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Practical value of the complex analysis of sentinel lymph nodes in colorectal carcinomas

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Abstract

Background: Despite modern factors, which seem to predict outcome, lymph node (LN) status remain the main prognostic factor, which also shows the need for complex oncotherapy in colorectal carcinomas (CRC). Sentinel lymph nodes (SLNs) mapping is a very controversial method, which can increase the number of identified LN. *Materials and Methods*: In 28 patients who underwent surgical intervention between December 2009 and December 2010, we performed *in vivo* SLNs mapping followed by *ex vivo* examination at 1, 10, and 48 hours. All blue nodes were separately included. In cases without LN metastases (pN0) five multilevel sections and immunohistochemical stain with cytokeratin 20 were performed in SLNs. *Results*: Two cases were excluded because they were in pT4 stage. In one case the diameter of lymph nodes was about 10 mm and we obtained a false negative result (negative SLNs with positivity in the non-SLNs). From the other 25 cases, 13 do not presented LN metastases or micrometastases, nine had metastases only in the SLNs and the other three in both SLNs and non-SLNs. Mean identified number of LNs was 15. The blue dye intensity increased after formalin fixation and some nodes with metastases were blue stained only after 10 hours. *Conclusions*: SLNs mapping is a simple and inexpensive technique, which can improve the management of CRC. All *in vivo* and *ex vivo* blue LNs should be considered SLNs. Ultrastaging of SLNs is an expensive method, with uncertain results. High diameter of LNs seems to be an exclusion criterion for SLNs mapping.

Keywords: colorectal carcinoma, sentinel lymph node, lymph node metastases, prognosis.

Introduction

The concept of "sentinel lymph node" was first applied in 1960, by Gould EA *et al.* [1], in parotid carcinoma and by Cabanas RM in penile carcinoma [2]. Melanomas [3] and breast cancer [4] were the other two lesions in which sentinel lymph nodes (SLNs) mapping and removal seem to limit postoperative morbidity.

In colorectal cancer, many researchers admit the prognostic value of lymph node metastases [5] and nodal status predicts the need for postoperative oncotherapy. High rate of recurrence are reported in the cases diagnosed with pN0 (lack of lymph node metastases) which are not allowed for adjuvant chemotherapy. Up to 30% of patients with negative nodes recur or die from distant metastases [6]. Some authors agree the possibility of false negative staging due to tumor cell deposits, which can be present in lymph nodes but cannot be diagnosed with conventional histopathologic techniques or to insufficient number of lymph nodes, which are removed from the surgical specimen. The majority of researchers agree that 12 lymph nodes are necessary for a correct nodal staging but this number is under discussions [7, 8]. In other cases, aberrant lymph node drainage can lead to improper removal of lymph nodes during surgery.

Based on these concepts about importance of lymph nodes (LNs) in therapy and patients' survival, the sentinel lymph node mapping in colorectal cancer is performed in order to decrease the false-negative results and to increase the number of identified LNs. One of the first description of this concept was proposed in 1999 by Joosten JJ *et al.* [9]. The mapping can be performed in vivo or ex vivo with different substances: blue dye, radioactive or fluorescent tracers [10]. These techniques identify first 2–4 nodes that have drainage from the colorectal tumor. *In vivo* SLNs mapping is the same accurate as the *ex vivo* technique [11], one of the disadvantages for *ex vivo* mapping being the impossibility of detection aberrant lymphatic drainage. On the used substances, the blue dye and radioactive tracers have same false-negative rate [12]. The number of identified SLNs is reported to be between 1 to 8 [13, 14].

The most recent studies regard the factors which affect false-negative rate on lymph node mapping and also the practical importance of micrometastases or tumor isolated cells in patients' prognosis and therapy [15, 16]. Lymph node micrometastases include

metastases between 0.2 and 2 mm, which are detected either multilevel sections or immunohistochemically and cluster metastases or isolated cell metastases refer to cluster tumor cells smaller than 0.2 mm [17]. Both of them are not accepted by AJCC (*American Joint Committee on Cancer*) and WHO (*World Health Organization*) like upstaging methods.

The aim of the present study was to quantify the benefits of SLNs mapping in colorectal cancer and to identify the proper method for their detection.

Materials and Methods

Patients

Twenty-eight patients who underwent surgical intervention between December 2009 and December 2010 were enrolled for the study. All of patients do not received pre-operative radiotherapy. In every case open surgery was performed to remove the colorectal tumor. The agreement of the local ethical committee and the patient's consent have been obtained.

In vivo lymph nodes mapping

In all 28 cases, *in vivo* sentinel lymph nodes mapping was performed. The 1–2 mL blue dye was injected by a tuberculin syringe, subserosally (Figure 1), around the tumor, in a circumferential manner, respecting the Saha S *et al.* recommendations [18]. The first lymph nodes, which became blue in first 5–10 minutes were marked as sentinel lymph nodes (SLNs), they have been removed and have been sent to the pathologist in separate boxes. Once the SLNs were identified, the standard surgical intervention was performed. Fresh surgical specimens were sent to the pathologist, for histo-pathological and immunohistochemical examination.

Ex vivo analysis of lymph nodes

In fresh specimens, at one hour after surgical intervention, we removed the other palpable lymph nodes, around and at distance of tumor. These lymph nodes were fixed in neutral formalin in separate boxes. The surgical specimen was also fixed in formalin. At 10 hours after surgical intervention we reevaluated the surgical specimens and removed the blue lymph nodes, if they were present in the peritumoral fat. The dissection of surgical specimen was performed at 48 hours after surgical intervention (Figure 1).

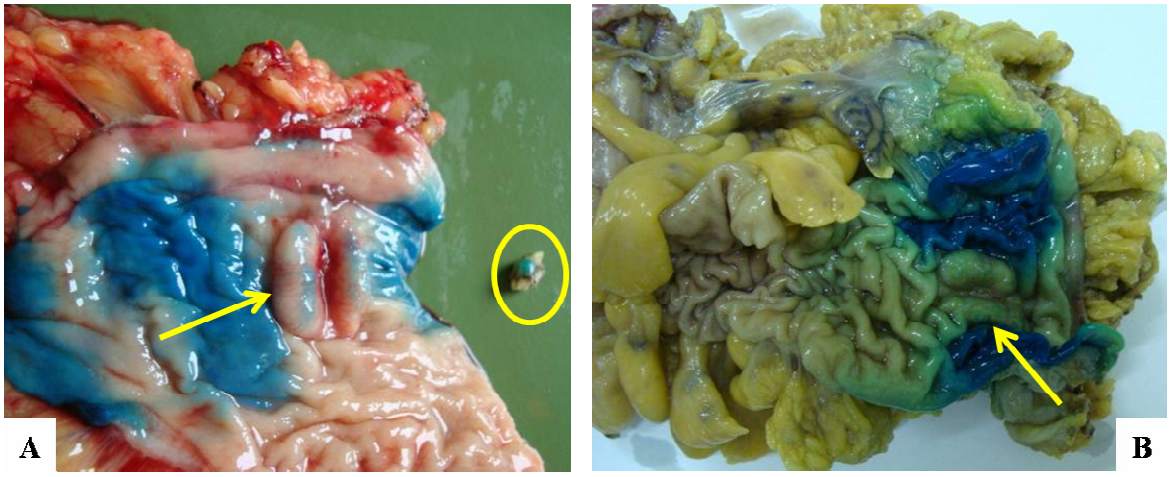

Figure 1 – ***One surgical specimen with colorectal carcinoma: (A) Fresh specimen, with subserosally injection of blue dye and the sentinel lymph node removed in vivo; (B) The specimen after 48 hours fixation in neutral formalin.***

Histochemical and immunohistochemical evaluation

In all cases, we performed a microscopical examination and the pTNM staging was in according with the "AJCC (American Joint Committee on Cancer) Cancer Staging Manual", 7th edition, 2010 [19].

All SLNs and non-SLNs were marked according to the location and time-mapping, and were included in separate boxes. The metastases were identified with Hematoxylin–Eosin (HE). In the cases with negative lymph nodes, five serial sectioning of 5-µm were performed in the SLNs. All the five sections were stained with Hematoxylin–Eosin. If the SLNs was also negative, immunohistochemical reactions with the anti-Cytokeratin-20 (CK20) antibody have been performed.

For immunohistochemical reactions, UltraVision system by LabVision, in formalin-fixed, paraffin-embedded tissues was used. The sections were deparaffinized, incubated at 100^{0}C in EDTA solution, at pH 9, and washed with distilled water before the hydrogen peroxide incubation. After this, they were washed with Tris Buffered Saline (TBS) and were incubated with primary antibodies for 60 minutes, then were washed with TBS and were covered by Streptavidin Peroxidase Solution for 5 minutes. After this, they were washed with TBS and were covered with Biotinylated Goat Anti-Polyvalent Solution for other 5 minutes. The development was performed with substrate-chromogen solution (DAB) for 3–5 minutes. The nuclei were colored with Mayer's Hematoxylin.

Tumor cell deposits between 0.2 and 2 mm were considered micrometastases and those smaller than 0.2 mm were considered clusters of isolated tumor cells. The separate tumor deposits and macrometastases (tumor clusters bigger than 2 mm) were included in the category of lymph node metastases, according with AJCC criteria [19].

Statistical analysis

For statistical analysis, we used the Statistical

Program Graph Pad In Stat 3. We used the one simple *t*-test and the contingency tables to establish the mean and median values.

Results

Exclusion criteria

From the 28 cases, two cases were diagnosed in pT4 and were excluded. From the other 26, in one case we obtained a false-negative result. It was about a male, 58-year-old, which presented a sigmoidian tumor with invasion in mucosa, submucosa and muscularis (pT2) layers and metastases in three of seven identified lymph nodes (pN1b) but no metastases in the three SLNs. The diameter of all 10 lymph nodes was about 10 mm (Figure 2). In our study, the false-negative rate was 3.84%.

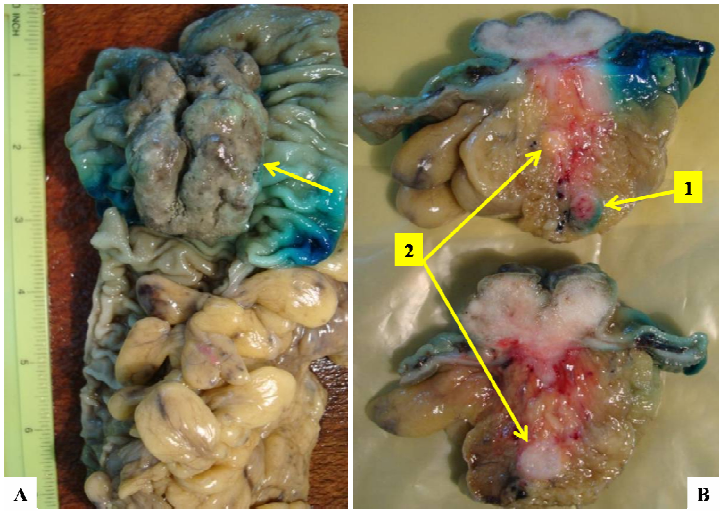

Figure 2 – *A bulky sigmoidian tumor (A) with blue sentinel lymph node without metastases (B-1) and metastases in the non-sentinel lymph nodes (B-2). All lymph nodes have more than 10 mm diameter.*

Clinicopathological features

The clinicopathological characteristics of the 25 patients enrolled in study are summarized in Table 1.

Ten of them were males and 15 were females. The mean age was 55.76±7.92-year-old (minimum 41 and maximum 76 years).

Ten cases were rectal tumors and the other 15 were localized on the other colon segments.

The median number of identified lymph nodes was 15 (minimum 4 and maximum 83). The case with 83 lymph nodes regarded a 47-year-old male which presented familial adenomatous polyposis and the total colectomia was performed, only one polyp from the ascendant colon presenting malignant transformation. The median number of SLNs was 3 (minimum 1 and maximum 7).

Table 1 – *Clinicopathological features of the patients enrolled in study. F = female, M = male, LNs = lymph nodes, SLNs = sentinel lymph nodes, pT = tumor extension, pN = lymph node status, meta = macrometastasis (tumor cell clusters >2 mm)*

No.	Gender	Age [years]	Tumor location	pT	pN	Total LNs identified	Meta only in SLNs	Meta in SLNs + other LNs
1.	F	55	rectum	pT2	pN0	8	no	no
2.	F	52	sigma	pT3	pN0	19	no	no
3.	M	66	sigma	pT3	pN0	12	no	no
4.	M	59	transverse colon	pT3	pN0	37	no	no
5.	M	47	ascendent colon	pT3	pN0	83	no	no
6.	F	50	rectum	pT2	pN0	15	no	no
7.	F	61	sigma	pT3	pN0	27	no	no
8.	F	56	sigma	pT3	pN0	10	no	no
9.	F	51	sigma	pT3	pN0	6	no	no
10.	F	64	transverse colon	pT2	pN0	4	no	no
11.	F	48	caecum	pT3	pN0	5	no	no
12.	M	54	caecum	pT3	pN0	19	no	no
13.	M	54	rectum	pT3	pN0	16	no	no
14.	M	65	transverse colon	pT3	pN1a	13	yes	n
15.	F	53	sigma	pT3	pN1a	14	yes	no
16.	M	55	rectum	pT2	pN1a	6	yes	no
17.	F	49	sigma	pT3	pN1a	20	yes	no
18.	F	55	recto-sigma	pT3	pN1a	6	yes	no
19.	M	49	rectum	pT3	pN1a	25	yes	no
20.	F	62	rectum	pT3	pN2a	13	yes	no

No.	Gender	Age [years]	Tumor location	pT	pN	Total LNs identified	Meta only in SLNs	Meta in SLNs + other LNs
21.	F	41	sigma	pT3	pN2a	17	yes	no
22.	F	76	rectum	pT2	pN1b	20	yes	no
23.	M	59	rectum	pT3	pN2b	25	no	yes
24.	M	67	rectum	pT3	pN2b	12	no	yes
25.	F	46	rectum	pT3	pN1b	24	no	yes

Indispensable steps for a proper SLN mapping

During surgery, one or two SLNs were identified. In fresh specimens, ex vivo, we removed the palpable lymph nodes either they were not blue-stained and included them in separate boxes. In two of the cases, we observed a peculiar aspect. Even the lymph nodes were uncolored in fresh specimens they became blue 10 hours later, after formalin fixation (Figure 3). At the same time, in both cases, the SLNs which were *in vivo* removed were negative but lymph nodes macrometastases have been identified only in these *ex vivo* blue nodes and were considered SLNs. The other lymph nodes were uncolored and without metastases.

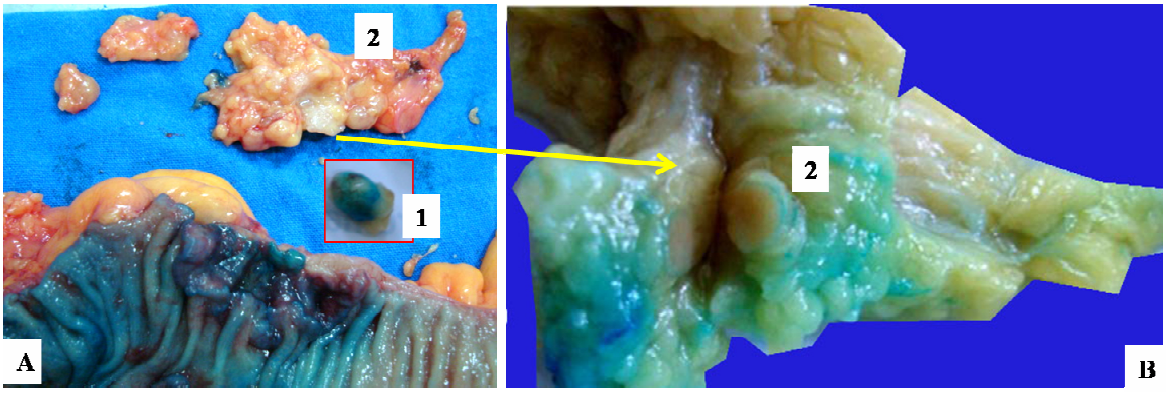

Figure 3 – ***The sentinel lymph node removed in vivo (A-1) do not presented metastases. In the fresh tissue, the palpable lymph nodes, uncolored, were removed at one hour (A-2). One of them became blue after 10 hours in formalin fixation (B-2) and was the only metastatic lymph node in this case.***

Identification of micrometastases and tumor isolated cells in SLNs

In all the 22 cases with lymph node metastases, the tumor cells were identified after one lymph node section and Hematoxylin–Eosin stain. In the other 13 cases without lymph node metastases, we made multilevel sections and immunohistochemical reactions in all SLNs to identify the micrometastases. Forty-six SLNs were examined but no isolated cells were observed.

Discussion

The aim of this study was to identify the benefits and the proper method for SLNs mapping in colorectal cancer. The reasons why this mapping may be necessary are the following: it is an inexpensive and simple method; it can lead to increase of the number of identified lymph nodes; it may lead to decrease the false-negative rate of negative lymph nodes and it reduces the time for pathologist identification in the peritumoral fat.

In our study, nine from the 25 cases (36%) presented metastases only in SLNs, in six of them only one node being affected. Because the majority of positive lymph nodes had less than 5 mm in size these small nodes could be easily loosed. In one of our previous unpublished study, which was performed on 507 cases with colorectal carcinomas diagnosed between 2002 and 2006, without SLNs mapping, we revealed that 61.12% of cases do not present lymph node metastases. In this study, the rate of negative lymph node cases was 52% (p=0.0001). At the same time, in the previous study the median number of identified LNs was 9.56 (minimum 2, maximum 20) compared with 15 in our study (p=0.003). The number of cases is too small to admit that the difference is significant but these are only the preliminary results. Other authors identified a higher rate of patients without node positive disease after SLNs mapping – 53.8% [20].

The majority of authors agree the idea of SLNs mapping in colorectal cancer for many reasons: identification of aberrant lymphatic drainage [15], the simplicity of technique with possible prognostic and therapeutically impact and no side effects [20], a more accurate assessment of nodal status with the ability of upstaging [11].

One of the new aspects revealed by our study is the proper method for SLNs mapping. Other authors reported a rate of false-negative SLNs (skip metastasis) from 6% [20] to 12% [21] and either 31% [16]. In our study, only one of the cases presented a skip metastasis (3.84%). This very low rate was obtained after re-evaluation of surgical specimen at one hour, 10 hours and 48 hours. We considered all blue nodes like SLNs and the results confirm the importance of this *in vivo* mapping with *ex vivo* control. Because the intensity of blue stain increased after formalin fixation and the diameter of lymph node was usually very small, we

considered that the SLNs could be removed intra-operatory, if they were identified, and a reevaluation was necessary to be performed in the Pathology Department. Because in fresh specimens not all SLNs were colored and some small nodes can be damaged through palpation, we tend to believe that it is not necessary the reevaluation at one hour. The best and complex method seems to remain the proper collaboration between surgeon and pathologist, with lymph node mapping in operatory room and re-evaluation in the laboratory, at 48 hour, with separate embedding of every blue lymph node. This technique does not need supplementary time, is easily, inexpensive and can have benefit for patients' prognosis and therapy.

The only false negative case from the 26 it was also the only case with high diameter of lymph nodes (10 mm). These lymph nodes can be easily identified but it seems that they do not allow the proper passage of blue dye. High lymph node diameter can be an exclusion criterion in SLNs mapping together with the other conditions reported to be associated with false-negative findings: pT4 stage, large tumors, high body mass index, tumor location in the extraperitoneal rectum [16].

Another aspect involves the possibility of ultra-staging of SLNs using multiple level sections and immunohistochemistry in the cases with negative lymph nodes and negative SLNs. In our study, none of the 13 cases showed isolated tumor cells or micrometastases. In other studies, 1 from 55 patients [22], 8 from 28 [23], 3 from 25 [11], 2 from 17 [8] respectively 1 from 200 cases [20] presented micrometastases. This small number of cases with possible but non-accepted upstaging shows that this technique is too expensive and has a little benefit. Not all authors agree this idea, some of them reported that 3% of non-metastatic cases present occult tumor cells [24] and ultrastaging has a prognostic impact.

Conclusions

SLNs mapping seems to have a real impact in management of diagnosis and therapy of patients with colorectal cancer. The practical importance of this technique is those that a simple and inexpensive supplementary method could reduce the rate of false-negative results regarding status of lymph nodes, one of the main prognostic and predictive factors of this lesion. In order to realize the proper mapping a good collaboration between surgeon and pathologist is necessary. It can reduce the time for lymph node identification in the Pathology Department, can increase the number of these LNs and, not last, can help patients and can easily identify the cases in which the post-operative therapy is necessary. All *in vivo* and *ex vivo* blue nodes should be considered SLNs.

The high diameter of lymph nodes seems to be an exclusion criterion in SLNs mapping.

The ultrastaging of SLNs in colorectal malignant lesions remains an unelucidated, expensive and time-consuming method with uncertain value.

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