1. KT (truncated)

Chapter 4

RT-PCR and immunohistochemical evaluation of sentinel lymph nodes after in vivo mapping with Patent Blue V in colon cancer patients

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μm paraffin-embedded tissue section. In brief, tissue was incubated in lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 2% SDS) and treated for 12 hours with 500 μg/ml proteinase K at 60˚C. Proteinase K was inactivated for 5 minutes at 95˚C, and RNA was extracted with 1/10 volume of 3M NaAc, 1/5 volume of chloroform, and 1 volume fenol. RNA was precipitated using an equal volume of isopropanol and 1 ul carrier glycogen (Roche). Total RNA was treated with DNAse I using the TURBO DNA-*free* kit™ according to manufacturer's instructions (Ambion,). RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Paisley, UK) in a volume of 20 μl using random hexamers (300 ng). An Assay-on-Demand Gene Expression Product™ (Applied Biosystems) was used for analysis of *CEA* (Hs 00237075_m1). Primers (Invitrogen) and probe (Eurogentec, Seraing, Belgium) for *GAPDH* were developed using primer design software (Applied Biosystems, Foster City, CA, USA). Primers used were: *GAPDH*F 5'-ccacatcgctcagacaccat-3', *GAPDH*R 5'-gcgccaatacgaccaaat-3'. Probe sequence labeled 5' with the FAM reporter dye and 3' with the TAMRA quencher dye molecules was: *GAPDH* 5'-cgttgactccgaccttcaccttccc-3'. Reactions were performed in 384-well plates (Applied Biosystems) in a volume of 20 μl containing real-time PCR mastermix (Eurogentec), 900 nM of each primer, 200 nM of an individual probe and 5 ng cDNA. PCR amplifications were performed using the ABI prism 7900HT sequence detection system (Applied Biosystems). Standard cycling conditions were used including a pre-amplification step of 50˚C for 2 min, 95˚C for 10 min, followed by amplification of 40 cycles of 95˚C for 15 s and 60˚C for 1 min. All samples were analyzed in triplicate. Mean cycle treshold values (Ct) and standard deviations (SD) were calculated.

Results

A total of 30 patients were included in the study, 14 women and 16 men. The mean age at the time of surgery was 69 years (48-85). The tumor characteristics are shown in table 1. A median number of 14 lymph nodes were harvested, with a mean number of 2,7 (range 1-4) sentinel nodes. The procedure was performed successfully in 29 patients (97%). The patient in whom the procedure failed, had a carcinoma of the sigmoid within an area of diverticulitis. Aberrant lymphatic drainage was seen in 3 patients (10%): to the splenic flexure in right-sided tumors (n=2) and a para-aortic node in a recto-sigmoid tumor (n=1). This resulted in a more extended resection. No patient developed toxicity associated with the use of Patent Blue.

SLN examination was negative for metastases by H&E and IHC in 18 patients (62%). In 16 of these patients, the non-sentinel nodes were also tumor-negative. This leads to a negative predictive value of 89%. One of the two patients with a false-negative SLN had extranodal

disease in the non-SLN's. The other failure occurred in a patient with a tumor in the ascending colon with H&E proven micrometastases in a small, peritumoral lymph node. Overall, the accuracy of the procedure in our study was 93% (27/29). In 11 patients (11/29, 38%) we found metastases in the SLN's. In 4 of these patients the SLN's were positive on H&E examination and in 7 patients the SLN's showed metastases after immunohistochemistry. In 5 of these 7 patients we only found isolated tumor cells. In 6 out of 29 patients (21%) the nodal stage could be identified by conventional H&E examination. In 13 patients the combination of H&E and IHC lead to a positive lymph node result (45%), leading to an upstaging of 25%. All sentinel nodes found by detection of aberrant drainage were negative in this study.

RT-PCR for CEA was performed on the paraffin embedded sentinel nodes for 12 out of 16 cases. At every run, we checked positive as well as negative controls. Every time, positive controls turned out positive, and negative controls turned out negative. The mean Ct-value for the housekeeping genes was 27,92 (26,3-29,7) with a mean standard deviation of 0,097 (0,034-0,27) indicating that the RNA quality and quantity was similar for all cases. This analysis revealed 3 patients with increased CEA levels indicating the presence of metastases in the SLN's. Taking this into account, we found a total of 16 out of 29 patients (55%) to be node positive: two patients had H&E positive non-SLN's and negative SLN's, 4 patients had H&E positive SLN's, 7 patients had IHC proven metastases, and 3 patients had metastases after RT-PCR examination. This leads to an upstaging of 33% by IHC and RT-PCR in our group.

We also looked at the stage of the primary tumor in relation to the occurrence of IHC or PCR detected metastases in H&E negative patients. In stage I patients ($T_{1/2}N_0$) we found micrometastases or a positive PCR result in 1/6 patients (17%). In stage II patients ($T_{3/4}N_{0)}$ there were 8/17 patients with IHC or PCR detected metastases (47%). The numbers are too small to obtain any significance from these results.

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2. RMF-OGS2009

The role of sentinel node in the treatment of colon cancer

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The problem of sentinel* lymph *node analysis (SLN) was taken into account regarding the colorectal cancer cases, too, after revealing substaging due to the existent micrometastases. Thus, the extent of lymphatic dissection may be established, and sometimes the thorough assessment of the entire tissue sample may be minimised through SLN isolation.The aim of the study is to evaluate the technique of SLN detection and its correlation with one of the primary tumour's characteristics. The SLN detection is performed through intraoperative peritumoral injection of methylene blue dye and the detection of metastatic spread to the lymphatic nodes is performed by haematoxylin-eosin staining (H/E). The sentinel lymph node detection was performed on 20 patients who underwent surgery for colon cancer and standard lymph node dissection was performed. The dye was injected into the peritumoural serosa on 14 patients in vivo and on 6 patients ex vivo. SLN detection was performed on 95% of the patients (in 13/14 examined* patients *using the in vivo technique and in 6/6 examined patients using the ex vivo technique). A total number of 275 lymph nodes from the samples were examined, with an average of 13,75 lymph nodes/ sample (between 3 and 32 sampled lymph nodes). A number of 41 were marked as the SLN (an average of 2,05); the presence of metastases was detected in 6 patients. Due to the absence of metastases in the SLN on a number of 14 patients, all the other examined lymph nodes were negative (100% specificity). Limiting the histological analysis to the SLN can not replace the histological examination of all the sampled lymph nodes. The thorough examination of SLN sections may emphasise the presence of some micrometastases and thus, shifting a patient's results from NO stage to N+ stage. The methylene blue dye injection method is applicable both for in vivo and ex vivo usage. The preliminary results indicate that false negative results are very likely to occur.

Key words: sentinel lymph node, cancer, colon

The colorectal cancer is the most encountered type of cancer in Romania (18,55/100.000 inhabitants), observing, in the last 20 years an important increase of the incidence from 10,1 in 1989 to 18,55 in 1999. The invasion to the lymph nodes is one of the major prognostic factors. The survival rate up to 5 years in stage III patients with lymph node invasion is much lower than in stage I/II patients without lymph node invasion. The adjuvant chemotherapy administered in cases with lymph node invasion highly improves the survival rate.[2,3] Approximately 30% of the initially stage I/II patients develop a systemic metastatic spread, a significant part of them being substaged. Lymph node dissection in colonic cancer is a very studied and disputed issue[5], but it has been proved that staging is as accurate as the number of the examined lymph nodes is increased.[6,7] The TNM classification for malignant tumours requires the histological assessment of at least 12 lymph nodes from the sampled tissue[8]. In 1977, Cabanas introduces the concept of the sentinel lymph node in the treatment of penile cancer and proved the connection between lymph node analysis and tumo metastatic spread.[10] According to this concept, the SLN is the first lymph node involved in the tumour's lymphatic drainage and presents with the highest risk of metastasis. In 1990, Morton introduces a new this concept, applying the SLN detection technique I malignant melanoma of the skin.[11] In 1994, Giulian and al. mention the first cases in breast cancer. [12] The impact of SLN detection in colorectal cancer cases is still a disputed subject. In 2000, Saha and al. present the first favourable results and confirms the method's validity in colorectal cancer cases, too.[16]

MATERIAL AND METHOD

We included in the analysed group a number of 20 patients diagnosed with colonic cancer, who underwent surgery within the First Surgical Clinic of The County

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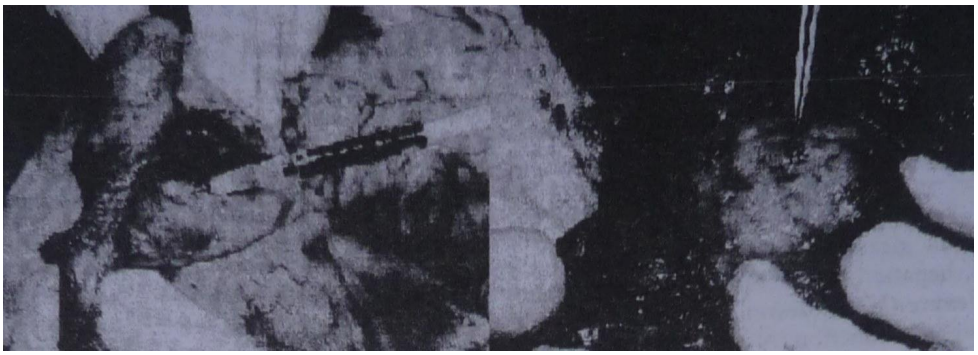

Figure 1. In vivo SLN technique

Figure 2. Ex vivo SLN technique

Clinical Hospital Targu-Mures, from August 2007 until February 2009, whom we included in to a SLN detection and examination protocol. The data were included into a data base study within The First Surgical Clinic, also based on the pathological reports. The exclusion criteria were: preoperative stage IV, metastatic spread to lymph nodes and close organs, emergency surgical in terv en tions, preoperativ e radiotherapy. The patients with intraoperative lymph node metastases detection, peritoneal carcinomatosis and liver metastases were also excluded. There were used two methods of SLN detection, the *in vivo* and *ex* vivo techniques. The *in vivo* technique was used on the most cases, in case the tumour was situated above the rectosigmoid junction. After tumour and subsequent mesentery exposure, approximately 2 cc of methylene blue are injected with a 22 gauge need le into the peritumoural submucosa, in the four cardinal points[19] (Figure 1).

After 5 minutes, without handling the tissue, we notice a blue thread on the mesocolic wall, which shows us the tumour's lymphatic drainage towards the SLN.

The first identified lymph node is marked and separately sent postoperative for pathological analysis. The extent of the lymph node dissection is established by the oncological resection limit, depending on the location of the colonic tumour. The *ex vivo* technique was used especially for the cases where the tumour was located at the rectosigmoid junction and are less mobile. The SLN detection was performed after the removal of the tissue sample, through injection of 2 cc of methylene blue into the peritumoural submucosa, and approximately after 5 minutes the SLN is detected and is removed without opening the mesocolon, in order to avoid damaging the following lymphatic mapping [25] (Figure 2).

A protocol was used for the sampled SLN. The SLN is fixed with formaldehyde solution for 24 hours and after they are examined: those under 3mm in diameter are fully examined, and those over 3mm through multiple sections from paraffin wax embedded blocks (3 sections of 5 µm at intervals of 150 pm), stained with H/E. The rest of the wax block is saved for the following immunohistochemical examination. The presence of the metastases identified through H/E staining between 0,2 and 2 mm in diameter, lead to staging the case as pNl. Concealed metastases (micrometastases) are defined as the existence of some isolated tumour cells or tumour cell clusters measuring under 0,2 mm in the lymph nodes, their presence qualifies the case as pNO.[32] If the SLN is negative on standard examination, it may be examined through immunohistochemistry (cytokeratin).

RESULTS

A number of 20 patients were included into the study, 8 men and 12 women, with ages between 49 and 79 years old (the average age of 61,45) (Table I).

Table 1.Gender and average age

Patient's gender	Number of patients	Average age
male	8	62,25
female	1 2	60,91
Total	20	61,45

The tumour location was at: cecum (N=2), right colon (N=1), hepatic flexure (N=1), transverse colon (N=1), left flexure (N=1), descendent (N=2), sigmoid (N=6), rectosigmoid junction (N=6) (Table II).

Table II. Tumour location

Tumour location	N=20	%
Cecum	2	10
Ascendent colon	1	5
Hepatic flexure	1	5
Transverse colon	1	5
Splenic flexure	1	5
Descendent colon	2	10
Sigma	6	30
Rectosigmoid junction	6	30

The pathologic diagnostic was well defined adenocarcinoma - 8, moderately differentiated - 6, poorly defined - 4 and undefined - 2. The postoperative classification was pT1 - 4, pT2 - 8, pT3 - 5 , pT4 - 3, pN0 - 14 patients, with pN1 - 4 patients and pN2 - 2 patients, in Dukes staging system. The SLN detection was possible in 19/ 20 patients (95%), in one patient with the in vivo technique it could not be detected. A total number of 275 lymph nodes from the samples were examined, with an average of 13,75 lymph nodes/sample (between 3 and 32 sampled lymph nodes). A number of 41 were marked as the SLN (an average of 2,05). The presence of metastases was detected in 6 patients, for the other 13 patients where the SLN was negative; the other lymph nodes from the surgical sample were also negative (Table III).

Table III. Examined lymph nodes

	n r	%
Total number of patients	20	100
Total number of patients with SLN detected	19	95
Total number of patients with positive SLN	6	30
Total number of patients with positive SLN and the other lymph nodes positive	6	30

The following surgical interventions were performed: 4 right hemicolectomies, 1 transverse colon segmental resection, 3 left hemicolectomies, 6 sigmoid colon segmental resections (Raybard) and 6 rectosigmoid resections (Dixon). Standard lymph node dissection was performed after oncological principles. Without taking into account the SLN examination (41 marked lymph nodes), there was no spread to the lymph nodes in 14 patients (70%) and were staged as pNO. Out of the 6 patients (30%) with positive SLN, 4 (20%)

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Table IV. The analysis of the examined lymph nodes

The analysis of the exam. lymph nodes	SLN(%)	nonSLN(%)
Total lymph nodes = 275	41(14,9)	234(85,1)
Negative lymph nodes = 185	9	176
Positive lymph nodes = 90	32(11, 6)	58 (21, 1)

DISCUSSION

The intraoperative SLN detection was applicable in most patients (95%), consistently with the data from the specialty literature, the failures were due to technical errors[5], insufficient experience[15,35], the use of insufficient quantity of dye[36], the failure rate may be increased due to preoperative radiation therapy.[37] In comparison with the *in vivo* technique, the *ex vivo* technique has the advantage of decreasing the intraoperative time and avoids possible allergic reactions.[38] In case of the *in vivo* technique, the colon is mobilised only after the ligation of the vascular pedicle, also, there is a known fact that in some cases the lymphatic drainage is not directly towards the SLN, but there are different paths, which may lead to false negative results. Recently, Japanese authors describe a method which uses endoscopic dye injection in small sized tumours.[39,40] Saha and al. observed that the SLN is single only in 42% of the cases.[16] The SLN examination accuracy in order to evaluate tumour extension is susceptible of false negative rate, which may vary from 4 to 40%. The use of other methods for SLN analysis like immunohistochemistry, molecular biology, may decrease the number of false negative results, but implies high costs, unaffordable especially for our country[22,23,24], thus, SLN detection can not replace standard pathological examination of all the surgically sampled lymph nodes.[19,25,41] The method may offer additional information for stage NO patients, where a closer examination (immunohistochemistry) may increase their grading and in consequence, change the attitude of the complementary oncological treatment represented by chemotherapy.[16,17,19, 22, 23, 24, 25, 26, 35]

CONCLUSIONS

In conclusion, we may state that after the technique is learned and becomes a routine, it is an applicable and cheap method, if methylene blue is used as dye (the cost of other dyes is high in order to be used in our country). Also, the use of only H/E dye on multiple sections, may offer enough information, and a part of the cases will be staged from pNo to pN I or pN2 and may later benefit ot chemotherapy treatment, with a major impact on the survival rate. The SLN analysis may influence the extent of colon resection (especially in the case of location on the transverse colon and on flexures level). In order to avoid an increased rate of false negative results, a thorough examination of the whole surgical sample is needed.

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ORIGINAL ARTICLE

3. AMM2011 (truncated)

Immunohistochemical Evaluation of Sentinel Lymph Nodes in Colon Cancer

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Background: Lymph node status is the most important predictive factor in the treatment of colic cancer. As sentinel lymph node (SLN) biopsy might upstage stage II colon cancer, it could have therapeutic consequences in the future.
Aim: To investigate and evaluate nodal microstaging and ultrastaging using cytokeratin immunohistochemistry.
Material and methods: In 20 consecutive patients operated on First Surgery Clinic of the County Hospital Mures for colon cancer, subserosal injection with Patent Blue dye was used for SLN detection. In searching for occult micrometastases, each SLN was examined. In tumor-negative SLNs at routine hematoxylin-eosin (H&E) examination (pN0) we performed cytokeratyn(CK) immunohistochemistry (IHC).
Results: The procedure was successful in 19 out of 20 patients (95%). The SLN was negative in 12 patients detected by H&E and IHC, in 10 patients the non-SLN was also negative, leading to a negative predictive value of 89% and an accuracy of 93%. In 6 patients with SLN negative by HE was pozitive by IHC, leading to a 33% value of upstaging.
Conclusions: The SLN concept in colon carcinoma using Patent Blue V is feasible and accurate. It leads to upstaging of nodal status in 6 cases (33%) when IHC techniques are involved. The clinical value of the method will be evaluated by postoperative chemotherapy efficiency.

Keywords: sentinel lymph node, colon, carcinoma

Introduction

Colorectal carcinoma is the most common gastrointestinal malignancy. Lymph node status as the most important predictor of outcome indicates the use of adjuvant chemotherapy.The reported 5-year survival rate is 70–80% for patients with node negative disease (st. I–II), but only 45–50% for those with node positive tumors (st.III) [1]. Adjuvant chemotherapy significantly improves the 5-year survival in patients with node positive disease. Despite the favorable prognosis of patients with localized colon cancer without regional lymph node metastasis, 20–30% of these patients will develop recurrent disease, after apparently curative resection. It is therefore necessary to perform a more detailed histological examination of negative lymph nodes by histological examination with haematoxylin-eosine staining (HE) and immunohistochemistry with cytokeratin (CK).Understaging may be the result of inadequate numbers of examined lymph nodes, missing some metastases [1,2,3]. For adequate staging and treatment of patients with colon cancer, meticulous examination of at least 12 nodes harvested by pathological analysis is mandatory [4].

Sentinel node technique was described by Cabanas in 1977 in penile cancer, and Morton Giuliano introduced the method for melanoma and breast cancer. In colon cancer the sentinel lymph node is defined as the first tumor draining lymph node, with the highest potential to harbor metastatic disease [5,6,7]. This allows a targeted examination of a smaller number of nodes that can be examined with multiple sections and immunohistochemistry for the accurate detection of metastases and micrometastases and to provide a better staging of colon cancer.

We used methylene blue dye to identify sentinel lymph nodes and examined them with haematoxylin-eosine staining and immunohistochemical technique with cytokeratin. In tumor-negative SLN's at routine hematoxylin-eosin (H&E) examination (pN0) we performed CK8/CK18 immunohistochemistry (IHC).

Material and methods

Only patients with histological proven primary colon carcinoma were included in the study. Patients with distant metastases or gross lymph node involvement as shown by preoperative examinations or palpation during surgery were excluded.

Sentinel lymph node mapping was carried out through an open procedure by injection of 1–3 ml Blue Dye with a tuberculin syringe and 29 gauge needle subserosally in 4 quadrants around the tumor. The subserosal injection was carried out prior to vascular ligation. Within 5 to 10 minutes after the blue dye injection, the SLN's could be identified by following blue stained lymphatic vessels leading to the blue stained sentinel node [8–12]. These nodes were tagged with a long suture. Sentinel nodes were defined as the first four bluestaining nodes seen within the regional basin. After marking of the SLN's, routine resection was performed.

Table I. Sex and age distribution

Patient sex	Number of patients	Average age
Male	8	62.25
Female	12	60.91
Total	20	61.45

The tumor and all lymph nodes were examined according to standard guidelines. If the SLN's were negative after routine hematoxylin-eosin (H&E) staining, they were sectioned at 150 µm intervals and examined at 3 levels with H&E as well as immunohistochemistry on cytokeratins (CK8/CK18). Metastases between 0.2 mm and 2 mm were referred to as micrometastases. Metastases smaller than 0.2 mm were described as isolated tumor cell 13.

A total of 20 patients were included in the study, 8 men and 12 women, with ages between 49 and 79 years old (the average age of 61.45) (Table I).

Results

The procedure was successful in 19 out of 20 patients (95%), but failed in one patient. The SLN was negative in 12 patients detected by H&E and IHC, in 10 patients the non-SLN was also negative, leading to a negative predictive value of 89% and an accuracy of 93% (Table II).

A total number of 275 lymph nodes from the samples were examined, with an average of 13.75 lymph nodes/sample (between 3 and 32 sampled lymph nodes). A number of 41 were marked as the SLN (an average of 2.05). The presence of metastases was detected in 6 patients, SLN was negative; the other lymph nodes from the surgical sample were also negative.

Without taking into account the SLN examination (41 marked lymph nodes), there was no spread to the lymph nodes in 14 patients (70%) staged as pN0. Out of the 6 patients (30%) with positive SLN, 4 (20%) were staged as pN1 and 2 (10%) as pN2.In 6 patients of the other 13 patients where the SLN was negative by HE, it was evidenced as pozitive by IHC, leading to a 33% value of upstaging. (Table III).

Discussions

Unlike the validated SLN concept in breast cancer and melanoma mandating lymphatic dissection, the main reason for SLN mapping in colon cancer is to focus pathologic IHC evaluation of SLN after in vivo mapping with patent blue. In colon cancer patients examination of the SLN's, will increase the accuracy of nodal staging, resulting in a higher percentage of node-positive patients, who may benefit from adjuvant chemotherapy [14,15,16].

Upstaging by H&E conventional examination is difficult to measure. It might be explained by the focused examination of blue stained nodes, because these blue nodes can be very small nodes and would otherwise not have been detected. The IHC in our study was performed on cytokeratins. Most studies performed sectioning with intervals of 500 µm or immunohistochemistry on 1–4 levels in total. Increasing the number of slices for immunohistochemistry probably improves the detection rate of micrometastases smaller than 2 mm. A variety of results on this subject have been described [19,20,21] (Table IV).

We found an upstaging by immunohistochemical staining in 33% of patients. We should wait for the results after follow up in a large group of patients before assesing the real impact. If future results confirm the importance of microstaging and ultrastaging in CRC, the sentinel node concept can help the pathologist to focus the examination on one or two sentinel nodes in H&E negative cases. The detection of micrometastases might then select a subgroup of patients who could benefit from adjuvant treatment.

Table II. Examination of SLN

	No.	%
Total number of patients	20	100
Total number of patients with SLN detected	19	95
Total number of patients with positive SLN at HE	6	30
Total number of patients with negative SLN at HE	13	75
Total number of patients with negative SLN at HE, and positive at IHC	6	30
Total number of patients with positive SLN and the other lymph nodes positive	6	30

Table III. Analysis of the examined lymph nodes

The analysis of the examined lymph nodes	SLN(%)	nonSLN(%)
Total lymph nodes = 275	41 (14.9)	234 (85.1)
Negative lymph nodes at HE = 185	9	176
Positive lymph nodes at HE = 90	32 (11.6)	58 (21.1)
Positive lymph nodes at HE + IHC = 108	37	61

Conclusions

The sentinel node concept in colon carcinoma using Blue Dye is feasible and accurate.It leads to an upstaging of nodal status in 33 % of patients when IHC techniques are combined and may detect aberrant lymphatic drainage. This procedure can be performed in a multi-center study under adequate supervision during the learning curve and may have diagnostic and therapeutic consequences in the future.

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