

Immunohistochemical Study of Sentinel Lymph Node in Colon Cancer

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ABSTRACT: Identification of sentinel lymph node (SLN) in colon cancer is very important in order to increase the accuracy of lymph node staging. The number of examined lymph nodes represents a significant predictor of survival. This study aims to show the importance of SLN histological and immunohistochemical examination in adjuvant oncological treatment. The study includes 23 patients with colon cancer (44% women and 56% men) who came in our clinic for surgical intervention. In all cases, the SLN was identified and prepared for histological examination. In 13 of the cases, micrometastases were found on haematoxylin-eosin (HE) staining, there were 5 cases with positive immunohistochemistry using antibodies anti-p53, anti-VEGF-C, anti-CD34, and 5 cases with SLN negative both for HE and immunohistochemistry. Altogether we had a detection rate of 92%, an accuracy of 78,2%, a sensitivity of 90%, a false negative rate of 10% and a negative predictive value of 71,4%, good values according to the literature. Four (17,3%) patients had micrometastases exclusively in the sentinel lymph node, after performing additional histological examination, using multilevel section and immunohistochemistry. After assessing the SNL on our patients, we concluded that it is a reproducible practice for lymph node analysis.

KEYWORDS: *Immunohistochemistry, sentinel lymph node, colon cancer*

Introduction

In colon cancer, which still represents one of the most common malignancies worldwide [1], the primary treatment remains the surgical one with resection of all masses and removal of all mesenteric lymph nodes. After surgery, the most important step for disease prognosis is the histological assessment of the resected lymph nodes, forming the basis of the multidisciplinary decision for receiving or not the adjuvant chemotherapy [2]. Also, the total number of examined lymph nodes represents a prognostic factor. The current guidelines recommend to be evaluated a total number of minimum 12 lymph nodes to adequately assess the tumor stage [3]. Regarding the examination of the lymph nodes, those node-negative patients are nowadays excluded by postoperative treatment [4]. There is still the problem of stage migration that means the migration from node-negative to node-positive colon cancer based on a better assessment of the lymph nodes using sentinel

node techniques and immunohistochemistry [5]. However, stage migration can happen also to patients with stage III colon cancer, with positive nodes, reflected by the survival rates on long term. In order to reduce stage migration, it was suggested to use lymph nodes ratio obtained by dividing the total number of positive lymph nodes to the number of examined lymph nodes [6,7].

This study is a multidisciplinary experience and includes patients with colon cancer with node positive undergoing curative surgery and adjuvant chemotherapy.

Materials and Methods

The study includes patients with colon cancer histological certified undergoing curative surgery defined as removal of all tumoral masses, absence of microscopic residual tumor, negative resection margins in histological exam and extended lymphadenectomy [8].

From January 2014 to June 2015, 109 patients with colon cancer were referred to

our surgical department for appropriate treatment. Patient exclusion criteria in the study were: presence of T4 tumours and absence of distant metastases identified in preoperative or intraoperative evaluation, emergency cases, patients with no possibility of tumour resection, all the cases with invasion of lymph nodes in the moment of the intervention, patients with synchronic colon and rectal cancer, patients with previous abdominal surgical treatment because of the adhesions. In this study we were interested of the cases with colon cancer so that in a further study we will approach the sentinel lymph node (SLN) for the rectal cancer patients.

The study protocol was approved by the local Ethics Committee of the University of Medicine and Pharmacy from Craiova, and all patients were fully informed before giving their written consent to the procedure.

After applying all these criteria, the study includes 25 patients who came in our department to follow the surgical treatment and to whom was identified the sentinel lymph node by injecting 1-2ml of blue dye into the subserosa, around the tumor. For the first 5 cases it was practiced an ex vivo technique in order to apprehend the technique and the others were performed following an in vivo procedure. After inoculation, infiltration points were massaged for 5min until the first lymph nodes were seen, ranging from 1 to 4, and they were considered SLN (Fig. 1).



Fig. 1. Sigmoid colon tumour and blue stained sentinel lymph node

Each SLN or sections were included in paraffin blocks and marked according to standard procedure. Sections with a thickness of 5µm were cut from each block at 3 levels; and for each level, 2 sections were stained with haematoxylin and eosin, and a third one was kept for the immunohistochemical analysis, which was performed if the conventional analysis turned out negative. For the immunohistochemical study, 3µm thick sections were routinely prepared from the paraffin blocks. Separated biological material was collected on special histological plates coated with poly-L-lysine (Sigma). The immunohistochemical protocol consisted in dewaxing and hydration of the sections, antigen retrieval by boiling them in a sodium citrate solution, pH 6, for 21 minutes (7 cycles of 3 minutes), in a microwave oven, blocking the endogenous peroxidase by incubating the slides in 3% hydrogen peroxide for 30 minutes at room temperature followed by washing in distilled water for 10 minutes and washing in 1×phosphate buffered saline (PBS) for 5 minutes. Blocking of non-specific sites was achieved by passing the sections into a 2% skim milk solution for 30 minutes. Thereafter, the sections were incubated with the primary antibodies for 18 hours (overnight) in a refrigerator at 4°C, and the next day a biotinylated secondary antibody was applied for 30 minutes at room temperature (Avidin-Biotin System, Dako, Glostrup, Denmark). Next day the protocol continued with washing of the biological material in 1× PBS followed by a streptavidin-HRP solution for 30 minutes at room temperature and then the slides were washed again in PBS 3 times for 5 minutes each time. The signal was detected using 3,3'-diaminobenzidine (DAB) (Dako) and the reaction was stopped in 1× PBS. The sections were contrasted with Mayer's hematoxylin, dehydrated, cleared in xylene and mounted using a xylene-based medium DPX (Fluka).

In our research we used the following markers:

- anti-p53, clone DO-7, 1/50 (Dako);
- anti-VEGF-C, clone VG1, 1/25 (Dako);
- anti-CD34, clone EP373Y, 1/100 (Abcam);
- Anti-pancytokeratin, clone AE1-AE3, 1/50 (Dako)

An immunohistochemical examination of the colon tumours themselves was also performed (Fig. 2). Micrometastasis was considered as tumour areas less than 2mm.

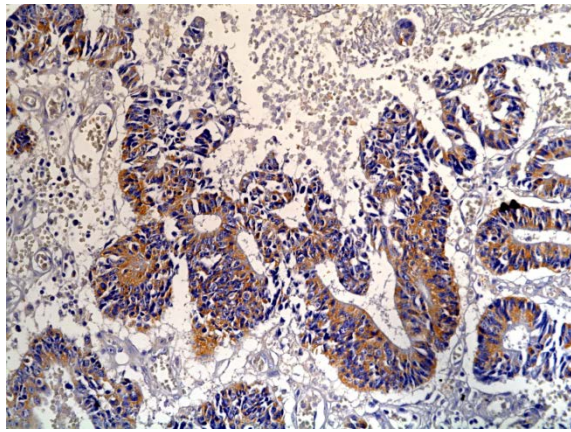


Fig. 2. Immunohistochemical study of the colon tumour with intense reaction to VEGF, $\times 4$ objective

After dye was infiltrated, bowel resections were performed as standard open procedure.

Micrometastasis were the nodal tumours, which measured less than 0.2cm in the larger diameter, or it was only detectable by immunohistochemistry. Micrometastases were considered in the SLN upstaging benefit [9].

Lymph nodes were sliced up to maximally 2mm and processed to paraffin blocks for hematoxylin and eosin staining.

In tumor-negative SLNs at routine hematoxylin-eosin (HE) examination (pN0) we performed immunohistochemistry (IHC).

Tumor cell deposits larger than 0.2mm but smaller than 2mm in diameter were classified as micrometastases (MM).

Tumour cell clusters up to a diameter of 0.2 mm or single cytokeratin-positive cells were classified as "isolated tumour cells" (ITC) according to Hermanek et al. [10] and the UICC/AJCC [11].

The following definitions were used for tumour assessment calculations:

- Detection rate;
- Sensitivity;
- False-negative rate;
- Negative predictive value;
- Accuracy.

Results

The procedure was successful in 23 out of 25 patients (92%), with identification of at least one sentinel lymph node, which represents the detection rate. No patient showed adverse reaction to the blue staining.

All the characteristics of the patients being evaluated were collected in a database containing the age, gender, topography, complications after surgery, number of

examined lymph nodes and all these data are presented in a table (Table I).

Table 1. Patients characteristics

	No.	%
Number of patients	25	100
Number of patients with SLN identified	23	92
Patients with positive SLN at HE	13	52
Patients with negative SLN at HE and IHC	5	20
Patients with negative SLN at HE, and positive at IHC	5	20

In 2 patients from 25 studied were identified macro metastases in the lymph nodes and were considered nodal positive (pN1 or pN2). These 2 patients were not included any further in the study of SLN.

The histopathological examination revealed that most of them had an adenocarcinoma structure respectively 88%, 8% of the cases presented a mucinous carcinoma and in one case it was a mucinous carcinoma with signet ring cells.

After the sentinel lymph node was dyed, it was realized first the histopathological examination with haematoxylin-eosin (HE) resulting a number of 13 lymph node positive with this coloration (Fig. 3), to the other 10 SLN haematoxylin-eosin negative tissues we continued with the immunohistochemical analyses using the above mentioned antibodies.

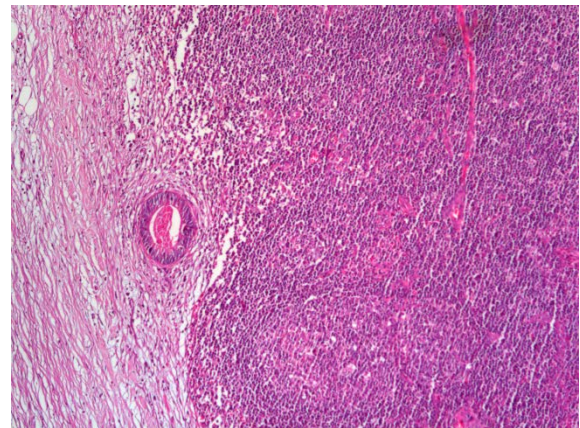


Fig. 3. Sentinel lymph node coloured in haematoxylin-eosin, $\times 10$ objective

In 5 cases the SLN, there was no micro metastasis detected after the immunohistochemical examination and in 5 cases it was determined the presence of the SLN micro metastases (Fig. 4 a,b).

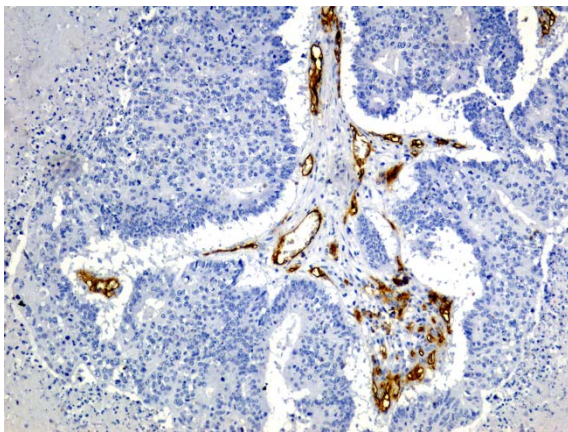


Fig. 4a. Sentinel lymph node with angiogenesis vessels developing in stroma, anti-VEGF, × 10 objective

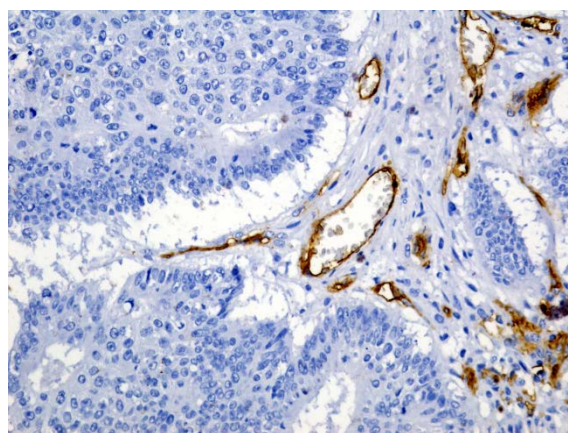


Fig. 4b Detail from the previous figure, × 20 objective

Table 2. SLN examination

Parameter	Value
Number of patients	25 (100%)
Female	11 (44%)
Male	14 (56%)
Age (years)	42-84
Topography	
Right colon	12
Transverse colon	1
Left colon	12
Length of stay in hospital (days)	7- 16
Complication rate	1 (fistula)
No. of examined lymph nodes per patient	12-19

The study revealed a detection rate of 92%, an accuracy of 78,2%, a sensitivity of 90%, a false negative rate of 10% and a negative predictive value of 71,4%.

Upstaging refers to an upward change in pathological staging, which may then alter patient treatment if there is a shift from node-negative (pN0) to node-positive (pN1 or pN2). This is because node-positive patients will

receive chemotherapy, while node-negative patients may not. There were discovered 4 cases of micro metastasis and an upstaging rate of 17,3% (Fig. 5).

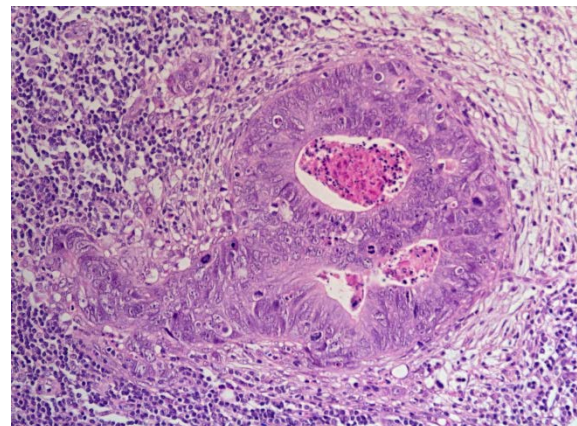


Fig. 5. Lymph node micrometastasis in HE coloration in colon cancer, × 20 objective

Result at 6 months and one year

All 23 patients were investigated after surgery at 6 months and one year. At 6 months they were only examined by CT, while at one year they were also investigated through colonoscopy.

At 6 months, from all 23 patients, one patient with SLN positive at HE died from a heart disease but no colon postoperative complications. The others 12 patients HE positive followed an oncologic postoperative treatment, while the others SLN negative did not have another therapy than the surgical one.

At one year one of the patients with SLN positive at HE came to the emergency room with symptoms of intestinal occlusion and intraoperative was diagnosed with metachronous right colon cancer after a left colectomy.

At one year, the CT examination revealed at one of the patients with positive SLN a hepatic metastasis on left side of the liver which was suitable for surgical resection.

Colonoscopy of the patients did not show to any of the them recurrent tumour.

CT examination of the patients which were SLN negative with no postoperative oncological treatment did not revealed peritoneal metastases, only one patient presented 2 mesenteric lymph nodes of 0,5cm and respectively 1 cm diameter which was proposed for exploratory laparoscopy to evaluate the lymph nodes extension and if was possible to remove them.

Evaluation of the patients was possible because of the good collaboration between surgeon, oncologist and radiologist.

Discussions

The main reason for identifying the SLN on the patients with colorectal and gastrointestinal cancers is simply, for increasing the accuracy of lymph node staging, this way limiting detailed examination only to “critical” lymph nodes [12-14]. The number of lymph nodes removed during colon tumour resection represents a significant predictor of survival and therefore the extent of lymphadenectomy will not be altered by SLN biopsy. Discussing about SLN in patients with melanoma and breast cancer, the goal of SLN biopsy is to provide prognostic information while minimizing the extent of lymph node dissection [15,16].

The number of lymph nodes examined is very important for staging, and directly influences treatment and prognosis of patients operated for colon cancer. To obtain reliable pathology, the pathologists should examine at least 10-12 lymph nodes per patient. Many studies have found that when are examined less than 10 lymph nodes the survival at five years is approximately 73%, 80% when there are examined 11-20 lymph nodes, and 87% with more than 20 lymph nodes examined [17]. The American Joint Committee on Cancer (AJCC) recommends that at least 7-14 lymph nodes should be examined [18].

In our study, we found a mean of 12.3 lymph nodes per patient and a success rate in identifying sentinel lymph nodes in colon cancer of 92%. In literature worse results are influenced by: flaws in the injection; advanced stage of the lesion; mucinous histological type and we had this type of adenocarcinoma in 8% of the cases, neoadjuvant radiotherapy [19-21].

Several studies reported a SLN identification rate between 58% and 100% and the greatest percentage was reached in case series with the largest number of patients. Upstaging obtained with SLN analysis varies greatly, ranging from 6% to 60%, yielding non-conclusive results, in our study being a percentage of 17,3% [14,22].

In the specific case of sentinel lymph node study for colon tumours, the adverse findings with high rates of false negative did not interfere with the results because the therapeutic radical lymphadenectomy is always maintained, regardless of the presence or absence of metastases in regional lymph nodes.

We had a rate of 10% false negative results during this study. According to a review, the average overall rate of false negative rate is 33%, ranging from zero to 63% [23,24]. According to the validation of this study, the

sensitivity rate that we obtained was of 90%, while in the literature it ranges from 40 to 100% [25-27].

Van der Zaag et. al [28] made a interesting study of 908 lymph nodes from 58 patients with pN₀ carcinomas, three serial sections (cut at 500µm intervals) of all (according to standard evaluation on HE stained slides) were examined with three different antibodies [directed against pancytokeratin (Cam5.2), cytokeratins 20, and Ber-EP4]. The examination revealed occult tumour cells in 33% (19 of 58) of histologically pN₀ patients (12% micro metastases and 21% isolated tumour cells). Occult tumour cells were predominantly found in sentinel nodes with an overall sensitivity of sentinel mapping for occult tumour cells of 88%.

In this study, four (17,3%) patients had micro metastases exclusively in sentinel lymph node, after performing additional histological examination, using multilevel section and immunohistochemistry. These 4 cases could benefit from the investigation of sentinel lymph node because the disease is diagnosed when the chances of providing a cure with adjuvant chemotherapy would be greater by treating the tumour in a more incipient phase [29].

Comparing this study with a previous one [30], the sentinel lymph nodes were examined only by HE after 6 serial sectioning and considering the short period of the study and the limited number of the patients, we can state that the percentages of accuracy, sensitivity and upstaging have a higher rate, in our opinion being influenced by the immunohistochemical examination.

Conclusion

Lymph node staging is a major prognostic factor in colorectal cancer and seems to be the most important criteria in selection of the patients for adjuvant treatment.

After applying SNL procedure on our patients, we concluded that it is a reproducible practice for lymph node analysis, without significant time or cost increase and this is a technique that may be able to upstage 17,3% of patients who were classified as N0 under the conventional technique, which could lead to changes in adjuvant treatment guidelines.

Many other studies should be conducted on a greater number of patients, thus multicentre, and standardising the technique applied, regarding the marker used, surgical approach (laparoscopy vs. laparotomy), and “in vivo” or “ex vivo” lymph node dissection.

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