Original article

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Ex vivo sentinel lymph node mapping in colorectal cancer using a magnetic nanoparticle tracer to improve staging accuracy: a pilot study

J. J. Pouw*, M. R. Grootendorst*†[1], J. M. Klaase†, J. van Baarlen‡ and B. ten Haken*

*MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands, †Department of Surgery, Medisch Spectrum Twente, Enschede, The Netherlands and ‡Laboratorium Pathologie Oost Nederland, Hengelo, The Netherlands

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Abstract

Aim Nodal status is the most important prognostic factor in colorectal cancer (CRC). Small occult metastases may remain undetected on conventional histopathological examination, potentially resulting in undertreatment. *Ex vivo* sentinel lymph node mapping (SLNM) can be used to improve the accuracy of nodal staging, but the currently used tracers suffer from drawbacks, which hamper implementation of the technique in routine clinical practice. Magnetic tracers are the optimal size for sentinel lymph node (SLN) retention and allow objective quantitative selection of SLNs; they therefore have great potential for SLNM in CRC. The study evaluates the feasibility of *ex vivo* magnetic SLNM and compares the performance of this technique with blue dye SLNM.

Method Twenty-eight *ex vivo* SLNM procedures were performed in 27 histological node-negative patients with CRC using a magnetic tracer and blue dye. A magnetometer was used to select magnetic SLNs after formalin fixation of the CRC specimen. Both magnetic and blue SLNs were subjected to serial sectioning and immunohistochemical staining to reveal occult metastases.

Results At least one SLN was successfully identified in 27/28 (96%) and 25/28 (89%) of the cases with the magnetic technique and blue dye. Isolated tumour cells were detected in 10 patients. This was predicted with 100% sensitivity and accuracy using the magnetic technique, and with 91% sensitivity and 96% accuracy using the blue dye technique.

Conclusion This study demonstrates that *ex vivo* magnetic SLNM is a feasible technique for use in routine clinical practice, improving nodal staging accuracy of CRC patients.

Keywords Superparamagnetic iron oxide, sentinel lymph node mapping, magnetic tracer, colorectal cancer, staging

What does this paper add to the literature?
This study is the first to use a magnetic tracer and handheld magnetometer for sentinel lymph node mapping in colorectal cancer. The procedure improves the accuracy of nodal staging by detecting small occult metastases, potentially allowing improved treatment. The *ex vivo* procedure can easily be implemented in routine clinical practice.

Introduction

Worldwide over 1.3 million patients a year are diagnosed with colorectal cancer (CRC). In the more developed regions of the world, CRC is the second most common cause of cancer-related death, accounting for over 300 000 deaths in 2012 [1]. Treatment with curative intent consists of *en bloc* resection of the affected colorectal segment and adjacent lymphatic drainage. Current guidelines demand that at least 12 lymph nodes (LNs) should be retrieved from the specimen and examined with haematoxylin and eosin (H&E) staining of a single cut surface for the presence of metastasis [2]. The presence of nodal metastasis is the most important prognostic factor, and determines whether adjuvant chemotherapy is indicated. Up to 30% of node-negative patients, however, develop recurrent disease within 5 years of surgery with curative intent [3,4]. Several retrospective studies

Correspondence to: J. J. Pouw and B. ten Haken, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, PO Box 217, 7500AE, Enschede, The Netherlands.
E-mails: J.J.Pouw@utwente.nl (JJP); B.tenHaken@utwente.nl (BTH).

[1]Present address: King's College London, Division of Cancer Studies, Breast Cancer Surgery Group, London, UK

attribute this high recurrence rate to the presence of occult nodal involvement, which remains undetected on conventional histopathological analysis [5–7]. Patients suffering from occult nodal involvement may therefore be understaged, potentially leading to under-treatment [6–8]. Although these small micrometastases (0.2–2 mm) and isolated tumour cells (ITCs; < 0.2 mm) can be detected using so-called 'ultrastaging' (serial sectioning and immunohistochemical staining or molecular pathology techniques), this is too expensive and time-consuming to perform on the large number of LNs retrieved from colorectal specimens in routine clinical practice. Since ultrastaging is not performed routinely, occult nodal involvement is missed. This results in inadequate staging and potentially inadequate treatment of a large group of patients. The goal of the present study is to provide a method to improve the accuracy of staging of conventional node-negative CRC patients that can be easily implemented in routine clinical practice.

Sentinel lymph node mapping (SLNM) allows identification of lymph nodes that receive direct lymphatic drainage from the tumour area – the so-called sentinel lymph nodes (SLNs). Since occult nodal involvement is predominately found in SLNs [5], their status is representative of the entire colorectal nodal basin. By subjecting only the SLNs to ultrastaging, the presence of occult metastasis can be accurately detected without considerably increasing cost and labour, thus allowing improved nodal staging.

At present, SLNM is performed by injecting a blue dye and/or radioisotope tracer peritumourally, followed by visual and/or gamma probe detection of discoloured and/or radioactive SLNs. The procedure can be performed *in vivo* or *ex vivo*, with a similar detection rate and accuracy [9,10]. Aberrant lymphatic drainage is reported in only about 1–10% of cases, and this is therefore a limited drawback when an *ex vivo* approach is used for SLNM. An important advantage of the *ex vivo* approach is that the patient is not exposed to the tracer and therefore not at risk of developing an allergic reaction [11,12]. Furthermore, an *ex vivo* procedure does not add time to the surgery. Although the colloidal properties of radiotracers are beneficial for SLN detection [13,14] they are generally not used in an *ex vivo* setting because the use of radioisotopes is strictly regulated [15]. Blue dye is readily available and not limited by radiation safety legislation, but due to the small size of the blue dye particles, they rapidly distribute through the lymphatic system, not only colouring the true SLNs but also higher-echelon nodes. In routine clinical practice, all LNs are dissected from the formalin-fixed tissue by the pathologist, usually on the day after surgery. Because of the distribution to higher-echelon nodes and dilution of the blue dye in formalin, the identification of SLNs must be performed shortly after injection of the tracer. Widespread clinical implementation of SLNM in CRC patients requires a tracer that allows detection of SLNs after formalin fixation during routine pathological analysis, without the regulatory and logistical barriers that hinder its day-to-day use.

Magnetic tracers have several advantages for use in SLNM in CRC. We previously demonstrated that they accumulate in the LNs in CRC after *ex vivo* administration using nonclinical laboratory equipment [16]. Recently the use of magnetic tracers was also evaluated for SLN biopsy in patients with breast cancer [17]. These tracers consist of superparamagnetic iron oxide (SPIO) nanoparticles with a hydrodynamic size of 50–150 nm, which is favourable for retention in the SLN [18]. The colloidal properties of the tracer also prevent dilution from formalin fixation. Another advantage is that the presence of a magnetic tracer in LNs can be easily quantified with a handheld magnetometer [18], allowing objective discrimination between SLNs and higher-echelon nodes. In addition, magnetic tracers do not suffer from the regulatory and logistical barriers associated with radioactivity, thus providing flexibility to perform SLNM. Because of these advantageous, magnetic SLNM has great potential to improve nodal staging accuracy in CRC patients in routine clinical practice. In this study we evaluate the feasibility of using *ex vivo* magnetic SLNM in a routine clinical work flow and compare this novel technique with *ex vivo* blue dye SLNM with SLN identification after formalin fixation.

Method

Patients with clinical Stage I/II CRC scheduled for curative surgery were eligible for inclusion. The study was performed in agreement with the local ethics committee and all patients gave written informed consent. Exclusion criteria included the presence of distant metastasis, intra-operative gross nodal involvement and preoperative neoadjuvant chemoradiotherapy. Forty *ex vivo* SLNM procedures were performed in 39 patients, including one patient who had two tumours resected. All patients underwent a standard oncological resection and *en bloc* lymphadenectomy according to the location of the tumour. The presence of nodal metastasis was detected by conventional histological analysis in 12 of the 39 patients. Since the goal of the study was to improve the accuracy of staging of those patients without nodal metastasis on conventional histological analysis, these 12 patients were excluded from further study. The remaining 27 node-negative patients (28 procedures) comprised the group investigated in the study.

Sentinel lymph node mapping procedure

Immediately after resection, the fresh surgical specimens were sent to the pathology department. Colonic specimens were opened antimesenterically by the pathologist or technician, and rectal specimens were opened on the anterior border opposite to the mesorectum, leaving the mesorectum intact. The tumour was located, and the diameter determined.

First, 2 ml of magnetic tracer was injected submucosally at four sites around the tumour. In the first 10 procedures an MRI contrast agent, Endorem® (Guerbet B.V., Gorinchem, The Netherlands), was used as the magnetic tracer. In the remaining 18 procedures, a magnetic tracer optimized for SLN localization [18], Sienna+® (Endomagnetics Ltd, Cambridge, UK), was used. Endorem® consists of SPIO nanoparticles with a mean hydrodynamic diameter of 111 nm and contains 11.2 mg iron (Fe)/ml. Sienna+® has a concentration of 28 mg Fe/ml and the nanoparticles have a mean hydrodynamic diameter of 59 nm. The original tracer was changed because a new tracer specifically designed for SLN localization became commercially available during the course of the study. Since production of the MRI contrast agent was discontinued, it was used as an alternative. A gentle massage of the injection sites was performed for 3 min to promote distribution of the *ex vivo* tracer. Subsequently, an optimized dose of 0.5–2 ml of Patent Blue V (Guerbet B.V.) was injected at the same sites (0.5 ml/cm of tumour diameter [19]), followed by massaging for 5 min. The magnetic tracer was injected first, because it was anticipated that distribution of the viscous magnetic tracer would be more difficult than that of the more fluid blue dye.

After administration of the tracers the specimen was fixed in formalin (10%) for 24–72 h. Following fixation, all LNs were harvested from the specimens according to the standard histopathological protocol. All harvested LNs were individually placed on the probe of a handheld magnetometer (SentiMAG®; Endomagnetics Ltd) to select the LNs that had taken up magnetic tracer (Fig. 1). The probe was balanced in air (away from any magnetic material) before each measurement to compensate for any drift of the signal. The signal is proportional to the amount of magnetic tracer [18,20] and was used to quantify tracer uptake. A SLN was defined as a lymph node with uptake of magnetic tracer. If more than three nodes with tracer uptake were identified, those with the highest SPIO uptake were designated as SLNs. Blue nodes were also defined as SLNs, if more than three blue nodes were detected the first three blue nodes closest to the tumour were selected as SLNs. This definition is similar to that used by Faerden *et al.* [21]. A procedure was considered successful if at least one SLN was identified. If ITCs and/or micrometastasis was found in a LN, but not in one of the SLNs, this was considered to be a false negative result.

Figure 1 Selection of lymph nodes with uptake of magnetic tracer. A blue formalin-fixed lymph node is placed on the handheld probe (covered with a surgical glove) of the magnetometer with plastic tweezers. The numerical display of the system is used to quantify the uptake of magnetic tracer.

Histopathological analysis

All lymph nodes were embedded in paraffin and stained with H&E. In addition to this conventional staging, SLNs of the node-negative patients were subjected to ultrastaging. The SLN was additionally sectioned at 250-μm intervals, and coloured with Cam5.2, an antibody against keratin, and H&E. Positive cells were categorized as isolated tumour cells (< 0.2 mm) or micrometastasis (0.2–2 mm). Rare single positive-staining cells with immunohistochemical staining lacking cytological characteristics of malignancy were considered negative. In the first 10 procedures ultrastaging was performed on all resected nodes, to rule out false negative results. If none was detected, ultrastaging of the non-SLNs would be omitted in the remaining procedures. Ultrastaging of the non-SLNs was not performed in the vast majority (about 83%) of studies reported in literature [5], and omission was therefore justified.

Statistical analysis

Means and range were calculated for continuous variables. The number of SLNs identified with both techniques were compared using a paired *t*-test. Statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, Illinois, USA), and $P < 0.05$ was considered significant.

Results

Twenty-eight *ex vivo* SLNM procedures were performed in 27 patients who underwent intended curative surgery for CRC and were lymph node negative on routine histological staining. Patient and tumour characteristics are provided in Table 1. At least one SLN was successfully identified in 27/28 procedures with the magnetic tracer and in 25/28 procedures with the blue dye, resulting in an identification rate of 96% for the magnetic technique and 89% for the blue dye technique. The results of the SLNM procedure are given in Table 2.

A total of 607 LNs were dissected, of which 199 (mean 7.4, range 1–16) were magnetic and 205 (mean 8.2, range 3–36) were blue. More than three LNs with tracer uptake (blue and/or magnetic) were found in all but three procedures. The uptake of magnetic tracer in the LNs could be easily quantified with the handheld magnetometer, and allowed an objective selection of three SLNs with the highest uptake in all procedures. From all LNs with magnetic tracer uptake, the three with the highest uptake per patient were designated to be magnetic SLNs: the LNs with lower uptake were considered to be non-SLNs. Of the blue lymph nodes, the three closest to the tumour were designated to be blue SLNs, the other blue LNs located further away were considered to be non-SLNs. It is therefore possible that a node might demonstrate tracer uptake (blue and/or magnetic), but would not have been considered as a SLN. A total of 120 SLNs were selected from all nodes with tracer uptake (blue and/or magnetic).

Of the 120 SLNs, 33 were selected as both magnetic and blue SLNs, 46 were selected as magnetic SLNs only and 41 were selected as blue SLNs only (Fig. 2). Only 11 magnetic SLNs were not coloured blue and only 11 blue SLNs were not magnetic.

Ultrastaging of the SLNs by serial sectioning and immunohistochemical staining showed the presence of ITCs in 10/27 conventional node-negative patients.

Table 1 Patient and tumour characteristics of the included patients

Characteristic	*n*
Gender	
Male	13
Female	14
Age (years), mean ± SD (range)	73 ± 6 (63–84)
Tumour size (cm), mean ± SD (range)	4.3 ± 2.7 (1–11.5)
Tumour location	
Caecum	5
Ascending colon	7
Transverse colon	1
Descending colon	1
Sigmoid colon	11
Rectum	3
Depth of tumour invasion	
T1	4
T2	6
T3	15
T4	3

Table 2 Sentinel lymph node mapping procedure and ultrastaging results

Characteristic	*n*
Identification rate	
Magnetic	27/28 (96%)
Blue	25/28 (89%)
Number of dissected nodes, mean ± SD (range)	21.7 ± 11.6 (7–54)
Magnetic	7.4 ± 4.1 (1–16)
Blue	8.2 ± 6.6 (3–36)
Total number of SLNs	120
Magnetic and blue	33
Magnetic	46
Blue	41
Histology after ultrastaging	
Negative	18
Isolated tumour cells	10
Micrometastasis	0
False negatives after ultrastaging	
Magnetic	0
Blue	1

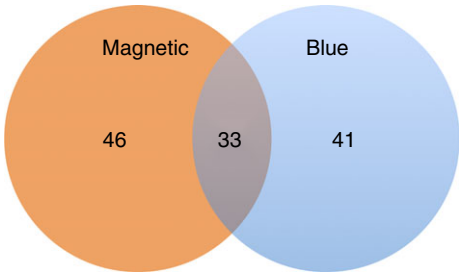

Figure 2 Number of magnetic and/or blue sentinel lymph nodes identified by the different techniques.

No micrometastases were detected. On a node-to-node basis, ITCs were detected in 11 SLNs that were both magnetic, six solely blue SLNs and five solely magnetic SLNs. Isolated tumour cells were found in two non-SLNs from one patient. On a patient-to-patient basis the magnetic technique accurately predicted the presence of occult tumour cells in all patients, but in one patient ITCs were found solely in a magnetic SLN that was not blue, resulting in a false negative result with the blue dye. No false-negative results were identified when ultrastaging all the non-SLNs of the first 10 patients, therefore ultrastaging of the non-SLNs was omitted in the remaining procedures of this pilot study.

Discussion

The presence of nodal metastasis is the most important prognostic factor in CRC and is used to determine whether a patient should receive adjuvant treatment. Accurate nodal staging is therefore of great importance. SLNM can be used to improve the accuracy of nodal staging by identifying the nodes at risk of harbouring occult metastasis, and subjecting these nodes to ultrastaging. This study demonstrates the feasibility of a magnetic technique for *ex vivo* SLNM to improve the accuracy of nodal staging in routine clinical practice. The use of a magnetic nanoparticle tracer and handheld magnetometer was recently evaluated for *in vivo* SLN biopsy in breast cancer patients and shown not to be inferior to the standard technique [17]. Our group previously explored the potential of using magnetic nanoparticles for *ex vivo* SLNM in CRC [16,22]. To our knowledge, the current study is the first to evaluate the magnetic technique for *ex vivo* SLNM in CRC in a routine clinical workflow. An identification rate of 96% was achieved with the magnetic tracer, which is in line with the previously reported average identification rate of 94% with blue dye and/or radioisotopes [15,23].

The identification rate using the magnetic technique was higher than with the blue dye. There are two possible explanations for this difference. First, the magnetometer is able to detect very low amounts of tracer, whereas small amounts of blue dye are difficult to distinguish visually. Secondly, formalin fixation of the tissue can dilute the blue dye and thus lower its concentration so that it is no longer visible, resulting in a false negative. In contrast, the colloidal properties of the magnetic tracer prevent dilution once it has taken up by the LNs. Therefore, the magnetic technique does not suffer from dilution of the tracer, allowing identification of SLNs through the presence of small amounts of magnetic tracer, even after formalin fixation.

Identification of SLNs with blue dye is generally performed immediately after surgery [15] to prevent washout of the dye after formalin fixation. This procedure does not, however, fit easily into a routine clinical workflow. Identification after formalin fixation, as described by Faerden *et al.* [21] and Smith *et al.* [24], allows the procedure to be performed in routine clinical practice. Since the goal of this study was to develop a method for the identification of SLNs in routine clinical practice, this approach was used for both the blue dye and magnetic tracer.

Although the magnetic tracer consists of larger particles, there was no significant difference in the mean number of blue nodes and the mean number of nodes with magnetic tracer uptake. This is also most likely because of the high sensitivity of the magnetometer for small amounts of magnetic tracer, as it could result in detection of higher-echelon nodes with very limited tracer uptake. Most non-SLNs with uptake of magnetic tracer (77/120 non-SLNs) displayed a signal less than 10% of that of the most magnetic SLNs, supporting the assumption that higher echelon nodes taking up small amounts of magnetic tracer were also detected. This results in a large number of magnetic lymph nodes. In our study, the three most magnetic LNs were designated as SLNs, and the 10% criterion was not used to discriminate between SLNs and non-SLNs. Future studies should determine whether the 10% criterion can be used to further reduce the number of magnetic SLNs. A unique property of the magnetic technique is that the quantitative signal allows the objective selection of the SLNs. Furthermore, unlike the blue dye, a standardized dose of 2 ml of SPIO was used whatever the tumour diameter. Dose optimization of the magnetic tracer is likely to reduce the number of magnetic nodes as well, because it will restrict tracer distribution to higher-echelon nodes.

A large discordance between the nodes selected as SLNs was observed between the magnetic and blue techniques. Of the 120 SLNs, 33 were selected as both magnetic and blue SLNs, 46 were selected as magnetic SLNs only and 41 were selected as blue SLNs only. However, only a small proportion of magnetic SLNs (11/46) were not coloured blue and a similar small proportion of blue SLNs were not magnetic (11/41). The observed discordance is therefore likely to arise from differences in the definitions used for the selection of the magnetic and blue SLNs, the former being based on an objective functional definition the latter on a subjective anatomical definition. In some procedures it was difficult to determine which lymph nodes were closest to the tumour, emphasizing the subjective nature of this definition.

Ultrastaging of the SLNs revealed ITCs in 10/27 patients (37%), which is concordant with previous studies [5]. The presence of occult metastasis was accurately predicted by the magnetic technique in all 10 patients, and in 9 out of 10 by the blue dye technique. Ultrastaging did not show any micrometastasis. The true false negative rate cannot be determined because ultrastaging of the non-SLNs was only performed in the first 10 patients. Because no false negative results were obtained in these patients, and ultrastaging was generally limited to the SLNs only [5], our results indicate that the magnetic technique accurately predicts the presence of occult metastasis. Ultrastaging of the non-SLNs was limited to the patients in whom Endorem® was used as tracer. Since a different tracer was used in the remaining patients this could have influenced the results. Both tracers, however, were very similar in terms of constituents and particle size distributions [18], and this is therefore unlikely. To date no prospective trials have been performed to determine whether patients with ITCs/micrometastasis would benefit from adjuvant treatment, but a recent meta-analysis concluded that patients with micrometastasis have a worse prognosis than node-negative patients and patients with ITCs [25]. The clinical relevance of only finding ITCs and not micrometastasis therefore seems limited. Although no micrometastases were detected, the feasibility of the technique to accurately select positive lymph nodes was demonstrated. Based on these results, we advise that a study should be carried out with more patients to determine the clinical relevance of the magnetic technique.

An alternative technique for SLNM that uses a near-infrared (NIR) fluorescent tracer and imaging system has been described previously. This allows visualization of tracer distribution from the injection sites to the sentinel nodes, and has been used *in vivo* and *ex vivo* in CRC [26–28]. Schaafsma *et al.* [27] and Hutteman *et al.* [26] used an *ex vivo* approach and reported sensitivities of 80% and 92%, respectively, for macrometastasis. However, they did not perform ultrastaging on the SLNs to detect the presence of occult tumour cells; therefore their results cannot be compared with ours. A limitation of this technique is that fluorescent tracers do not allow the detection of SLNs after formalin fixation due to migration to higher-echelon nodes [27]. This makes the method less suitable for colorectal SLNM to improve the accuracy of staging in routine clinical practice.

The present study has demonstrated that the use of a magnetic tracer for *ex vivo* SLN selection is feasible. Rather than improving the accuracy of staging, the goal of an *in vivo* procedure is to allow for a limited resection of the increasing number of early stage tumours [29] or patient-tailored determination of the extent of the lymphadenectomy [30]. Since the magnetic tracer Sienna+® is approved for *in vivo* use, and the magnetometer has a favourable detection depth of 2–3 cm, it could be used for this purpose, although no magnetometer for laparoscopic use is yet available. A limitation of the current study is the small number of patients, in particular the small number with occult metastasis. Larger studies are needed to validate the advantages of the magnetic technique over the blue dye technique.

In conclusion, this study has demonstrated that *ex vivo* magnetic SLNM is a feasible technique for use in routine clinical practice. The magnetic tracer can be detected in LNs after formalin fixation, and the quantitative signal of the magnetometer can be used to objectively distinguish SLNs from higher-echelon nodes. The magnetic technique has achieved an excellent identification rate, sensitivity and accuracy, and is superior to the blue dye technique. The technique can be used to improve staging accuracy of CRC patients in routine clinical practice.

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Conflicts of interest

None.

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