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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Abstract:

Background: Lymph node status is the most important predictive factor in the treatment of colorectal cancer. As sentinel lymph node (SLN) biopsy might upstage stage II colon cancer it could have therapeutic consequences in the future. Therefore we studied the feasibility of in vivo SLN detection with Patent Blue V dye and evaluated nodal microstaging and ultrastaging using cytokeratin immunohistochemistry and RT-PCR methods.

Patients and Methods: In 30 consecutive patients operated for colon cancer, subserosal injection with Patent Blue dye was used in the SLN detection in 4 different hospitals under supervision of one regional coordinator. In searching for occult micrometastases each SLN was examined at three levels. In tumor-negative SLN's at routine hematoxylin-eosin (H&E) examination (pN0) we performed CK8/CK18 immunohistochemistry (IHC) and RT-PCR for CEA.

Results: The procedure was successful in 29 out of 30 patients (97%). The SLN was negative in 18 patients by HE and IHC. In 16 patients the non-SLN were also negative, leading to a negative predictive value of 89% and an accuracy of 93%. Upstaging occurred in 10 patients (33%); 7 by IHC and 3 by RT-PCR. Aberrant lymphatic drainage was seen in 3 patients (10%).

Conclusions: The SLN concept in colon carcinoma using Patent Blue V is feasible and accurate. It leads to an upstaging of nodal status in 33 % of patients when IHC and PCR techniques are combined. Therefore, the clinical value of SLN should be subject of further studies

Introduction

Colorectal carcinoma (CRC) is the most common gastro-intestinal malignancy and the second leading cause of cancer related deaths in the Western World. Lymph node status as the most important predictor of outcome indicates the use of adjuvant chemotherapy in these tumors. The 5-year survival rate is 70-80% for patients with node negative disease (stage I/II), but only 45-50 % for those with node positive tumors (stage III).[1] Adjuvant chemotherapy significantly improves the 5-year survival in patients with node positive CRC. 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. This might be explained by abstinence of adjuvant treatment in case of pathological understaging at the time of resection. Understaging may be the result of inadequate numbers of examined lymph nodes, missing some metastases.[2,3] For adequate staging and treatment of patients with colon cancer, meticulous examination of at least 12 nodes harvested by pathological analysis is warranted according to the Dutch National Cancer Centre Guidelines and international guidelines.[4] Moreover, intensive pathologic examination of lymph nodes by immunohistochemical staining for cytokeratin or reverse transcriptase-polymerase chain reaction (RT-PCR) may reveal micrometastases that would be missed by routine hematoxylin & eosin (H&E) examination. Although these staging techniques are time consuming, labor intensive and costly, several authors have reported a decreased survival rate when nodal micrometastases are detected in CRC.[5,6] For optimal staging, examination of SLN's may therefore be helpful.

The technique of the sentinel node biopsy was first described and performed by Cabanas (1977) in penile carcinoma.[7] However, it was Morton et al. and Giuliano et al. who introduced the sentinel node biopsy for staging patients in general practice in melanoma and breast cancer.[8,9] In CRC the SLN's are defined as the first one to four blue-stained nodes with the most direct lymph drainage from the primary tumor. They have the greatest potential to harbor metastatic disease when present, enabling focused examination with multilevel microsectioning of the SLN's to provide a more efficient and cost-effective detection of micrometastases. In addition, patterns of aberrant lymphatic drainage can be visualized with sentinel lymph node mapping, which may lead to a more extended resection. Most reported studies of the SLN concept in CRC showed good results with isosulfan blue (Lymphazurin). [10-22] However, Lymphazurin is not registered for clinical use in Europe. The results with Patent blue-V, that is commonly used in Western Europe, are variable with only one study showing comparable results to the isosulfan blue studies.[23-28]

Besides, only few sentinel node studies in colon carcinoma have been performed in a multi-center setup. We tested the utility of Patent Blue-V in vivo to identify SLN in colon cancer patients in four different hospitals and evaluated our experience with immunohistochemical and RT-PCR techniques in detecting occult micrometastases on routine H&E negative SLN's.

Patients and Methods

Patients

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 pre-operative examinations or palpation during surgery were excluded. The local scientific ethics commission approved this study and all patients had given informed consent.

Surgical procedure

This feasibility study was performed by one surgeon in each of the four different hospitals. The first 5 procedures of each surgeon were supervised by one coordinating surgeon (J.T.P). Sentinel lymph node mapping was carried out through an open procedure by injection of 1-3 ml Patent Blue 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. These nodes were tagged with a long suture. Sentinel nodes were defined as the first four blue-staining nodes seen within the regional basin. After marking of the SLN's, routine resection was performed.

Pathology

The tumor and all lymph nodes were examined according to standard guidelines.[2] 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 cells.[2] From 12 out of 18 SLN-negative patients (of which 2 were false-negative), enough paraffin embedded material was available to perform real-time PCR for CEA on one level of the sentinel node. This method has been described earlier.[29] Total RNA was isolated from one 4

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.

concept in CRC. This study suggests that it may be possible to perform an in vivo SLN procedure in a multi-center study with adequate supervision during the learning curve. Recently, Bertagnolli performed the sentinel node procedure in 13 different hospitals with 25 different surgeons on 79 patients and concluded that the sentinel node was a poor predictor of lymph node status.[30] However, a mean number of three patients per surgeon may be insufficient to perform this procedure adequately and will probably lead to a relatively high number of technical failures. Read et al. also failed to obtain good results with this procedure. However, they included a relatively high number of stage III and IV patients (30%) which could have disturbed the normal lymphatic distribution resulting in a non-reliable SLN node procedure.[31] Other studies, including that of Bilchik and Saha performed at 3 different hospitals reported excellent results.[16,32] Paramo et al. found a stabilization of the learning curve of the SLN procedure in colon carcinoma after 5 operations.[13]. In our study the sentinel node procedure failed only once. The surgeons appreciated the presence of a supervising instructor at their first two to four procedures. The overall accuracy of 93 % is comparable to that found in larger, previous studies.[13,16,18,19,22,32]

The one failure in SLN detection can be explained by the presence of diverticulitis around the tumor. Diverticulitis could disturb the normal lymphatic distribution, thereby interrupting the movement of dye from the tumor to surrounding lymph nodes. In one of our two false negative SLN procedures the non-SLN's showed extra-nodal tumor invasion. It is well known that grossly involved lymph nodes or large bulky tumors with direct tumor invasion through the bowel wall can lead to obstruction of lymphatic channels and skip-metastases. These skip metastases (false negative SLN's) are reported in 18-25% depending on the use of ultrastaging methods.[13,18,20,22,24] Usually the dye-mapping affects the pericolic LN's directly around the bowel, assuming that they are first to be reached by metastatic disease. Sometimes intermediate and apical nodes just proximal of the main vessels are stained blue, suggesting that large bowel segments should be resected to obtain optimal regional control. In very rare cases direct lymphatic drainage to para-aortic nodes is seen, suggesting that an even more extended resection should be performed .

Aberrant lymphatic drainage was found in 10% of cases. This is according to the reported rate of 2-9% in the literature.[10,13,15,16,18,19,32] In all these cases we performed an extended resection. None of these sentinel nodes contained tumor cells. The importance of this aberrant drainage for staging can only be established in larger series with a long follow up.

Unlike the validated SLN concept in breast cancer and melanoma which affect the need for lymphatic dissection, the main reason for SLN mapping in CRC is to focus pathologic

examination on the SLN's, which will increase the accuracy of nodal staging, resulting in a higher percentage of node-positive patients, who may benefit from adjuvant chemotherapy.[4,5,19] 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. The reason to test for cytokeratins, is the known and widely used protocol in Dutch hospitals for the SLN procedure in breast cancer using IHC for cytokeratins. Several studies described the PCR examination of lymph nodes in colon carcinoma using CEA or CK 20 as a marker.[6,33-37] All reported upstaging, and 4 studies reported an adverse effect of upstaging on prognosis.[6,35-37] We use CEA because it is a disease specific marker that is present in the majority of colon carcinomas.

The answer to the crucial question regarding the impact of occult nodal metastases detected by serial step sectioning combined with immunohistochemistry and RT-PCR examination remains unclear. We found an upstaging by immunohistochemical staining in 25% of patients, including micrometastases in two cases, and isolated tumor cells in five cases. Upstaging of the nodal status with multilevel pathologic sectioning and the use of immunohistochemistry has been described in 11-19 % of cases.[10,13-15,18-20,23] The higher percentage in this study can be explained by the difference in methods used for immunohistochemistry in previous studies. Most studies performed sectioning with intervals of 500µm or immunohistochemistry on 1-4 levels in total, while we used standard intervals of 150 µm at 3 levels standard in this study. Increasing the number of slices for immunohistochemistry probably improves the detection rate of micrometastases smaller than 2 mm, but the prognostic significance of these small deposits still has to be cleared in large studies. A variety of results on this subject have been described.[5,6,38-41] Some studies using immunohistochemistry on cytokeratins reported no effect of micrometastases on survival, while others described a worse survival in patients with micrometastases. [34,38-40 5,41,42]

Liefers et al. examined lymph nodes in colorectal carcinoma using RT-PCR on CEA. They found a significant survival difference in patients with and without tumor cells in lymph nodes.[6] This could mean that our detection of isolated tumor cells in five patients and a positive PCR-result in another three with an upstaging to 35% is important.

The quantitation of gene expression in formalin-fixed, paraffin embedded tissue has been subject to serious limitations in the past. RNA isolated from paraffin embedded tissue blocks is of poor quality due to extensive degeneration during the formalin fixation process.

Moreover, formalin fixation causes cross-linkage between nucleic acids and proteins and covalently modifies RNA by the addition of mono-methylol groups to the bases, making subsequent RNA extraction, reverse transcription and quantitation analysis problematic.[43]

The method we used to perform RT-PCR on formalin-fixed tissue, has been described and validated before.[29] For this method it is crucial to use an RNA extraction protocol that provides only minimally cross-linked RNA. In addition, small target sequences should be selected (60-100 basepairs) enabling the detection of fragmented and degraded RNA. The Ct values and standard deviations obtained for the housekeeping gene indicated that the quality and input amount of RNA is comparable for the different paraffin blocks.

We should wait for the results after follow up in a large group of patients before we can estimate 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.

Conclusion

The sentinel node concept in colon carcinoma using Patent Blue V is feasible and accurate. It leads to an upstaging of nodal status in 33 % of patients when IHC and PCR techniques are combined and may detect aberrant lymphatic drainage (10%). 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.