Value of RT-PCR Analysis of Sentinel Nodes in Determining the Pathological Nodal Status in Colon Cancer

WENDY KELDER[1,2], ANDRIES BRAAT[3], ANKE VAN DEN BERG[4], INGE PLATTEEL[4], HARRY HOLLEMA[4], HENK. GROEN[5] and JOHN PLUKKER[1]

Departments of [1]Surgery, [4]Pathology and [5]Medical Technology Assessment, University Medical Center Groningen, University of Groningen; [2]Department of Surgery, Martini Hospital, Groningen; [3]Department of Surgery, Isala Clinics, Zwolle, The Netherlands

Abstract. *Background: Pathological examination of sentinel lymph nodes (SLNs) and non-SLNs in colon cancer is frequently not performed to the same extent. We examined whether non-SLNs were truly negative in tumors with tumor-negative SLNs using reverse transcriptase-polymerase chain reaction (RT-PCR). Patients and Methods: RT-PCR with carcinoembryonic antigen (CEA) was performed in hematoxylin-eosin (H&E) and immunohistochemical (IHC) tumor-negative SLNs. In RT-PCR negative SLNs, we also performed RT-PCR on non-SLNs. Statistical analyses indicated the requirement for a minimum of 72 accurate comparisons of non- SLNs and SLNs, which could be fulfilled using tissues from 12 patients. Results: Negative and positive controls were performed. In nine of the 12 colon tumors, H&E and IHC-negative SLNs were also negative with CEA-RT-PCR. A total of 102 lymph nodes, including 99 non-SLNs were retrieved in these nine specimens and none of the non-SLNs were CEA RT-PCR-positive. Conclusion: In this study, all CEA RT-PCR tumor-negative SLNs correctly reflect the tumor-negative status of the non-SLN's in primary colon tumors. The reliability of this method in colon cancer seems promising.*

In approximately 80% of all colon cancer patients, the tumor is in a stage for which a curative treatment will be possible. Lymph node status still is the most important predictor of outcome after a radical resection of the tumor. The 5-year survival rate is 70-80% for patients with node-negative disease (stage I/II), but is only 45-50% for those with node-positive tumors (stage III) (1). Adjuvant chemotherapy significantly improves the 5-year survival, by 10-15% in patients with node-positive colon cancer (2). Despite a favorable pathological outcome, 20-30% of patients with localized colon cancer without regional lymph node metastases will develop recurrent disease after an apparently curative resection (1). It is possible that small tumor metastases are missed or not detectable, so called occult lesions, leading to understaging of these tumors (3). For adequate staging and treatment of patients with colon cancer, meticulous examination of at least 12 nodes harvested at pathological examination is warranted according to international guidelines (4). Immunohistochemical (IHC) staining or reverse transcriptase-polymerase chain reaction for carcinoembryonic antigen (CEA) or cytokeratin may reveal micrometastases missed on routine haematoxylin and eosin (H&E) examination. Several authors reported a decreased survival rate in colon cancer patients with nodal micrometastases (5, 6). However, ultrastaging techniques are time-consuming, labor intensive and costly. For optimal and efficient staging, focused examination of only the sentinel lymph nodes (SLNs) may be helpful in detecting the presence of micrometastases.

In colon cancer, the SLNs are defined as the first one to four blue-stained nodes with the most direct lymph drainage from the primary tumor, after peritumoral injection with Patent Blue (7, 8). They are the most likely to harbor metastatic disease when present, enabling focused examination with multilevel microsectioning to provide a more efficient and cost-effective detection of micrometastases.

To validate a procedure in which it would be sufficient to examine only the SLNs with ultrastaging methods instead of all H&E-negative lymph nodes, we performed a highly sensitive RT-PCR method for CEA on H&E and IHC-negative SLNs as well as the other, so-called non-SLNs.

Correspondence to: John Th.M. Plukker, MD, Ph.D., Department of Surgery, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands. Tel : +31 503612317, Fax: +31 503614873, e-mail: j.t.m.plukker@chir.umcg.nl

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Patients and Methods

Patient selection. Only patients with histologically proven primary colon carcinoma, without pre- or intraoperatively visible distant metastases or gross lymph node involvement were included. This study was approved by the local scientific ethics committee and all patients gave informed consent. Based on statistical power measurements, a total of 72 accurate comparisons of non-SLN status to SLN status were required for a reliable pathological examination of the SLNs with a 95% confidence interval of the concordance rate (range 0.95 to 1.7). If one comparison were inaccurate, for example: a negative sentinel node with a positive non-sentinel node, 109 comparisons would need to be performed. Assuming a mean of at least 12 lymph nodes per specimen, the analyses could be performed in nine to ten patients, if one sentinel node was false-negative.

Sentinel lymph node technique. SLN mapping was carried out through an open procedure. With a tuberculin syringe and 29 gauge needle, 1-3 ml Patent Blue was injected subserosally in 4 quadrants around the tumor prior to any vascular ligation in the mesocolon. Within 5 to 10 min after the blue dye injection, the blue-stained SLNs were identified by following the blue-stained lymphatic vessels. After tagging these nodes with a long suture, routine resection was performed. The tumor and all lymph nodes were examined histologically according to standard guidelines (9). If the SLNs were negative after routine H&E staining, they were sectioned at 150 μm intervals and examined at 3 levels with H&E, as well as immunohistochemistry of cytokeratins (CK8/CK18). Metastases between 0.2 mm and 2 mm were described as micrometastases and those smaller than 0.2 mm were referred to as isolated tumor cells.

Quantitative RT-PCR-analysis. As a positive control, tumor tissue samples from lymph nodes containing metastatic tumor were used. As a negative control, tissue samples were obtained from lymph nodes of histologically benign resected colon specimens. Sentinel nodes that were negative after H&E and IHC staining were examined with quantitative real-time PCR (qRT-PCR). Real-time, quantitative PCR applications include gene expression and are able to detect sequence-specific PCR products as they accumulate in "real-time" during the PCR amplification process. Using qRT-PCR we could detect their accumulation and quantify the number of substrates present in the initial PCR mixture before amplification.

Before the RT-PCR procedure, all lymph nodes were carefully dissected from the surrounding tissue to prevent false-positive results due to admixture of non-lymph node tissue. All SLNs of the 12 patients were tested for the presence of (micro)-metastases with RT-PCR and subsequently the non-SLN were analyzed in case of a negative SLN. In nine patients, the RT-PCR analysis of the sentinel node was negative and in these patients all non-SLNs were tested to determine the reliability of our concept. Glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) was chosen as the housekeeping gene because the cycle threshold (Ct) value was comparable to or slightly less than the number of cycles needed to get a positive result from positive CEA controls in a previous study (8). This indicates that the expression level of CEA is higher than or similar to the expression level of GAPDH.

Total RNA was isolated from one 4 μm paraffin-embedded tissue section using the Specht method (10). 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 h with 500 μg/ml Proteinase K (Qiagen, Benelux) at 60˚C followed by Proteinase K inactivation for 5 min at 95˚C. RNA was purified by extraction with 1/5 volume of chloroform and 1 volume phenol. RNA was precipitated using 1/10 volume of 2 M NaAc, an equal volume of isopropanol and 1 ul carrier glycogen 10 mg/ml (Roche, Mannheim, Germany). Total RNA was treated with DNAse I using the TURBO DNA-*free* kit™ according to manufacturer's instructions (Ambion Inc., Austin, TX, USA). 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, Foster City, CA, USA) was used for analysis of *CEA* (Hs 00237075_m1). Primers (Invitrogen, Paisley, UK) and probe (Eurogentec, Seraing, Belgium) for *GAPDH* were developed using primer design software (Applied Biosystems). Primers used were: *GAPDH*F 5'-ccacatcgctcagacaccat-3' forward, *GAPDH*R 5'-gcgccaatacgaccaaat-3'reverse. The 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, Seraing, Belgium), 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 sec and 60˚C for 1 min. All samples were analyzed in triplicate. Mean cycle threshold values (Ct) and standard deviations (SD) were calculated. The amount of target gene was normalized relative to the amount of GAPDH ($\Delta Ct = Ct_{(CEA)} - Ct_{(GAPDH)}$) and the SD of the ΔCt (SD(ΔCt)) was calculated as: $(SD(\Delta Ct) = \sqrt{((SD_{CEA})^2 + (SD_{GAPDH})^2)}$. The factor difference was also calculated ($2^{-\Delta Ct}$). For each run, positive and negative controls were included. A Ct value of >30 for the housekeeping gene indicates that the RNA input and/or quality was poor; these lymph nodes were excluded from further analysis. Only good quality samples were used to calculate the relative expression levels of CEA.

Lymph nodes were considered negative when the Ct value of GAPDH was less than 30 and the $\Delta Ct \geq 10$, indicating a relative expression level of 0.001 or less as compared to GAPDH. Lymph nodes were considered positive when ΔCt was <5 indicating a relative expression level of 0.03 or more as compared to GAPDH.

Results

As a control, all five tissue samples obtained from metastatic lymph nodes were positive with Ct values varying from 23-31. The ten negative control lymph nodes stayed negative, even after 40 cycles. The relative expression levels for the positive controls varied from 0.084 to 0.330.

The SLNs of the 12 patients without lymph node metastases from our aforementioned study were examined. Tumor characteristics and RT-PCR results are shown in Table I. These 12 patients had a total of 29 sentinel nodes, with a mean of 2.2 (range 1-4). Three out of the 12 patients

Table I. *Overview of lymph node status.*

No.	Tumor site	T-stage	Number of LN	Number of SLN	PCR SLN	Relative CEA expression level ($2^{-\Delta Ct}$)	PCR non-SLN
1	Right colon	3	13	3	positive	0.084	
2	Sigmoïd colon	3	23	3	positive	0.470	
3	Sigmoïd colon	3	5	1	positive*	0.004	
4	Sigmoïd colon	3	10	3	negative	<0.001	negative
5	Right colon	2	4	1	negative	<0.001	negative
6	Right colon	2	11	2	negative	<0.001	negative
7	Left colon	3	10	1	negative	<0.001	negative
8	Right colon	3	19	3	negative	<0.001	negative
9	Right colon	3	14	4	negative	<0.001	negative
10	Right colon	3	15	1	negative	<0.001	negative
11	Sigmoïd colon	3	14	4	negative	<0.001	negative
12	Sigmoïd colon	2	8	3	negative	<0.001	negative

*Positivity is doubtful. Relative expression level is in the range between the definitions of positive and negative values.

with negative SLNs on H&E and IHC examination showed positive results in at least one of the SLN after CEA RT-PCR. The relative expression levels for these SLNs were 0.084, 0.470 and 0.004, respectively. According to our definitions, the latter sentinel node could not be defined as either positive or negative. Therefore, this patient was not included in the non-sentinel node analysis. All positive PCR results were found in T3 tumors. None of the three T2 tumors showed lymph node metastases on PCR.

The remaining nine patients had a negative SLN status on H&E, IHC and RT-PCR examination. The resected specimens in these nine patients had a total of 102 lymph nodes, with a mean number of 11.3 examined lymph nodes per patient. In each run, positive controls turned out positive, with Ct values varying from 23.95 to 29.59. Negative controls turned out negative with Ct values of 40 or occasionally with 1 out of 3 Ct values of more than 37. The mean Ct-value for the housekeeping genes was 27.92 with a mean standard deviation of 0.0707, indicating that the RNA quality and quantity was similar for all cases. Three lymph nodes had GAPDH Ct values of 31 or 32, indicating that the RNA quality and input for these samples was poor. However, Ct values for CEA for these three samples were >40, suggesting a negative result. None of the other 99 non-sentinel nodes showed positive results on RT-PCR.

Discussion

Contrary to the SLN method in breast cancer and melanoma aiming to limit the surgical procedure, the rationale for SLN in colon cancer patients is mainly to stage tumors upwarts by identifying micro-metastatic nodal disease. If the SLN does not contain (micro)-metastatic disease it is unlikely that metastatic disease will be detected in the other regional nodes. Using the SLN method for proper pathological staging, a proportion of node-negative tumors on conventional pathological examination will be upstaged and this subset of patients may benefit from adjuvant treatment. The SLN procedure will not alter the surgical resection in colon cancer patients. This concept is clinically relevant if identification of nodal micro-metastasis affects the prognosis.

Studies on the SLN concept in colorectal carcinoma demonstrated varying results usually depending on different techniques used (11-28). Most studies performed cytokeratin IHC on the SLNs, whereas the non-sentinel nodes were only examined with conventional H&E staining. In these cases, enhanced detection of metastatic tumor in the sentinel lymph node may only reflect the more intensive histopathological technique rather than the biological significance of the sentinel node. One study validated the procedure by examining both the sentinel nodes and non-sentinel nodes with IHC (29). They found a false-negative rate of the sentinel node procedure of 13% with IHC on all lymph nodes in an unselected population that represented the early experience with dye-directed lymphatic mapping in colon cancer. The authors also considered cases with single cytokeratin-positive cell node-negative because these may lack specificity in the setting of colorectal neoplasms. In our study, we used CEA RT-PCR on lymph nodes in a selected population that was part of a larger study on the sentinel node biopsy (8). Patient material was selected based on node-negative status after H&E examination of sentinel and non-sentinel nodes. In addition, the sentinel nodes were negative using IHC. We used qRT-PCR to detect CEA transcript levels because it is a disease specific marker that is present in the majority of colon carcinomas (30).

Several studies described the PCR examination of lymph nodes in colon carcinoma using CEA or CK 20 as a marker (6, 31-35). A disadvantage with RT-PCR is the false-positivity that may occur (35-37). On the other hand, the consequences of RT-PCR node-positivity is still not clear. RT-PCR nodal positivity may occur because of a very small tumor burden which has the ability to metastasize, or a single mRNA copy in a cell without metastatic potential. In addition, some non-tumor cells bear a few copies of CEA and might result in a positive RT-PCR result when enough cycles are performed.

Because the aim of our study was to determine whether the sentinel node is truly the lymph node most likely to harbor metastatic tumor and to assess the true histologically false-negative rate of the SLN-procedure, we were interested in the most sensitive technique to detect tumor cells. As RT-PCR is more sensitive than IHC, it appeared to be the best technique to use. In our study design, false-positive results are not really a problem because we macro-dissected all lymph nodes from the surrounding tissue. We only saw three positive RT-PCR results in sentinel nodes, and these patients were excluded from the non-sentinel lymph node analysis. The sentinel nodes that were negative on H&E, IHC and CEA RT-PCR examinations in our study indeed represented the node-negative status of the lymphatic basin of the primary tumor in all 102 examined non-sentinel nodes. This shows that the sentinel node procedure is indeed a reliable concept in colon cancer and seems to be useful in selecting high-risk groups. We would like to mention that the RNA quality of our samples was good enough to perform RT-PCR in most cases, with only three out of 102 non-sentinel nodes having Ct values for GAPDH of >30 indicating poor quality RNA. Therefore, it is indeed possible to perform RT-PCR on paraffin-embedded lymph nodes as demonstrated previously (10).

This study does not present any evidence in terms of prognosis for the routine use of qRT-PCR examination of (sentinel) lymph nodes in colorectal cancer. However, some reports do suggest that micrometastatic and/or molecular evidence of tumor in lymph nodes does influence survival (5, 6, 33-35, 38, 39). Two RT-PCR studies confirmed the negative influence on survival of RT-PCR proven metastases in colon cancer (6, 35). Recently, a meta-analysis was presented in which micrometastases detected retrospectively with RT-PCR correlated with overall survival more than did IHC and thus carried a significant prognostic value (40). Prospective studies are needed to evaluate the potential benefit of systemic chemotherapy in patients with these micrometastases. A reliable sentinel node procedure might facilitate intensive pathological examination by allowing a focused qRT-PCR/IHC examination of only the sentinel node(s), with routine H&E examination of the non-sentinel nodes.

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