

Colorectal cancer lymph node staining by activated carbon nanoparticles suspension *in vivo* or methylene blue *in vitro*

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Received: June 16, 2012 Revised: August 2, 2012

Accepted: August 14, 2012

Published online: November 14, 2012

Abstract

AIM: To investigate whether activated carbon nanoparticles suspension (ACNS) or methylene blue (MB) can increase the detected number of lymph nodes in colorectal cancer.

METHODS: Sixty-seven of 72 colorectal cancer patients treated at our hospital fulfilled the inclusion criteria of the study which was conducted from December 2010 to February 2012. Seven patients refused to participate. Eventually, 60 patients were included, and randomly assigned to three groups (20 in each group): ACNS group (group A), MB group (group B) and non-stained conventional surgical group (group C). In group A, patients received subserosal injection of 1 mL ACNS in a 4-quadrant region around the mass. In group B, the main artery of specimen was identified and isolated after the specimen was removed, and 2 mL MB was slowly injected into the isolated, stretched and fixed vessel. In group C, no ACNS and MB were injected. All the mesentery lymph nodes were isolated and removed systematically by visually inspecting and

palpating the adipose tissue.

RESULTS: No difference was observed among the three groups in age, gender, tumor location, tumor diameter, T-stage, degree of differentiation, postoperative complications and peritoneal drainage retention time. The total number of detected lymph nodes was 535, 476 and 223 in the three groups, respectively. The mean number of detected lymph nodes per patient was significantly higher in group A than in group C (26.8 ± 8.4 vs 12.2 ± 3.2 , $P < 0.001$). Similarly, there were significantly more lymph nodes detected in group B than in group C (23.8 ± 6.9 vs 12.2 ± 3.2 , $P < 0.001$). However, there was no significant difference between group A and group B. There were 50, 46 and 32 metastatic lymph nodes dissected in 13 patients of group A, 10 patients of group B and 11 patients of group C, without significant differences among the three groups. Eleven of the 60 patients had insufficient number of detected lymph nodes (< 12). Only one patient with T_{4a} rectal cancer had 10 lymph nodes detected in group B, the other 10 patients were all from group C. Based on the different diameter categories, the number of detected lymph nodes in groups A and B was significantly higher than in group C. However, there was no statistically significant difference between group A and group B. The metastatic lymph nodes were not significant different among the three groups. Similarly, tumor location, T stage and tumor differentiation did not affect the staining results. Body mass index was a minor influencing factor in the two different staining methods. The stained lymph nodes can easily be identified from the mesenteric adipose tissues, and the staining time for lymph nodes was not significantly different compared with unstained group. None of the patients in groups A and B had drug-related complications.

CONCLUSION: Both activated carbon nanoparticles suspension *in vivo* and methylene blue *in vitro* can be used as tracers to increase the detected number of lymph nodes in colorectal cancer.

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Key words: Nanotechnology; Activated carbon nanoparticles suspension; Methylene blue; Lymph nodes; Colorectal cancer

Peer reviewer: Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46, 1088 Budapest, Hungary

Cai HK, He HF, Tian W, Zhou MQ, Hu Y, Deng YC. Colorectal cancer lymph node staining by activated carbon nanoparticles suspension *in vivo* or methylene blue *in vitro*. *World J Gastroenterol* 2012; 18(42): 6148-6154 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i42/6148.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i42.6148>

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death among men and women in the United States with an estimated 143 460 new cases and 51 690 deaths in 2012 according to the statistics of American Cancer Society^[1]. Accurate lymph node metastasis staging is of prognostic and therapeutic importance in patients with CRC. Previous researches have found that the number of lymph nodes evaluated after surgical resection was positively associated with the survival of CRC patients^[2,3]. However, population-based data suggest that lymph node evaluation is not adequate in the majority of patients with CRC^[4,5]. In addition, computed tomography (CT)^[6], even positron emission tomography^[7], are not efficacious enough in identifying nodal status. Therefore, a variety of techniques, including lymph node staining or radionuclide scan^[8], have been applied to make lymph nodes retrieval more efficient.

The biological application of nanoparticles is a rapidly developing area of nanotechnology^[9,10]. Particles have been observed passing through the lymphatic vessels but not the blood capillaries mainly due to the difference in permeability. Activated carbon nanoparticles suspension (ACNS), using smooth carbon particles at a diameter of 21 nm added with suspending agents, is a stable suspension of carbon pellets of 150 nm in diameter. ACNS is obviously inclined to the lymphatic system. After macrophage phagocytosis, ACNS quickly gathers in the lymph nodes and dyes them black. This unique selective bio-distribution is being extensively studied in recent years, such as sentinel lymph node staining, drug carriers and thermotherapy^[11-13].

In our previous study^[14], 62 patients with CRC were divided into two groups. The experimental group, using a simple lymphatic staining method, was injected with methylene blue (MB) into the regional main blood vessels immediately after specimens were resected *in vitro*. More and smaller lymph nodes could be detected, which significantly improved the lymph node harvest of resected colorectal specimens. However, the detection

sensitivity for lymph node metastasis was low and the staining could not be done *in vivo* before the destruction of the lymphoid structures. Therefore, we conducted a randomized controlled trial to test whether MB and ACNS as tracers can increase the detected number of lymph nodes in the systematic nodal dissected tissues from CRC resection and compare the staining effect of the two methods in order to choose the best one for further clinical application.

MATERIALS AND METHODS

Patient selection

This trial was performed in the Department of Surgical Oncology, Second Affiliated Hospital Zhejiang University College of Medicine, China from December 2010 to February 2012. The study was approved by the Ethics Committee of Zhejiang University. Informed consent was obtained from all the patients. Inclusion criteria were as follows: 18-80 years of age; endoscopic biopsy confirmed; performance status of 0-1 on the Eastern Cooperative Oncology Group scale; good compliance; able to tolerate radical resection; adequate hematologic function [white blood cell (WBC) count > 4000/mL, absolute neutrophil count > 1500/mL, platelet count > 100 000/mL, and hemoglobin > 10 g/dL]; normal hepatic function [bilirubin < 1.5 the upper-normal limits (UNL) and alanine aminotransferase or aspartate aminotransferase < 2.5 UNL]; and normal renal function (creatinine < 1.5 mg/dL). Exclusion criteria included: clinical stage IV CRC according to the American Joint Committee on Cancer (AJCC); patients received chemotherapy, radiotherapy or biological therapy prior to surgery; previous abdominal surgery; significant neurological or mental disorder. Of the 72 CRC patients, 67 fulfilled the inclusion criteria. Seven patients refused to participate. The enrollment was completed when 60 patients were included. The patients were randomly allocated to three groups (20 patients in each group): ACNS group (group A), MB group (group B) and non-staining conventional surgical group (group C).

Surgical technique

All surgical procedures were completed by the same team of surgeons. Each patient was administrated with 2 g cephalosporin for antibiotic prophylaxis within 30 min before surgery, and the same dose was repeated if the operation lasted more than 2 h.

In group A, patients received subserosal injection of 1 mL ACNS (Chongqing LUMMY Pharmaceutical Co., Chongqing, China) in a 4-quadrant region around the mass (Figure 1A). To avoid surgical destruction of the lymphatic system along the bronchi and vessels, we waited for 10 min after injection. In group B, MB was injected by the same methods (Jiangsu Jumpcan Medicines Group, Taixing, Jiangsu Province, China), however, staining effect was very poor partly because MB was quickly absorbed *in vivo* and then excreted with urine.

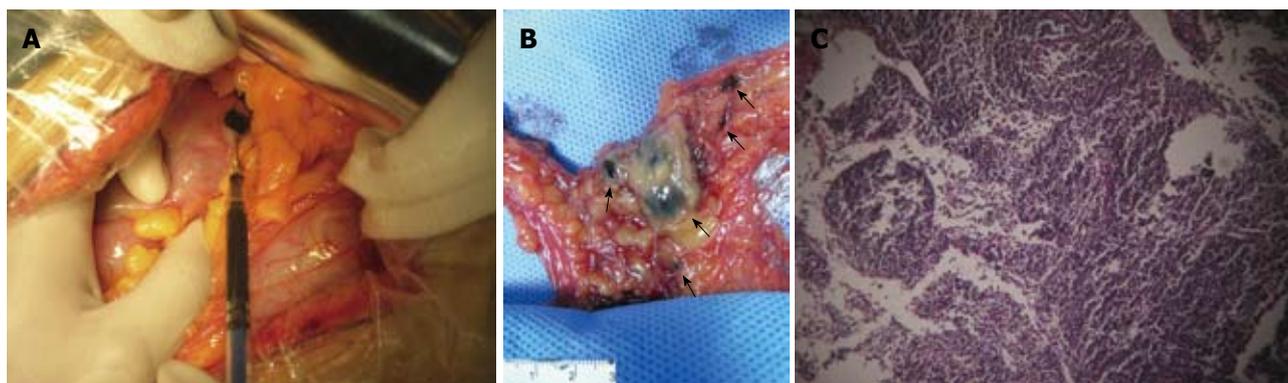


Figure 1 Activated carbon nanoparticles suspension *in vivo* is effective as a tracer in colorectal cancer lymph node detection. A: Subserosal injection of activated carbon nanoparticles suspension (ACNS) into a 4-quadrant region around the mass; B: Lymph nodes can easily be identified from the mesenteric adipose tissues, arrow points to the black dyed lymph nodes; C: ACNS migrating to the lymph node (hematoxylin and eosin, $\times 100$).

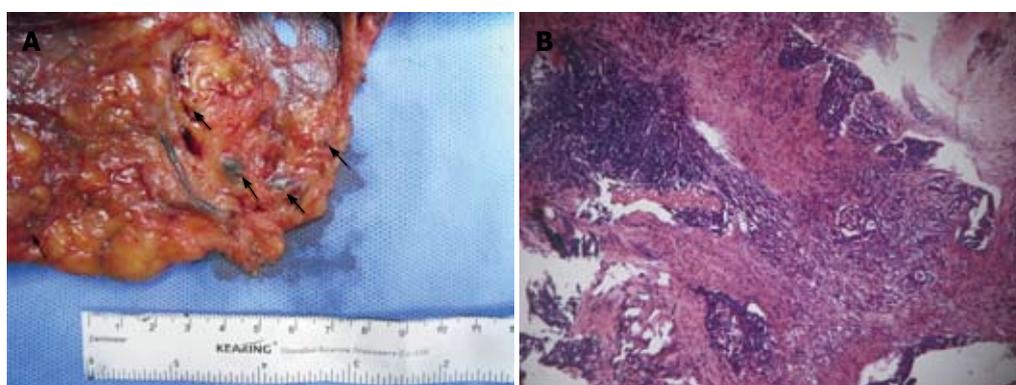


Figure 2 Methylene blue *in vitro* used as a tracer in colorectal cancer lymph node detection. A: The main artery of the specimen was isolated and methylene blue (MB) was injected into the vessel. Lymph nodes can easily be identified, arrow points to the blue dyed lymph nodes; B: MB migrating to the lymph node (hematoxylin and eosin, $\times 100$).

Therefore, we established a method for lymph nodes staining *in vitro*. After the specimen was dissected, we immediately identified the main artery of the specimen and isolated it at the root for 1 cm, and injected 2 mL MB slowly into the isolated, stretched and fixed vessel (Figure 2A). We also waited for 10 min after injection. In group C, neither ACNS nor MB was used for the staining.

All the mesentery lymph nodes were isolated and removed systematically by visually inspecting and palpating the adipose tissues. After identification and excision, all the black or blue nodes were collected for subsequent pathological examinations. Postoperative pain was relieved by intravenous opioid administration.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 for Windows (SPSS, Inc, Chicago, IL, United States). Factors considered to be possible determinants of the number of lymph nodes examined were first checked with the analysis of variance analysis and the influence of possible determinants was then tested in regression analysis. Kruskal-Wallis test was used when there was heterogeneity of variance. *P* value (two-tailed) of less than 0.05 was considered statistically significant.

RESULTS

Patient population

The 60 patients were randomly assigned to three groups. No difference was observed among the three groups in age, gender, tumor location, tumor diameter, T-stage and degree of differentiation. There was also no statistically significant difference in postoperative complications and peritoneal drainage retention time (Table 1).

Lymph nodes detected

The total number of detected lymph nodes was 535, 476 and 223 in the three groups, respectively. The mean number of detected lymph nodes per patient in group A was significantly higher than in group C (26.8 ± 8.4 vs 12.2 ± 3.2 , $P < 0.001$). Similarly, there were significantly more lymph nodes detected in group B than in group C (23.8 ± 6.9 vs 12.2 ± 3.2 , $P < 0.001$). However, there was no significant difference between group A and group B (26.8 ± 8.4 vs 23.8 ± 6.9 , $P > 0.1$) (Figure 3A). There were 50, 46 and 32 metastatic lymph nodes dissected in 13 patients of group A, 10 patients of group B, and 11 patients of group C, without significant difference among the three groups ($P > 0.1$). According to the

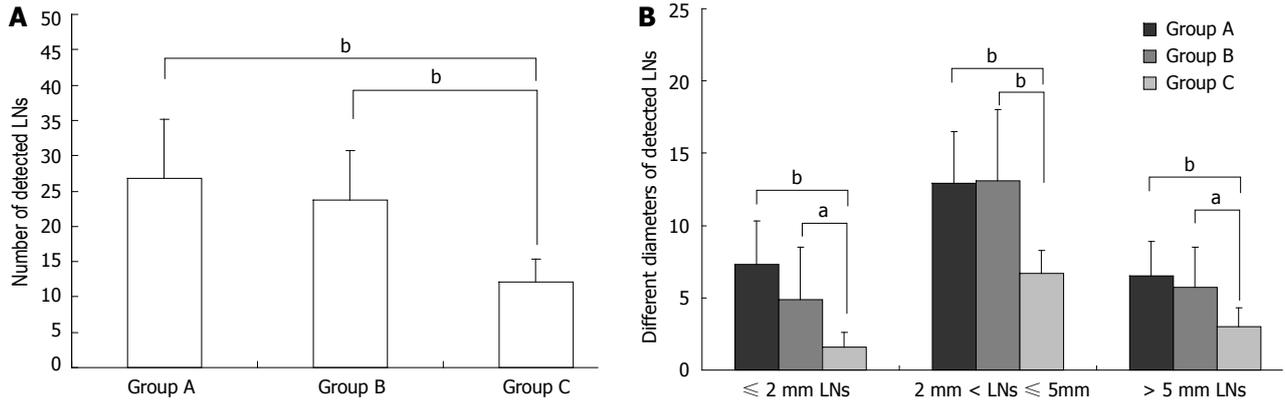


Figure 3 Lymph nodes detected in the three groups. A: The mean number of lymph nodes detected; B: Distribution of lymph node diameters. ^a*P* < 0.05, ^b*P* < 0.01 vs group A. LN: Lymph node.

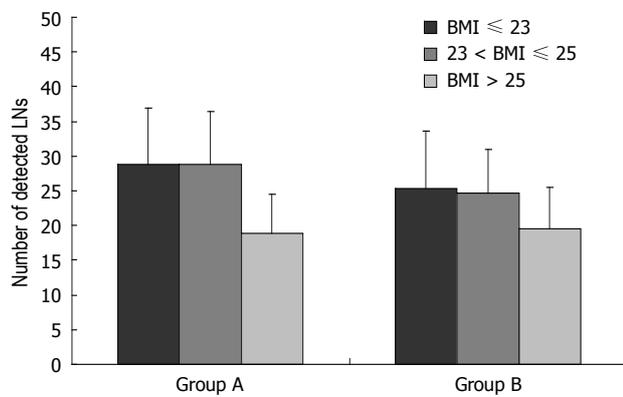


Figure 4 Body mass index as an influencing factor in the two different staining methods. LN: Lymph node; BMI: Body mass index.

AJCC guideline, more than 12 detected lymph nodes are required for the accurate clinical staging. In this study, 11 of the 60 patients had insufficient number of detected lymph nodes. Among them, only one patient in group B with T_{4a} rectal cancer had 10 lymph nodes detected, the other 10 patients were all from group C (*P* < 0.001). According to the different diameter range, lymph nodes (LNs) detected were divided into three categories: LNs ≤ 2 mm, 2 mm < LNs ≤ 5 mm, and LNs > 5 mm. We analyzed the different diameter categories of detected lymph nodes and metastatic lymph nodes in each group, and found that the number of detected lymph nodes was significantly higher in groups A and B than in group C. However, there was no statistically significant difference between group A and group B. Data is shown in Figure 3B. There was no significant difference in metastatic lymph nodes between each group (*P* > 0.05). Similarly, tumor location, T stage and tumor differentiation exerted no influence on the staining results (*P* > 0.05). Mesenteric hypertrophy may affect the lymph node detection by different staining methods^[15]. To explore whether the mesenteric hypertrophy affects the staining results in our trial, patients in the two staining groups were re-classified according to the Asian body mass index (BMI) criteria

Table 1 Clinicopathological details of the 60 patients in the three groups

	Group A	Group B	Group C
No. of patients	20	20	20
Age (yr)	57.5 ± 11.5	58.9 ± 17.8	64.9 ± 7.4
Gender			
Male	14	14	13
Female	6	6	7
Tumor location			
Right colon	2	2	4
Transverse colon	1	1	1
Left colon	5	1	1
Sigmoid	5	4	4
Rectum	7	12	11
Tumor diameter (mm)	5.0 ± 1.7	4.7 ± 2.3	3.7 ± 1.1
T-stage			
T1	0	1	0
T2	2	4	4
T3	6	7	7
T4	12	8	9
Degree of differentiation			
Well	7	7	8
Moderate	10	11	11
Poor	3	2	1
Postoperative complications			
Bleeding	1	0	0
Infection	0	1	0
Fistula	1	1	2
Abdominal tube drainage (d)	6.7 ± 2.8	6.1 ± 3.3	7.8 ± 3.6

as BMI ≤ 23 kg/m², 23 kg/m² < BMI ≤ 25 kg/m² and BMI > 25 kg/m². Statistical analysis showed that BMI was a minor influencing factor in the two different staining methods while mesenteric hypertrophy did not influence the staining results (Figure 4). Using either ACNS (Figure 1B) or MB (Figure 2A), stained lymph nodes can easily be visualized from the mesenteric adipose tissues, and the staining time for lymph nodes was not significant different compared with unstained group (*P* > 0.05). Hematoxylin and eosin stained micrograph confirmed that ACNS migrates to the lymph nodes (Figure 1C), so does the MB (Figure 2B). None of the patients injected with either ACNS or MB had drug-related complications.

DISCUSSION

Lymph nodes status of colorectal cancer played a vital role in tumor staging, classification, postoperative sequential treatment and prognosis. The number of detected lymph nodes is a significant prognostic factor in colon cancer patients^[16]. AJCC and College of American Pathologists recommend at least 12 lymph nodes detected for more accurate diagnosis of stage II CRC^[17]. However, recent studies indicated that lymph node detection rate was still low in CRC^[4], which can not accurately reflect the patient's disease status. There are many factors affecting the lymph node detection, including patient's age, gender, tumor grade, extent of surgical resection and the pathologist's expertise^[18]. Palpation is still the most important method for lymph node detection^[19]. Numerous studies have been conducted to improve the methods for lymph node detection. Cawthorn *et al.*^[20] used xylene alcohol clearance technique to facilitate the identification of lymph nodes (23.1 ± 1.18 vs 10.5 ± 0.6). Quadros *et al.*^[21] performed lymphoscintigraphy using technetium-99 m-phytate and patent blue to detect lymph nodes of rectal adenocarcinoma patients, which significantly increase the lymph nodes detection rate, particularly lateral pelvic lymph node metastasis. However, these techniques were not widely used in clinical practice because they are time-consuming, labor-intensive and toxic to doctors. This clinical trial used the novel nanomaterials ACNS and MB *in vivo* or *in vitro*. The results suggested that both staining techniques can significantly improve the lymph node detection compared with the conventional palpation method (26.8 ± 8.4 vs 23.8 ± 6.9 vs 12.2 ± 3.2). In our research, none of the patients had insufficient number of detected lymph nodes in ACNS stained group, only one patient in MB stained group, but 10 patients did so from unstained group C. Statistical results showed that in different diameter categories, the number of the detected lymph nodes was significantly higher in both the two stained groups than in the unstained group (Figure 4), especially in the ACNS group. This demonstrated the obvious advantages of the two staining methods in detecting the smaller diameter lymph nodes. Micrometastasis is defined as cohesive deposits of tumor cells of 2 mm or less, but larger than 0.2 mm. This definition has been extended in the AJCC 7th edition to include non-cohesive infiltrate of > 200 cells as micrometastasis^[22]. No definitive conclusions have been drawn about the role of sentinel lymph node biopsy and micrometastasis on the prognosis of CRC^[23,24]. Taking into account the advantage of the detection for lymph node micrometastasis in this study, our team will further study the effects of sentinel lymph node biopsy and micrometastasis in the prognosis of CRC patients. In this trial, we failed to find significant differences between the two staining methods with regard to the total number of detected lymph nodes, lymph node diameters or lymph node metastasis, which may be attributed to the limitations of sample size.

The biological application of nanoparticles is a rap-

idly developing area of nanotechnology that raises new possibilities in the diagnosis^[25] and treatment of human cancers^[26,27]. Nanoparticles are being developed for contrast at T1-weighted magnetic resonance imaging^[28], radionuclides for single photon emission computed tomography and positron emission tomography^[29], iodine for CT^[30] and gas-containing bubbles for ultrasonography^[31]. Several nanotechnologies have been used to improve the delivery of chemotherapeutic agents to cancer cells^[32,33], which promoted the microdosing clinical studies^[34]. A review by Schroeder *et al.*^[35] showed that nanoparticle therapies will improve the outcome for patients with metastatic cancer. Using ACNS combined with preoperative lymphoscintigraphy and intraoperative gamma probe detection, the detecting sensitivity was 100% for internal mammary sentinel node biopsy of breast cancer patients^[36]. A clinical trial from him^[37] showed that ACNS and MB both were effective as tracers in lymph nodes detection for non-small cell lung cancer. However, ACNS staining in colorectal cancer lymph nodes detection remains largely unexplored. MB is being widely used in clinical practice, however, when it was used in lymph nodes staining in CRC *in vivo*, it played a minor role in lymph nodes detection because it was absorbed and excreted too quickly. We thus used a modified method as described in the Surgical Technique, injected MB into the main blood vessels of the tumor drainage region *in vitro*, and achieved much better staining results in lymph node detection. An ideal tracer should possess the following properties: not influenced by external conditions, easy to perform, effective and free from side-effects. We found that T stage, degree of tumor differentiation, tumor location, and BMI had minor influence on the two staining methods. In this study, black-stained or blue-stained lymph nodes can be easily identified from the mesenteric adipose tissues, and no side-effect was found among patients, surgeons and pathologists. However, the results obtained in this study may be limited by the sample size. Further studies with a larger sample will be conducted.

In conclusion, both ACNS *in vivo* and MB *in vitro* are effective as a tracer in increasing the detected number of lymph nodes in CRC. They may play a key role in the studies of sentinel lymph nodes biopsy and micrometastasis in CRCs.

COMMENTS

Background

Accurate lymph node metastasis staging has important prognostic and therapeutic implications in patients with colorectal cancer (CRC). Activated carbon nanoparticles suspension (ACNS) is obviously inclined to lymphatic system. The unique selective biodistribution is being extensively studied in recent years, such as sentinel lymph node staining, drug carriers, and thermotherapy. Additionally, methylene blue (MB), as an efficacious and cost-effective tracer, was widely used in clinical practice.

Research frontiers

Numerous studies have been conducted to improve the methods of lymph node detection. The biological application of nanoparticles is a rapidly developing area of nanotechnology. However, ACNS as a tracer for lymph nodes detection

in CRC patients has not been reported. This study found that ACNS *in vivo* is effective in increasing the detected number of lymph nodes in CRC. Similarly, the authors used a modified method, injecting MB into the main blood vessels of the tumor drainage region *in vitro*, and achieved analogous clinical effects. Moreover, the two staining methods were mildly influenced by T stage, degree of tumor differentiation, tumor location, and body mass index.

Applications

This is the first research that compares the staining effects of ACNS and MB in colorectal cancer lymph nodes detection. Both ACNS *in vivo* and MB *in vitro* are effective as tracers in increasing the detected number of lymph nodes in CRC.

Terminology

ACNS is a stable suspension of carbon pellets with a diameter of 150 nm, which is obviously inclined to lymphatic system. After macrophage phagocytosis, ACNS quickly gathers in the lymph nodes and dyes them black.

Peer review

The topic is very interesting and is of great clinical importance as the number of removed and histologically analyzed lymph nodes does determine the prognosis of CRC patients. An easy, time- and cost-effective method to help the surgeons' work is needed.

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