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ORIGINAL RESEARCH ARTICLE

Ex vivo sentinel lymph node mapping in colorectal cancer using [^{99m}Tc]Tc-HMPAO-patent blue liposomes: A hybrid radiotracer/dye approach

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ABSTRACT

Introduction: To assess the effectiveness of [^{99m}Tc]Tc-HMPAO-Patent blue liposomes for sentinel lymph node (SLN) mapping in colorectal cancer (CRC) using combined visual and radiotracer detection in a single injection.

Methods: In this prospective study, 40 CRC patients underwent surgical resection. Post-resection, PEGylated or non-PEGylated [^{99m}Tc]Tc-HMPAO liposomes were injected peritumorally ex vivo. SLNs were identified by blue dye visualization and gamma probe detection, followed by pathological assessment.

Results: SLNs were not detected in 6 patients (15%). Among the 34 cases with SLN detection, 5 (14.7%) had metastatic involvement. In 2 cases with negative SLNs, non-sentinel nodes were positive, leading to pathological upstaging in 2 patients (5%). The overall detection rate was 85%, with sensitivities of 80% for colon and 50% for rectal cancer. The false-negative rate was 28%.

Conclusion: [^{99m}Tc]Tc-HMPAO-Patent blue liposomes offer an effective dual-modality approach for SLN mapping in CRC, enhancing staging accuracy, especially in colon cancer.

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INTRODUCTION

Colorectal cancer (CRC) is a significant global health burden, ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide. It is the fourth leading cause of cancer-related deaths worldwide, following lung, stomach, and liver cancers, with over 1 million new cases diagnosed annually [1, 2]. Lymph node (LN) status is a key prognostic factor in CRC, significantly influencing patient outcomes. The presence of positive regional LNs reduces 5-year survival rates by 25-30%, highlighting the critical role of LN evaluation in disease management [3-5].

Effective staging and treatment protocols are essential for improving CRC outcomes, as they have been shown to significantly reduce recurrence and mortality rates. Staging in CRC is guided by the TNM classification system. Stage I and II patients typically undergo surgical resection and lymphadenectomy without adjuvant therapy, whereas Stage III patients are treated with adjuvant chemotherapy in addition to surgery. Accurate LN assessment is vital in determining the need for adjuvant treatment and predicting long-term outcomes such as recurrence, disease-free survival, and overall survival [6-9].

The sentinel lymph node (SLN) represents the first LN to receive metastatic spread from the primary tumor. SLN biopsy has proven effective in staging and nodal evaluation for cancers like melanoma, breast cancer, and gynecological cancers [10-12].

The adoption of SLN mapping in CRC aims to improve staging accuracy by identifying key LNs for thorough pathological examination. This approach is particularly advantageous for high-risk Stage II patients, providing precise nodal staging without altering surgical protocols and offering critical guidance for adjuvant therapy decisions [13, 14].

The ex vivo SLNM allows precise dye injection post-resection, avoids injection failure, reduces risks, and doesn't extend surgery time nor increase complications [15].

Liposomes have become versatile carriers in diagnostic and therapeutic applications due to their exceptional ability to encapsulate and deliver bioactive substances effectively. Initially introduced in 1982 for lymphoscintigraphic lymph node localization, liposomes are primarily composed of phospholipids—amphiphilic molecules containing both hydrophilic and hydrophobic regions [16-18]. Recent advancements include a novel method using the lipophilic chelator hexamethylpropyleneamine oxime (HMPAO), which has demonstrated success in stabilizing [^{99m}Tc]Tc -labeled liposomes in murine models, offering significant improvements over conventional lymph node localization techniques [19].

Traditionally two methods have been used for sentinel node detection in surgical oncology: blue dyes and radiotracers [20]. Blue stained sentinel nodes are detected visually and radiotracer enhanced sentinel nodes are detected by gamma probes intraoperatively. In the previous studies (grant number: 900543) we have incorporated these two techniques using liposome bound Patent blue labeled with [^{99m}Tc]Tc-HMPAO in order to use both methods (visual and radiotracer) by a single injection [21]. Therefore, the aim of this study is to evaluate the feasibility of sentinel lymph node mapping in an ex vivo setting using two novel agents, [^{99m}Tc]Tc -HMPAO-non-PEG-NLs and [^{99m}Tc]Tc -HMPAO-PEGylated NLs, for assessing sentinel lymph node mapping in colorectal cancer.

METHODS

A prospective study included forty patients with colorectal cancer undergoing elective resection for intraperitoneal colon carcinoma. Twenty patients underwent open surgical resections, while the other twenty had laparoscopic-assisted procedures, all including lymphadenectomy. Specimens were promptly processed in the operating room post-resection.

Preparation of liposomal formulations

Hydrogenated soy phosphatidylcholine (HSPC), DSPE-mPEG-2000, and cholesterol were obtained from Avanti Polar Lipids (USA). Glutathione (GSH) and Sephadex G-25 were supplied by Sigma (USA). An HMPAO kit comparable to the CERETEC formulation (containing 0.5 mg HMPAO and 7.6 µg $SnCl_2$) was prepared in-house. Chloroform and methanol were purchased from Merck (Germany). PEGylated and non-PEGylated nanoliposomes were produced using the thin-film hydration method followed by high-pressure homogenization. The required lipids were dissolved in a chloroform–methanol mixture at the desired molar ratios, and the solvent was evaporated to create a thin lipid film. This film was hydrated with a glutathione solution containing patent blue dye, then mixed and sonicated at 65 °C. The resulting suspension was processed through a high-pressure homogenizer to obtain uniformly sized vesicles. Unloaded dye and glutathione were removed by dialysis, and the final liposomes were characterized for particle size and surface charge using dynamic light scattering. The mean particle size in this study was reported to be in the range of approximately 120 to 131 nm.

Liposomes (70 mM) were incubated with 370 MBq freshly prepared [^{99m}Tc]Tc -HMPAO for 30 min at

room temperature. Free radionuclide was separated using a PD-10 (Sephadex G-25) column eluted with 5% dextrose. Radiolabeling efficiency was calculated from the activity measured before and after purification.

The radiolabeled PEG-NL and NL formulations were incubated in human serum at 37 °C for up to 24 h. Radiochemical purity at 1, 2, and 24 h was assessed by ITLC-SG using methyl ethyl ketone as the mobile phase.

Sentinel lymph node mapping procedure

Following tumour excision, lymphatic mapping was performed via four peritumoral subserosal injections of [^{99m}Tc]Tc -HMPAO–Patent Blue–labeled liposomes (0.5 mCi in 0.2 mL total volume), with patients randomized to receive either non-PEGylated or PEGylated formulations. Sentinel lymph nodes were identified 30 minutes after injection using a combination of blue dye visualization and gamma probe detection. Nodes were defined as either visibly stained or exhibiting radioactivity at least 5–10 times above background, measured at a distance of ≥20 cm from the injection site. Subsequently, Both the entire sample and the SLNs were subsequently sent for pathological evaluation in separate groups. In this study, we first tagged HMPAO with [^{99m}Tc]Tc and then incubated it for 30 minutes with preformed liposomes containing Patent Blue. Following this, we used centrifugation to separate the labelled liposomes from any unbound Tc-HMPAO. The study utilized two types of Patent Blue Dye-containing liposomes: [^{99m}Tc]Tc -HMPAO PEGylated NLs and [^{99m}Tc]Tc -HMPAO NLs.

Figure 1 showed ex vivo injection of [^{99m}Tc]Tc -HMPAO-PEG-NLs in the peri-tumoral region.

In our study, we evaluated the success of SLN mapping using three key metrics:

1. Detection Rate: The proportion of patients in whom at least one sentinel lymph node was successfully identified during surgery.
2. False-Negative Rate: The percentage of cases where pathological examination revealed metastatic lymph nodes in the nodal basin, despite the sentinel nodes being classified as negative [22].
3. Upstaging rate: The percentage of cases with SLN involvement but no metastasis in non-sentinel lymph nodes.

This study was approved by the Ethics Committee of Mashhad University of Medical Sciences. 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. This research was conducted retrospectively and did not interfere with patients' therapeutic procedures.

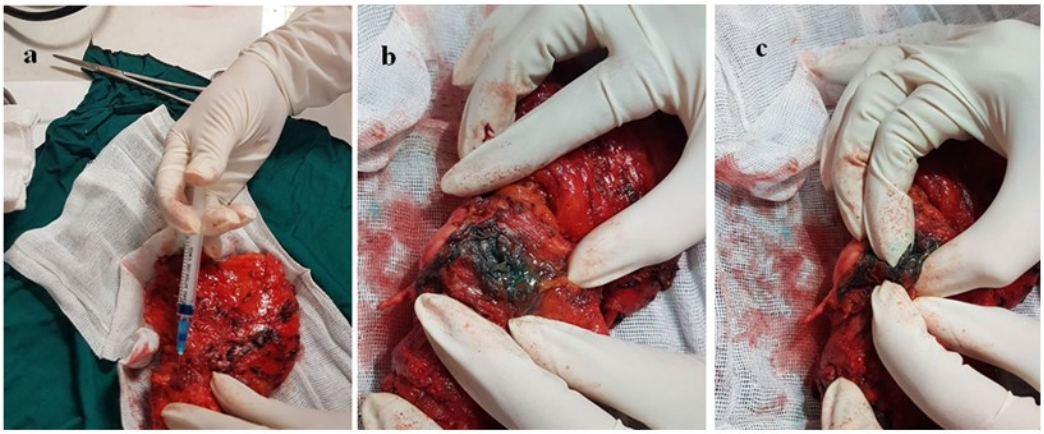

Figure 1. Ex vivo lymph node drainage following intratumoral injection of [^{99m}Tc]Tc -HMPAO-PEG-liposomes (34–74 kBq [1–2 mCi] in 0.5 mL). (a) Injection of [^{99m}Tc]Tc -HMPAO Patent Blue Liposomes into the peritumoral subserosal region. (b, c) Detection of the sentinel lymph node using visual assessment and a gamma probe

RESULTS

Pathological evaluation revealed adenocarcinoma in all patients. The surgery involved resecting a median of 11 lymph nodes (range: 7 to 16). Tumours were located in the colon in 19 patients (47.5%) and in the rectum in 21 patients (52.5%). SLNs were detected in 85% of cases. In 75% of patients, one sentinel node is detected, while in 10% of patients (four patients), two sentinel lymph nodes were found (two with colon cancer and two with rectal cancer), all using [^{99m}Tc]Tc -HMPAO PEGylated NLs as the radiotracer. The average tumour size in these cases was 2.87 cm. In six cases (15%), the SLN was not found; among these, four patients had colon cancer and two had

rectal cancer. Three of these cases used [^{99m}Tc]Tc-HMPAO NLs and the other three used [^{99m}Tc]Tc-HMPAO PEGylated NLs. The overall detection rate was 85%, with rates of 78% for colon cancer and 90% for rectal cancer, which were not significantly different based on sample size (Table 1). Sentinel lymph nodes were involved in five cases, with three showing tumoural involvement in non-sentinel lymph nodes as well. Four out of this five patients had colon cancer (sensitivity = 80%), while one patient had rectal cancer (sensitivity = 50%).

Two cases of patients whose sentinel lymph node was not involved, other lymph nodes (non-sentinel) were involved, as a result of this study, the false negative rate was 28% (2 from 7). This amount was 20% for the colon and 50% for the rectum.

In our study we identified two cases with SLN metastasis while non-sentinel nodes remained negative, leading to potential upstaging in 5% of patients.

Table 1. Histopathology of the SLN biopsy (SLNB) and lymph node dissection

		Lymph node dissection		
		Negative	Positive	Total
SLNB	Negative	27	2	29
	Positive	2	3	5
	No SN	6	0	6

DISCUSSION

In this study, we used [^{99m}Tc]Tc -HMPAO-labeled liposomes as a novel radiopharmaceutical to map sentinel lymph nodes through dual-modality imaging—blue dye visualization and gamma probe–guided radiotracer detection. The mean particle size of the nanoliposomes was approximately 120–131 nm, which falls within the biologically active range for lymphatic uptake and interaction with immune cells (14–150 nm) [21]. [^{99m}Tc]Tc -HMPAO-labeled liposomes demonstrated an 85% overall SLN detection rate, with 78% for colon cancer and 90% for rectal cancer. Both PEGylated and non-PEGylated liposomes yielded comparable detection rates, successfully identifying SLNs in 17 patients across both groups. These findings align with prior systematic reviews reporting a 92% detection rate in colorectal cancer, though with higher rates in colon cancer than rectal cancer [22]. While our detection rate is consistent with existing literature, it is important to note that our cohort was relatively small (n=40). Previous research highlights the impact of the learning curve, with more robust detection rates typically observed in studies involving over 100 patients [21-23]. Additionally, our results corroborate earlier animal model studies, which suggested that PEGylated liposomes exhibit faster lymphatic migration. Notably, in four patients receiving PEGylated liposomes, two SLNs were successfully detected, reinforcing their potential utility in lymphatic mapping [21].

Ex-vivo SLN evaluation provides key technical advantages. Injecting dye or tracer into the resected specimen eliminates the risk of tumor cell dissemination and allows free tissue handling without altering the surgical field. It also avoids lymphatic vessel trauma that can occur during in-vivo mapping, which may disrupt tracer flow and reduce SLN detection. This approach ensures reliable identification, supports the use of novel flow-independent tracers, and achieves high detection rates (≈90–100%), making it a feasible and robust method for focused ultra-staging and research purposes [24-27].

Sensitivity and comparative performance

The sensitivity of [^{99m}Tc]Tc-HMPAO liposomes was 80% for colon cancer and 50% for rectal cancer. False negative cases were particularly in patients with larger tumors (5 cm and 7 cm). In these cases, SLNs appeared histologically negative, yet subsequent evaluation of non-SLNs revealed metastatic deposits. This sensitivity profile is consistent with prior studies reporting an overall sensitivity of 69.6%, with colon cancer showing higher sensitivity (77.6%) than rectal cancer. Comparatively, traditional techniques such as blue dye (69.4%) and colloid-based lymphoscintigraphy (70.4%) exhibit lower sensitivity [20-22]. While our radiotracer demonstrates improved sensitivity, our findings—like those of previous studies—support the conclusion that SLN evaluation alone cannot replace complete mesenteric examination in colorectal cancer. Unlike in breast cancer or melanoma, where SN mapping guides therapeutic decisions, its role in colorectal cancer remains primarily staging-oriented [10, 28-31].

Upstaging on lymph node detection

Upstaging involves detecting metastatic involvement in lymph nodes, shifting a patient's

status from node-negative (N0) to node-positive (N+), which can significantly influence treatment decisions. Sentinel lymph node mapping has been shown to improve nodal staging accuracy in colorectal cancer, upstaging 10–18% of patients by identifying micrometastases that affect prognosis and therapeutic planning [31, 32]. In our study, 5% of patients had involved sentinel nodes while non sentinels were free of tumor which could potentially lead to upstaging in 5% of cases.

Impact of tumor stage and lymph node harvesting

Our study reaffirms that tumor size (T stage) significantly influences SLN detection accuracy, particularly in T1-T2 tumors. This underscores the critical need for harvesting an adequate number of lymph nodes (≥10–14) to avoid understaging [22, 28, 33, 34]. Furthermore, SN mapping in colorectal cancer has been shown to increase the proportion of N1 patients, thereby refining prognostic stratification and improving outcomes for the N0 subgroup. These findings reinforce the clinical value of SN mapping in colorectal cancer, particularly for identifying patients who may benefit from adjuvant therapy.

Limitations and future directions

The clinical significance of micrometastases in node-negative colorectal cancer remains controversial, with some studies linking them to higher recurrence and poorer survival, while others question their prognostic relevance. Although sentinel lymph nodes were successfully identified in 85% of patients in our cohort, confirming technical feasibility, the current false-negative rate of 28% indicates that SLN biopsy cannot currently replace standard lymphadenectomy in colorectal cancer. Therefore, these findings should be interpreted with caution and regarded as exploratory rather than definitive. Nevertheless, our novel radiotracer—by combining the visual guidance of blue dye with the sensitive detection capabilities of a radiotracer in a single injection—shows promise for identifying occult metastases, particularly in T1–T2 tumors. This approach may be especially useful for refining risk stratification in selected high-risk Stage II patients, although validation in larger studies is still required. This could be especially beneficial for high-risk Stage II patients and in minimizing unnecessary extended lymphadenectomies [9, 13, 35].

In our study, the median number of harvested lymph nodes was 11 (range: 7–16). Current international guidelines, however, recommend retrieval of at least 12 lymph nodes to ensure adequate N staging in colorectal cancer [36]. This point is particularly critical in the context of evaluating lymph node metastasis, as patients with a lower number of examined nodes (e.g., as few as 7) may be at risk of being understaged due to insufficient lymph node sampling rather than true absence of metastasis. Therefore, the relatively low lymph node yield in our cohort represents an important limitation of this study and should be taken into consideration when interpreting the findings. Moreover, the relatively small sample size further limits the statistical power and generalizability of our conclusions. Future studies with larger cohorts and long-term follow-up are warranted to correlate SLNB findings with recurrence and survival outcomes, and to further define the clinical role of this novel agent in nuclear medicine.

CONCLUSION

The use of patent blue-labeled liposomes tagged with [^{99m}Tc]Tc –HMPAO represents a promising strategy for SLN mapping in colorectal cancer. This dual-modality approach allows for concurrent visual identification using blue dye and radiographic detection via gamma probe from a single injection. Both PEGylated and non-PEGylated liposomes demonstrated comparable performance. Notably, the technique detected occult metastases in 5% of cases, leading to pathological upstaging. Sentinel node mapping with [^{99m}Tc]Tc -HMPAO-labeled liposomes enhances the accuracy of pathological staging and offers a high SLN detection rate, particularly in colon cancer, underscoring its potential value in surgical oncology.

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