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**Diagnosing occult tumour cells and their predictive value in
sentinel nodes of histologically negative patients with colorectal
cancer.**

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ABSTRACT

Purpose: Most studies on the sentinel node (SN) procedure in patients with colorectal cancer include immunohistochemical analysis of the SN only. To evaluate the real diagnostic accuracy of the SN-procedure with immunohistochemical analysis, the presence of occult tumour cells in all histologically negative lymph nodes was compared to the presence of these cells in SNs. Also the reproducibility of diagnosing occult tumour cells (OTC) and the sensitivity of three different antibodies was assessed.

Methods: Between November 2006 en July 2007, an ex vivo SN procedure was performed in 58 histologically N0 patients with colorectal cancer. All lymph nodes (n=908, mean 15.7) were step-sectioned and immunohistochemistry was performed using two antibodies against cytokeratins (Cam5.2, and CK 20) and one antibody against BerEp-4.

Results: OTC were identified in 19 of 58 patients, with micrometastases (0.2-2mm) in 7 and isolated tumour cells (ITC)($< 0.2\text{mm}$) in 12 patients. The overall agreement in diagnosing OTC between two independent pathologists was 86%. A SN was identified in 53 of 58 patients. All micrometastases were found in SNs. In two patients with negative SNs, ITC's were demonstrated in non-SNs (sensitivity 88%, and overall accuracy 96%).

Conclusion: Additional immunohistochemical analysis of histologically negative lymph nodes demonstrates occult tumour cells in 33% of the patients resulting in an upstaging rate of 12%. Occult tumour cells are predominantly found in the SN, therefore SN mapping has the potential to refine the staging system for patients with colorectal cancer.

Key words: Occult tumour cells, Sentinel lymph node, colorectal cancer

INTRODUCTION

Sentinel lymph node (SN) mapping is an increasingly popular technique to improve staging accuracy for patients with colorectal cancer by identifying nodes with the highest likelihood to harbour metastatic disease. This technique has shown promising results for colon carcinomas with SN mapping accurately predicting nodal status in 80-100%. [1-24] An advantage of SN mapping is the potential to use this technique for a more accurate and cost-effective pathologic assessment of lymph node status. The additional stepwise sectioning combined with immunohistochemical analysis of the SN has been demonstrated to detect occult tumour cells (OTC) in up to 40% of histologically negative patients with colorectal cancer [1-24], possibly identifying a patient group that may benefit from adjuvant systemic therapy.

However, previously reported results are difficult to interpret because most studies publishing SN results include immunohistochemical analysis of the SN only. This might result in flattering negative predicting values and sensitivity rates. Moreover, most studies published so far have not subdivided the finding of OTC into micrometastases and isolated tumour cells (ITC) on the basis of their dimensions as recommended by the American Joint Committee on Cancer (AJCC) staging manual. [25] This possibly results in an overestimation of the number of upstaged patients and makes the clinical significance of the detected OTC difficult to evaluate. The widespread use of SN immunohistochemical analysis in patients with breast cancer has demonstrated that stage migration due to SN mapping harbours the danger of potential misleading effects and artefacts in prognostication. [26] This highlights the need to differentiate correctly between clinically relevant (micro)metastases (0.2-2 mm)(pN1mi+) and immunohistochemically detected isolated tumour cells without prognostic significance (<0.2mm)(pN0itc+).

This is particularly so since the main purpose of SN mapping in patients with colorectal cancer is to establish a more accurate staging of patients at risk of recurrence due to lymphogenic spread and who could benefit from adjuvant chemotherapy.

For clinical application with therapeutical consequences, an immunohistochemical marker for OTC has to be both highly sensitive (i.e. detect the majority of tumour cells), and specific (i.e. absence of false positive staining of cells). Since most studies use different antibodies without a gold standard and uniform definition of OTC, the antibody of first choice for the detection of clinically relevant micrometastases in patients with colorectal cancer is still difficult to determine on the basis of published data.

We performed this study to evaluate the real diagnostic accuracy of upstaging N0 patients with colorectal cancer by SN-procedure with immunohistochemical analysis. Therefore the incidence of OTC in SNs was compared to the presence of these cells in all histologically negative lymph nodes of these patients. Two experienced pathologists independently assessed the presence of lymph node metastases. The detection of OTC was analysed by three different antibodies to determine the sensitivity and specificity of these antibodies. In addition, the presence of OTC was correlated to clinicopathological parameters.

PATIENTS AND METHODS

Patients

Between November 2006 and July 2007, an ex vivo SN procedure was performed in 100 patients with colorectal carcinoma who were operated on with curative intent. Exclusion criteria for SN mapping were invasion of other organs (T4 carcinomas), or two simultaneous colorectal carcinomas. Patients with locally advanced rectal cancer who underwent neoadjuvant chemoradiotherapy were excluded because of stage migration before surgical treatment. Of the 100 patients 42 patients were shown to have macrometastases in one or more lymph nodes. The remaining 58 patients were histologically staged as N0 (Dukes A or B), and they comprise the population of the current study. All patients with colon cancer underwent an oncological resection, including the mesocolon of the vascular trunk. Patients with rectal carcinoma underwent total mesorectal excision (TME) after preoperative short-course radiotherapy comprising five fractions of five Gray. In addition, the mesenteric lymph nodes of 6 patients were included who underwent colonic resection for benign disease (*e.g.* Crohn's disease or diverticulosis) as a negative control group.

The study was done in accordance with the guidelines of the local ethics committee.

Sentinel lymph node mapping

In this study, the ex vivo lymph mapping was used since only this technique can also be performed easily in patients with rectal carcinomas. The ex vivo technique has been demonstrated to be as accurate as the in vivo technique in identifying SNs[27, 28], and it facilitates standardised, uniform specimen processing and assessment, recognising that the principal limitation of this technique is its inability to detect a small percentage (1-10%) of cases with aberrant lymphatic drainage.[2, 16, 19] After standard resection, 0.5-2 ml (depending on the volume of the tumour) patent blue V (Guerbet, Gorinchem, The

Netherlands) was injected around the tumour with the colonic specimen left intact.[29] For colonic carcinomas, the mesocolon was inspected and the first one to four blue lymph nodes were identified as SNs and either dissected or marked with a suture. For rectal carcinomas the specimen was sent to the department of pathology immediately after resection in fresh state. The identification of blue nodes was performed at the department of pathology immediately, to keep the circumferential resection margin intact.

Immunohistochemical staining

The surgical resection specimens were analysed at the pathology department using a standardised protocol. All lymph nodes (SNs and non-SNs) were collected in separate boxes and marked according to location, then cut in two with both sides stained with hematoxylin and eosin and evaluated for tumour involvement. Of the 58 histologically N0 patients, serial sectioning was performed at 500µm intervals of all lymph nodes from formalin-fixed and paraffin-embedded archival tissue blocks. Three serial sections at three separate levels were immunohistochemically stained with three different monoclonal antibodies: the anti-epithelial cell antibody Ber-EP4 (DAKO, The Netherlands), was combined with two anti-cytokeratin antibodies: the anti-CK20 antibody, with its expression limited to gastrointestinal epithelial cells (Euro Diagnostica, Arnhem, The Netherlands), and the anticytokeratin marker Cam5.2 directed against cytokeratin 7 and 8 expressed in all epithelial cells (Becton and Dickinson, Alphen aan den Rijn, The Netherlands). Appropriate positive and negative controls were added on each automated run, to confirm the sensitivity and specificity of the antibodies (sections of colonic carcinoma tissue served as positive controls; negative controls were obtained by omitting the primary antibody). The staining procedures have been described in detail previously.[29]

Microscopic evaluation and definitions

All slides were reviewed by two independent experienced pathologists who were unaware of the clinical data. The type of involvement (single tumour cells or clusters), topography (lymph-angio invasion and/or lymph sinuses and/ or parenchyma) and extension of the lesion was assessed in each case (Figure 1).

Occult tumour cells were classified as either an ITC (diameter < 0.2 mm) or a micrometastasis (diameter between 0.2 mm and 2 mm). Patients were then restaged according to the AJCC classification.[25] OTC comprised of micrometastases (0.2-2 mm)(pN1mi+) and ITC (<0.2mm)(pN0itc+). In case of disagreement, the slides were re-evaluated in a consensus meeting. False-positive non-neoplastic haematopoietic cells (e.g. reticular cells and plasma cells which can also show staining for cytokeratins), were discriminated from ITCs on the basis of histopathologic features.

Statistical analyses

The following definitions were used for calculations (Figure 2). Identification rate is the number of patients with one or more SN identified (b) divided by the total number of procedures (a) x 100%. Sensitivity is the number of patients with an immunohistochemically positive SN (c) divided by the total number of patients with detected OTC (c+e) x 100%. Negative SNs were considered false negative if one of the other regional lymph nodes (non-SNs) demonstrated micrometastases or ITCs (e). The accuracy of the immunohistochemical SN analysis suggests a conformity of the SN status and the regional nodal status, i.e. the total number of patients with an immunohistochemically positive SN (c) plus the number of patients with a true-negative SN (f)/ the number of patients with an identified SN (b) x 100%.

All statistical analyses were performed using the Statistical Software Package version 14.0 (SPSS INC., Chicago, IL, USA). Associations between clinicopathological features and the presence of micrometastases were analysed using Student's t-test (continuous variables) or Chi-squared test (categorical variables). P-values of 0.05 or less were considered statistically significant.

RESULTS

Restaging patients by detection of occult tumour cells

Three serial sections of all 908 lymph nodes from 58 patients with N0 carcinomas were examined with three different antibodies (mean 15.7 lymph nodes per patient, range 6-42). In 27 patients none of the lymph nodes had metastases after immunohistochemical staining and in 8 patients one or more lymph nodes demonstrated false positive staining unanimously recognised by both pathologists. There was consensus on 6 patients with micrometastases and 9 patients with ITCs; overall agreement 86% (50 of 58 patients). For the remaining 8 patients there was discrepancy in the diagnosis by the two pathologists. In these 8 patients, one pathologist diagnosed one micrometastasis and 10 ITCs in 11 lymph nodes, whereas the other pathologist classified 2 lesions as micrometastases, 6 lesions as ITC and 3 lesions as false positive staining. It was noted that the discrepancy between micrometastases and ITCs was mainly due to differences in measuring multiple foci. According to the AJCC 6th edition of TNM classification of malignant tumours, clusters of tumour cells are considered as one focus if the nests are separated by a few cells, and when in doubt the lower category should be selected to prevent upstaging. In a consensus meeting the following was agreed upon: 52 lymph nodes with OTC were found in 19 of the 58 patients (33%); seven patients had micrometastases (12% pN1mi+) and 12 patients had ITCs (21% pN0itc+)(Table 1).

The presence of OTC (micrometastases and ITCs combined) was not related to any clinicopathological characteristic (Table 2). Although micrometastases and ITCs were found more frequently in tumours infiltrating the serosa and in tumours larger than 5cm, this was not statistically significant. Since micrometastases are thought to represent a different level of clinical significance, a correlation between pN1(mi+) versus pN0 (itc+/-) and clinicopathological characteristics was also analysed. The presence of micrometastases was

also not related to tumour infiltration depth, differentiation grade, and extent of tumour spread, but a significant relation with lymphangio-invasion could be demonstrated ($p < 0.001$).

Sensitivity and specificity of three different antibodies

No epithelial cells with atypical nuclear features characteristic of carcinoma cells were identified in lymph nodes from six control patients operated upon for benign disease, although occasional staining for keratin was observed in stellate cells located in the interfollicular regions of the nodes consistent with staining of reticulum cells. However, this pattern was easily distinguishable from the staining of carcinoma cells.

Each micrometastasis was detected with all three antibodies, as were 39 of 45 ITC-positive lymph nodes. All ITCs were detected with BerEP4. Six OTC were not detected with CK20 and two were missed with Cam5.2. It was noted that the intensity of staining with Cam5.2 and CK20 was related to differentiation of the primary tumour, and the undetected ITCs were found in lymph nodes of patients with a dedifferentiated tumour.

In 18% of the patients false-positive staining was detected with BerEP4 (*e.g.* reticulocytes, fibroblasts and mastcells) and this antibody yielded the most discordant classifications of ITCs and false-positive stained cells between the two pathologists. False-positive staining was less frequently encountered with Cam5.2 (although there was occasional staining of dendritic cells) and was hardly seen with CK20 immunohistochemical staining. These results would make Cam5.2 the antibody of first choice since it demonstrated the most consistent staining with high sensitivity and acceptable specificity.

Diagnostic value of SN mapping

The SN (median two SNs, range 1-4) was found in 53 of the 58 N0 patients (identification rate 91%). Of the five patients in which the SN could not be identified, two patients demonstrated OTC in lymph nodes; these cells were all ITC. The remaining 53 patients were

evaluated for the calculation of the diagnostic value of the SN. Overall, OTC were found in 17 patients; of which 15 were found in the SN and two in a non SN. Resulting in a sensitivity of 88% (15/17) and a negative predictive value of 94% (34/36)(Figure 2). OTC were found in 18 of the 105 SNs (17%), compared to 34 of 803 non-SNs (4%); this difference was statistically significant ($p<0.001$). Because all seven micrometastases were found in the SNs exclusively, the accuracy of the SN procedure in upstaging patients is 100%.

DISCUSSION

Value of SN procedure in refining staging of colorectal patients

Our study emphasises the potential value of SN procedure for optimising staging of patients with colorectal cancer. The examination of 908 lymph nodes by stepwise sections and immunohistochemical analysis revealed OTC in 33% of histologically N0 patients (12% micrometastases and 21% ITCs). OTC were predominantly found in SNs with an overall sensitivity of SN mapping for OTC of 88%.

A large number of studies already addressed the value of SN mapping for colorectal carcinomas (Table 3).[1-24] In reviewing the literature, most studies demonstrate identification rates and accuracy rates varying from 80 to 100%, and conclude that this technique can predict lymph node status with good accuracy rates. However, in these studies the percentage of histologically N0 patients is often more than 50% which makes the presented accuracy rates difficult to interpret.[1-7, 9-17, 19-23] This is merely because accuracy rates up to 90% may be accompanied by sensitivity rates and false negative rates of 50%.[3, 7, 16, 19-21]

For clinical use the SN procedure should be both highly sensitive and specific. Evaluating upstaging percentages in studies lacking immunohistochemically analysis of non-SNs harbours the danger of an underestimation of sensitivity rates and the presentation of flattering negative predictive values. The primary role of SN procedure in patients with colorectal cancer is to increase the accuracy of staging N0 patients by identifying and analysing those nodes with the highest likelihood to harbour metastatic disease in a cost-effective manner. Therefore, it is of great importance that OTC are predominantly found in SNs. We demonstrate that the incidence of OTC is more than four times higher in SNs compared to non-SNs. Our results are in agreement with two previous studies that showed SNs to have the highest probability of harbouring OTC.[23, 24]

Classification and detection of occult tumour cells

However, our study also emphasises that the classification of OTC remains difficult. Reliable and reproducible discrimination between micrometastases (pN1) and ITCs (pN0) is an essential component of adjuvant treatment planning. In analysing discrepant diagnoses for clinically relevant micrometastases between the two pathologists, we observed that the most challenging issue was whether multiple tumour clusters should be measured as one. A useful guideline in the AJCC system is that when controversy exists, the lower category should be selected. This recommendation was used during the consensus meeting. Another problem was the distinction between ITCs and false positively staining (non-tumour) cells. Although this has no impact on treatment recommendations, the inter-observer variation highlights the need for well defined antibodies.[31] Our data suggest that Cam5.2 would be the antibody of first choice for the detection of OTC. The anticytokeratin-marker BerEP-4 is more sensitive but has an almost 20% false positive rate with high inter-observer variation. The use of the highly specific anti-CK20 with its expression limited to gastrointestinal epithelial cells can not be recommended due to its loss of expression in dedifferentiated tumours, resulting in low specificity.

True upstaging rate of SN mapping and predictive value

Comparing the incidence of clinically relevant upstaging by SN mapping with immunohistochemical analysis to literature is difficult. Before the revised TNM classification was introduced in 2003 micrometastases were not distinguished from ITCs.[31] Only three studies discriminated micrometastatic disease from ITCs,[3, 7, 19] with low incidence of micrometastatic disease varying from 5 to 10%. Other studies reporting higher upstaging rates all included ITCs in their findings.[5, 10, 12, 13, 23, 24] This variation in definition of micrometastases makes it difficult to reach well-founded conclusions on the role of OTC and

could explain the controversial prognostic significance of OTC. So far, only two studies have analysed the prognostic significance of micrometastatic disease in SNs with a decreased disease-specific survival in the pN1(mi+) group.[10,11] All other studies commenting on prognostic significance of OTC did not perform SN mapping.[33] Most studies failed to demonstrate a relation between the presence of OTC and tumour recurrence or survival. Messerini et al. demonstrated that the inclusion of ITCs should probably be regarded as a confounding factor in these studies.[33] Although our study was not designed to comment on the prognostic significance of OTC, the relation between lymphangio-invasion of tumour cells and micrometastases is suggestive of clinical relevance. Especially since this relation could not be demonstrated between lymphangio-invasion and ITCs.

Conclusion

Occult tumour cells are predominantly found in the SN with a true upstaging rate of 12% in patients with pN0 colorectal cancer. For immunohistochemical staining Cam5.2 seems the antibody of choice with an overall agreement of pathologist of 88% in staging patients. The SN procedure has the potential to refine the staging system for patients with colorectal cancer. Further survival analyses should be awaited to reveal the real prognostic relevance of these findings.

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Table 1

Classification of immunohistochemical staining results by two independent pathologists of 58 histologically negative patients with colorectal cancer.

		Pathologist 1			
		Micrometastasis	Isolated tumour cells	False positive staining	Absent
Pathologist 2	Micrometastasis	6	1	0	0
	Isolated tumour cells	1	9	4	0
	False positive staining	0	5	8	3
	Absent	0	0	2	27
Consensus		7	12	10	29

Table 2

The presence of occult tumour cells correlated to tumour characteristics of 58 patients

Tumour characteristics	N=58	Occult tumour cells			p-value
		Micrometastasis (N=7)	ITCs (N=12)	Absent (N=39)	
Depth of invasion ^a	T1 (4)	0	0	4	ns
	T2 (18)	2	3	13	
	T3 (36)	5	9	22	
Differentiation grade	well (4)	0	0	4	ns
	moderate (45)	7	10	28	
	poor (9)	0	2	7	
Extend of tumour spread	< 5cm (34)	2	6	26	ns
	> 5cm (24)	5	6	13	
Lymphangio-invasion	absent (51)	3	12	36	0.001
	present (7)	4	0	3	

^aT1: tumour limited to the (sub)mucosa, T2: tumour infiltrates muscularis propria, but not adventitia, T3: tumour infiltrates adventitia.

Table 3

Predictive value of blue dye SN mapping in patients with colorectal cancer including immunohistochemical analyses (comparison of existing literature until may 2008; excluding duplicate series and princeps studies with updated publications).

Study	N	No LNs (mean)	N0 (%)	Identification (%)	Accuracy (%)	False neg (%)	Sensitivity (%)	Upstaging (%)	ITC ^a	Antibody
Immunohistochemical analysis of SN only										
Matter ¹	52	30 ^c	63	92	-	25 ^d	44 ^d	23	N	CK11/CEA /Ca19-9
Kelder ²	69	11	73	97	96	30 ^d	89 ^{d, f}	18	N	CK7/8/18
Bembenek ³	315	20	69	85	86	46	54	5/16 ^g	Y	MNF16
Stojadinovic ⁴	162	18	65	98	90 ^d	31 ^d	69 ^{d, f}	11	N	AE1/AE3 Cam5.2
Van Schaik ⁵	44	10.5	52	94	100	0	100	30	N	LU-5
Bianchi ⁶	22	22 ^c	73	100	95	17	83	13	N	CK?
Thomas ⁷	69	16	62	93	78	54	46 ^f	5/- ^{e, g}	Y	CEA/ CK
Smith ⁸	17	16	41 ^e	94	100	0	100	20 ^d	N	MNF116
Saha ⁹	500	15	50 ^d	98	96	10	90	26	N	AE1/AE3
Codignola ¹⁰	56	21	59	100	89 ^{e, f}	11 ^d	86 ^{e, f}	38 ^d	N	Cam5.2
Braat ¹¹	91	7.7 ^e	62 ^e	90 ^e	90 ^e	26 ^e	74 ^{e, f}	12 ^e	N	CK7/8
Cox ¹²	17	18	82	100	100	0	100 ^f	29	N	AE1/AE3
Smith ¹³	40	17	56	98	95 ^e	12	88 ^f	29 ^d	N	Cam5.2
Bell ¹⁴	58 ^b	30	57	97	84 ^e	16 ^d	64 ^e	4	N	CK
Paramo ¹⁵	55	12	67 ^e	82	98	3 ^d	93 ^e	11 ^d	N	Cam5.2
Fitzgerald ¹⁶	26	15	87 ^e	88	91 ^d	9 ^d	33 ^e	10	N	Cam5.2
Bendavid ¹⁷	20	-	55	90	-	-	-	25	N	AE1/AE3
Joosten ¹⁸	50	14 ^c	48	70	66 ^e	60	40	13	N	LU-5/ CK19
Bilchik ¹⁹	141	17	73	99	91	39	61 ^e	8/22 ^g	Y	CK
Bertagnoli ²⁰	72	17	66	92	80	54	46	-	N	AE1/AE3 CEA
Immunohistochemical analysis of all lymph nodes										
Faerden ²¹	199	13 ^c	68	93	85	47	53	30	N	Cam5.2
Yagci ²²	47	19	57	98	91 ^e	20	80	15	N	AE1/AE3 / CEA
Bembenek ²³	55	26 ^c	70	85	-	4	96	39	N	MNF 116
Turner ²⁴	51	14 ^c	55 ^e	100	88 ^e	26 ^e	74 ^e	29	N	AE1/AE3

^a Discrimination between micrometastasis and isolated tumour cells (N=no, Y=yes)

^b Specimens

^c Median

^d Discrepancy between reported value and calculated value (reported values are mentioned)

^e Calculated value

^f Immunohistochemistry results of SN included in sensitivity

^g % micrometastases (true upstaging)/ % isolated tumour cells

Figure 1

Representative examples of immunohistochemically detected occult tumour cells.

A: micrometastasis $> 0.2\text{mm}$. **B:** isolated tumour cell, cluster of cells $> 0.2\text{mm}$. **C:** isolated tumour cells, cluster of cells $< 0.2\text{ mm}$ with characteristic nuclear features. **D:** false-positively staining cell

A **B** **C** **D**

Figure 2

Flowchart. Predictive value of sentinel node mapping for occult tumour cells in histologically negative lymph nodes.