the most efficacious treatment approach for colorectal cancer, offering complete lesion removal and potentially life-saving outcomes². Lymph node metastasis is the primary and frequently encountered form of metastasis in colorectal cancer, contributing to tumor recurrence³. In patients lacking distant metastases, regional lymph node metastases represent a significant adverse prognostic factor⁴. Therefore, precise assessment of lymph node metastasis and thorough resection are crucial in colorectal cancer patients. Presently, there is a lack of a reliable and convenient technique for determining the extent of lymph node metastases during colorectal cancer surgery. Carbon nanoparticles (CNs) tracer, composed of nanosized carbon black particles with an average size of 150 nanometers, may offer a potential solution to this challenge. Instead of entering blood vessels, carbon nanoparticle tracers are taken up by macrophages, which exhibit a strong affinity and specificity for the lymphatic system⁵. In clinical practice, these tracers have been utilized to precisely visualize lymph nodes during colorectal cancer surgery to facilitate the retrieval of a greater number of lymph nodes⁶.

The objective of this investigation is to assess the correlation between the labeling of carbon nanoparticle tracers and the histopathological characteristics of metastatic lymph nodes. This study hypothesizes that carbon nanoparticles as a lymphatic tracer do not enhance the detection of metastatic lymph nodes but rather serve to increase the number of harvested lymph nodes, thereby aiding in more comprehensive pathological staging. While carbon nanoparticles facilitate lymph node localization, they do not selectively accumulate in metastatic lymph nodes, making them unsuitable as a direct tool for detecting lymph node metastases. Instead, their primary role is to assist in identifying more lymph nodes, which may contribute to a more accurate assessment of nodal status.

Materials and methods

Patients

We retrospectively collected data from consecutive colorectal cancer patients who underwent laparoscopic radical resection at a ward of Nanjing Drum Tower Hospital between January 2022 and December 2023. Inclusion criteria were as follows: (1) Preoperative pathology of endoscopic biopsy was primary colorectal cancer, (2) Lymph node metastases were confirmed by postoperative pathological examinations. (3) Carbon nanoparticle tracer was injected by the same group of physicians (4) All the operations were performed by the same group of surgeons. Exclusion criteria were (1) The postoperative pathological examination found no lymph node metastasis, (2) conversion to open colorectal resection, (3) clinical or pathological data was incomplete.

The patients were allocated into the carbon nanoparticles group (CNs group) and the control group (non-CN group). The allocation to each group was determined by the surgeon's clinical judgment, which considered factors such as tumor location, patient condition, and the complexity of the surgical procedure. Tumor marking was performed by carbon nanoparticles suspension under electronic colonoscopy, one day before surgery.

Lymph node analysis

During the lymphadenectomy for colorectal cancer, all pericolic lymph nodes, intermediate lymph nodes, and main lymph nodes were surgically separated and resected. All resected lymph nodes were fixed in a 10% neutral formalin solution for 24 h before being sent for pathological examination. All examined lymph nodes with a maximum diameter exceeding 2 mm were sectioned parallelly at 2 mm intervals. After undergoing dehydration, paraffin embedding, sectioning at a thickness of 3 μ m, and staining with hematoxylin and eosin (HE), the lymph nodes were examined microscopically to assess the presence of lymph node metastasis and carbon nanoparticle staining. The specific microscopic examination procedure is as follows: First, the presence of metastatic cancer in the lymph nodes was assessed in the HE-stained sections. Lymph nodes with suspected cancer metastasis or those in which the presence of cancer metastasis could not be clearly determined by HE staining were further verified by immunohistochemical staining (using pan-CK to mark metastatic cancer cells within the lymph nodes). Lymph nodes confirmed to contain cancer cells by the above pathological examination were considered metastatic lymph nodes (MLNs); otherwise, they were regarded as non-metastatic lymph nodes (non-MLNs). Second, the presence of carbon nanoparticle tracer staining was observed in all metastatic and non-metastatic lymph nodes⁷ (i.e., the presence of black carbon nanoparticles within the lymph nodes). The pathological sections of all lymph nodes were independently and blindly evaluated by two pathologists (X.Z. and Q.S.). For cases with inconsistent evaluation results, a final diagnostic consensus was reached through joint discussion under a multi-headed microscope. This procedure was designed to minimize interobserver variability and ensure the accuracy and consistency of lymph node evaluation. Finally, the relationship between the metastatic status of the lymph nodes and carbon nanoparticle tracer staining was analyzed.

Injection of carbon nanoparticles suspension

A preoperative electronic colonoscopy was performed on all patients in the carbon nanoparticles group. Carbon nanoparticle suspension (0.5 ml: 25 mg, trade name: Kanalin, Chongqing Lummy pharmaceutical Co., Ltd.) was injected into the cephalad and caudal margins of the tumor margin with 0.5 ml (25 mg) injections. Control group patients did not receive submucosal injections of carbon nanoparticles. As many lymph nodes as possible were manually extracted from fresh specimens following specimen removal by the pathology department. The pathological examination and nodal staging were conducted by experienced pathologists within the same group.

Surgical procedures

Laparoscopic radical resection of primary colorectal carcinoma was performed in all cases, including laparoscopic left hemicolectomy, laparoscopic transverse colectomy, laparoscopic right hemicolectomy, laparoscopic sigmoid

colectomy, and laparoscopic rectal resection. All patients also underwent D3 lymphadenectomy. As shown in Fig. 1A and B, location of tumor and black-stained apical lymph nodes were located under laparoscopy and identified. Surgeons from the same group performed the surgical procedures.

Statistical analyses

Data were expressed as frequency and proportion (%) for categorical variables, and mean and standard deviation for numerical variables. Statistics were carried out using IBM SPSS statistics 26. Comparisons between independent groups were conducted using Mann-Whitney U tests and Fisher's exact tests as appropriate. Asterisks indicate significant differences at the level of $P < 0.05$.

Results

Patient characteristics

As shown in Table 1, clinic characteristics of the study participants are summarized. The 119 cases were divided into a CNs group ($n = 80$) and a non-CN group ($n = 39$). There were no statistically significant differences observed in any of the parameters, including age, gender, body mass index (BMI), operation time, blood loss, tumor location, TNM Stage, T stage. The total number of harvested lymph nodes in CNs group was 1626, the mean number of resected lymph nodes in CNs group was 20.33 ± 7.42 ; The total number of harvested lymph nodes in control group was 606, the mean number of resected lymph nodes in control group was 15.54 ± 5.82 . In terms of the mean number of lymph nodes dissected, there was a significant difference between the two groups ($P = 0.001$). The total number of metastatic lymph nodes in CNs group was 209, the mean number of metastatic lymph nodes in CNs group was 2.61 ± 2.34 . The total number of metastatic lymph nodes in control group was 146, the mean number of metastatic lymph nodes in control group was 3.74 ± 2.99 . In terms of the mean number of metastatic lymph nodes, there was a significant difference between the two groups ($P = 0.026$).

Lymph node staining

In the CNs group, as shown in Table 2, a total of 1626 lymph nodes were examined, with 209 (12.85%) showing evidence of cancer metastasis and 1417 (87.15%) appearing normal. Of the metastatic lymph nodes, 27 (12.92%) were black stained with carbon nanoparticles while 182 (87.08%) were not. Among the normal lymph nodes, 787 (55.54%) were black stained and 630 (44.46%) were not. As shown in Fig. 2A, a normal lymph node was stained by carbon nanoparticles, while in Fig. 2B, a metastatic lymph node was not stained by carbon nanoparticles.

Relationship between staining and metastasis

In our study, as shown in Table 3, the black staining of lymph nodes using carbon nanoparticles was examined in each participant. Among the 80 patients in the carbon nanoparticles group, 19 individuals had positive lymph nodes that exhibited black staining, while negative lymph nodes did not show any black staining. Meanwhile, none of the patients exhibited black staining in positive lymph nodes, nor did they show any staining in negative lymph nodes. Additionally, among the 61 patients without black staining in positive lymph nodes, 56 individuals had negative lymph nodes that exhibited black staining, while 5 patients did not show any black staining in their negative lymph nodes. These findings indicate that carbon nanoparticles have limited utility in distinguishing metastatic lymph nodes.

Discussion

In addition to being one of the most common malignant tumors in the world, colorectal cancer also poses a serious threat to human life. Surgical treatment is the main clinical method for treating colorectal cancer. Surgical specimens are the most important indicator of prognosis based on their pathological findings⁸. After

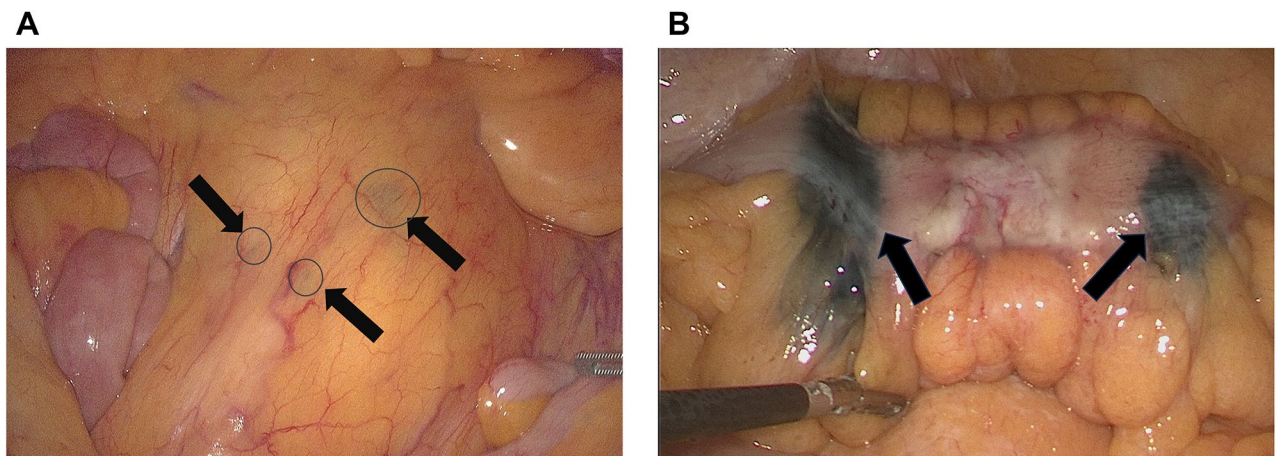


Fig. 1. CNs tracer-guided laparoscopic radical surgery. (A) Black stained apical lymph nodes under laparoscopy; (B) Tumor localization by CNs. The black arrow points to the location of the carbon nanoparticle staining.

	CNs group <i>n</i> = 80	non-CNs group <i>n</i> = 39	<i>P</i> value
Age (year)	62.29 ± 11.98	63.07 ± 11.99	0.736
Gender (male, %)	48 (60.0%)	24 (61.5%)	0.872
BMI (kg/m ²)	24.10 ± 3.58	23.18 ± 2.91	0.162
Operative time (min)	188.36 ± 55.30	199.23 ± 47.25	0.294
Blood loss (ml)	86.63 ± 63.66	92.82 ± 76.84	0.643
Tumor location			0.702
Ascending colon	12 (15.00%)	6 (15.39%)	
Transverse colon	1 (1.25%)	1 (2.56%)	
Descending colon	7 (8.75%)	5 (12.82%)	
Sigmoid colon	19 (23.75%)	8 (20.51%)	
Rectum	41 (51.25%)	19 (48.72%)	
Pathological stage			0.4963
III	79 (98.75%)	39 (100%)	
IV	1 (1.25%)	0	
Pathological T factor			0.596
1	3 (3.75%)	1 (2.56%)	
2	8 (10.00%)	4 (10.26%)	
3	62 (77.50%)	29 (73.36%)	
4	7 (8.75%)	5 (12.82%)	
Pathological N factor			0.0118
1	63 (78.75%)	22 (56.41%)	
2	17 (21.25%)	17 (43.59%)	
Total number of dissected lymph nodes	1626	606	
Mean number of dissected lymph nodes	20.33 ± 7.42	15.54 ± 5.82	0.001
Total number of metastatic lymph nodes	209	146	
Mean number of metastatic lymph nodes	2.61 ± 2.34	3.74 ± 2.99	0.026
Surgical margins positive	0	0	

Table 1. The clinicopathological characteristics and surgical outcome between the two groups.

	MLNs(<i>n</i> = 209)	non-MLNs(<i>n</i> = 1417)
Stained lymph node	27 (1.66%)	787 (48.40%)
Unstained lymph node	182 (11.19%)	630 (38.75%)

Table 2. The number of metastatic lymph node and the number of stained lymph node in the CNs group (Total lymph node *n* = 1626). MLNs: metastatic lymph nodes; non-MLNs: non-metastatic lymph nodes.

radical colorectal surgery, more than 45.3% of elderly patients develop lymph node metastases⁹. In patients with locoregional CRC, surgical resection of the tumor and removal of regional lymph nodes remain the mainstay of therapy¹⁰. An adequate lymph node examination is crucial in patients with CRC, as lymph node status is a strong indicator of long-term outcome¹¹. Colorectal cancer most commonly metastasizes to lymph nodes, during a radical resection of colorectal cancer (CRC), the National Comprehensive Cancer Network (NCCN) recommends that at least 12 lymph nodes should be examined. As Tepper suggests, it is often impossible to accurately stage lymph nodes from surgical specimens by pathologic assessment alone; further examination of more nodes is necessary to determine accurate nodal status; 14 nodes should be examined in order to determine nodal status accurately¹². Research by Destri and others has demonstrated that when the number of lymph nodes examined in stage II patients is fewer than 12, there is a significant reduction in the postoperative disease-free survival (DFS) rate¹³. A meta-analysis conducted by Chang et al. on colon cancer indicates that an increased number of lymph nodes is associated with improved survival rates in patients with stage II and III colon cancer¹⁴. Therefore, it is very important for the prognosis of patients with colorectal cancer whether the lymphatic dissection during the operation is thorough. Postoperative pathologists can also detect lymph nodes by observing the color changes of lymph nodes, thereby reducing the burden on pathologists.

If insufficient lymph nodes are removed and assessed, stage III CRC can be misdiagnosed as stage I. In order to determine the extent of surgical resection and identify patients eligible for surgery, a precise nodal status assessment is crucial. Since the 1990s, clinicians have begun to use endoscopic injection labeling for preoperative localization and lymph node tracing of CRC¹⁵. The black staining effect of carbon nanoparticles can be directly observed by the naked eye, which not only has good tumor localization and lymph node tracer effect, but also

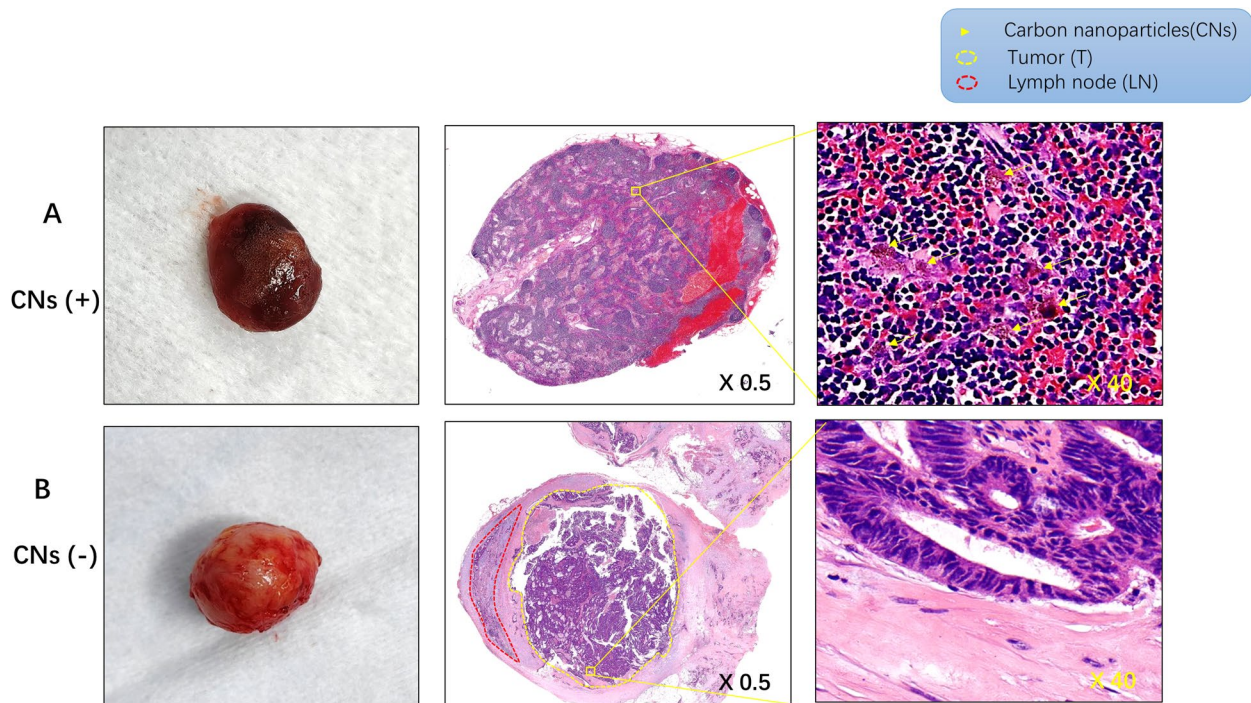


Fig. 2. Pathological sections of lymph node. (A) A normal lymph node was stained by carbon nanoparticles, while in (B), a metastatic lymph node was not stained by carbon nanoparticles.

	Patients with metastatic lymph node stained	Patients with metastatic lymph node unstained
Patients with normal lymph node stained	19 (23.75%)	56 (70.00%)
Patients with normal lymph node unstained	0 (0%)	5 (6.25%)

Table 3. Number of patients with lymph node metastasis and number of patients with lymph node staining ($n = 80$).

has high safety, strong tissue permeability, long retention time, and can not invade the blood system, becoming an ideal tracer¹⁶.

Currently, sentinel lymph node biopsy (SLNB) has been widely applied in the treatment of breast cancer and melanoma¹⁷, where it has been well validated. There have been many reports on sentinel lymph node biopsy for colorectal cancer from many researchers, and excellent results have been reported as an indicator for applying adjuvant therapy¹⁸. The use of carbon nanoparticles as a tattooing agent for tumor localization and lymph node tracing presents numerous benefits in clinical practice. Through their ability to accurately mark and stain lymphatic tissue, carbon nanoparticles improve the identification and detection of lymph nodes, thereby facilitating a more accurate staging process performed by pathologists. Moreover, the utilization of carbon nanoparticles for the identification and staining of tumor and lymph tissue has been shown to alleviate the difficulties faced during surgical procedures, leading to enhanced surgical resection rates for patients. This technology enables surgeons to achieve a more precise and prompt diagnosis of malignant areas requiring resection without the need for specialized equipment¹⁹. Additionally, enhanced visualization of lymph nodes during surgical procedures allows for safer operation by reducing the risk of injuries to surrounding vessels and nerves, as well as facilitating more precise dissection in the appropriate anatomical plane to achieve oncological benefits. Moreover, the use of carbon nanoparticles for tumor localization and lymph node tracing is considered to be a highly safe technique.

From a cost perspective, carbon nanoparticles are more expensive than traditional methods like tattooing ink. The production of carbon nanoparticles necessitates sophisticated nanotechnology, contributing to their higher cost. However, their long-term advantages, including enhanced lymph node detection rates and the potential for reducing secondary surgeries, may warrant the initial investment. In contrast, tattooing ink represents a cost-effective option; however, it is limited by its reduced visibility in lymph nodes and the potential necessity for further surgical interventions.

From a safety perspective, carbon nanoparticles exhibit outstanding biocompatibility, with minimal side effects and no significant local or systemic toxicity reported in contemporary studies. Although tattoo ink is generally regarded as safe, it can lead to local complications, including ink diffusion, granuloma formation, and

infections. Overall, carbon nanoparticles represent a safer and more effective alternative for long-term tumor and lymph node marking, albeit with increased cost implications.

The use of carbon nanoparticles for tumor localization and lymph node tracing is constrained by various limitations. Research has shown that lymph nodes that are entirely stained black demonstrate a significantly lower rate of tumor metastasis compared to those that are partially or unstained²⁰. This observation is thought to be linked to the necrosis of lymph nodes infiltrated by tumors, which is caused by insufficient blood supply resulting in structural damage. The presence of metastatic tumor tissue obstructing the lymphatic duct can impede the return of carbon nanoparticles through the lymphatic system, potentially hindering the complete or partial visualization of lymph nodes. Although carbon nanoparticles have a nanoscale particle size and can enter lymph nodes through small gaps or channels, if a cancer thrombus completely blocks the lymphatic vessels and no effective collateral circulation is formed, carbon nanoparticles will have difficulty entering the lymph nodes. This is because lymphatic vessels are the primary channels for lymph flow. Once they are completely blocked, lymph cannot flow normally, and carbon nanoparticles cannot reach the lymph nodes with the flow of lymph. In this case, carbon nanoparticles may accumulate near the blocked site or diffuse through other non-lymphatic pathways (such as interstitial spaces), but its efficiency of entering lymph nodes will be greatly reduced. Moreover, the presence of cancer thrombus not only physically blocks the lymphatic vessels but may also alter the microenvironment within the lymphatic vessels, such as causing inflammatory reactions and affecting lymphatic endothelial cell function. These changes may further hinder the entry of carbon nanoparticles²¹. In addition, lymph nodes have certain defense mechanisms that can recognize and eliminate some foreign substances. When carbon nanoparticles attempt to enter lymph nodes affected by cancer thrombus, it may be recognized and eliminated by immune cells within the lymph nodes, thereby reducing its effectiveness in entering the lymph nodes²². This reliance on carbon nanoparticles lymph nodes may lead to the inadvertent omission of positive lymph nodes, underscoring the importance of thorough evaluation during surgical interventions and subsequent pathological examination^{23,24}. Furthermore, an elevated quantity of excised indeterminate pathologic lymph nodes signifies an augmented caseload for pathologists, thereby imposing a substantial burden on their duties.

In our study, we found that the use of nano-carbon to locate tumors in laparoscopic colorectal cancer surgery can achieve better results, and the tumor can be accurately located and removed. Our research demonstrates that the employment of nano-carbon for tumor localization in laparoscopic colorectal cancer surgery yields superior outcomes, facilitating precise tumor identification and excision. While there is a suggestion that the utilization of carbon nanoparticle tracers in laparoscopic radical resection of colorectal cancer can effectively decrease surgical duration²⁰, our study did not confirm this. Furthermore, the utilization of carbon nanoparticle tracer in identifying lymph nodes has shown to result in a higher yield of lymph nodes; however, the control group exhibited a higher retrieval of metastatic lymph nodes compared to the CNs group. The staining rate of metastatic lymph nodes was found to be only 12.92%, while negative lymph nodes exhibited a staining rate of 55.54%. Additionally, our study revealed that none of the patients had metastatic lymph nodes stained while normal lymph nodes remained unstained, indicating a low accuracy of the carbon nanoparticle tracer. This suggests that relying solely on carbon nanoparticles staining for lymph node dissection may result in the potential oversight of lymph nodes with metastasis. Hence, there is a need for larger sample studies to enhance the effectiveness of nano carbon tracers or develop more precise targeted tracers, as this holds significant practical implications. While CNs do not reliably detect metastatic lymph nodes, they facilitate the retrieval of a greater number of lymph nodes during lymphadenectomy, thereby enabling more accurate pathological staging. This, in turn, aids in determining postoperative treatment strategies and improving patient prognosis.

A significant limitation of this study is the absence of a randomized controlled trial (RCT), which may introduce selection bias due to the non-random allocation of patients to the CN and non-CN groups. The lack of randomization poses a risk that unmeasured confounding variables, such as patient demographics or clinical characteristics, may have impacted the outcomes. This limitation compromises the internal validity of the study, thereby complicating the establishment of a definitive causal relationship between carbon nanoparticle staining and enhanced lymph node visibility. Furthermore, the external validity of the study is constrained, as the results may not be applicable to wider populations or diverse clinical environments. It is recommended that future large-scale RCTs be conducted to verify the efficacy of carbon nanoparticles and to address the potential biases inherent in observational studies such as ours.

The advancement of nanotechnology has led to the utilization of active targeting tracers, particularly active targeting CEA nano-tracers, in animal experiments and clinical trials for colorectal cancer treatment^{25,26}. The utilization of active targeting strategies in designing novel tumor localization and lymphatic tracers may yield improved therapeutic outcomes, warranting further investigation.

Conclusion

The utilization of carbon nanoparticles for tattooing in CRC surgery has been demonstrated to be a straightforward, secure, and advantageous technique for enhancing tumor localization and tracing lymph nodes without the need for specialized equipment. This underscores the significance of carbon nanoparticles in simplifying surgical procedures and increasing the yield of harvested lymph nodes in CRC surgery. Consequently, the significance of carbon nanoparticles in the surgical management of cancer warrants acknowledgment, and there is potential for their widespread adoption and utilization in the future, particularly in primary healthcare facilities. Nevertheless, the limited specificity and sensitivity of carbon nanoparticles in lymph node tracing necessitate caution in relying solely on this method for lymph node dissection. It is imperative to enhance the identification of specific targets for colorectal cancer, develop and produce novel active targeting nanomolecules, improve tumor localization and lymph node tracing, and enhance the efficacy of surgical treatment for CRC.

Data availability

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

Conceptualization, Q.Z.F., X.Z., S.Y.Y., Y.Q.F., S.F., Q.S. and M.F.; Methodology, Q.S.M.F. and Q.Z.F.; Data Curation, H.C., J.F.W. and Z.J.L.; Investigation, Q.Z.F., X.Z., S.Y.Y., Q.S. and M.F.; Writing—Original Draft, Q.Z.F., Y.Q.F., S.Y.Y. and X.Z.; Formal Analysis, Q.Z.F. and Y.Q.F.; Validation, X.Z., S.Y.Y., S.F. and Q.S.; Funding acquisition, Q.Z.F. and Q.S.; writing—review and editing, S.F., Q.S. and M.F.; supervision, Q.S. and M.F. All authors have read and agreed to the published version of the manuscript.

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Declarations

Ethics approval and consent to participate

Approval for this retrospective cohort study was obtained from the Ethics Committee of Nanjing Drum Tower Hospital, Medical School of Nanjing University. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All participants involved in this research have been provided with sufficient information regarding the purpose of our study. Additionally, the risks and benefits associated with the research were thoroughly explained by the author, and informed consent was obtained. Regular audits and oversight by the ethics committee ensured continuous compliance with ethical guidelines.

Consent for publication

All the individual person's data included in this study have consent for publication.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to S.F., Q.S. or M.F.

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