ORIGINAL ARTICLE

High prevalence of isolated tumour cells in regional lymph nodes from pN0 colorectal cancer

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Background: The prevalence of isolated tumour cells (ITCs) in regional lymph nodes from colorectal cancer (CRC) is controversial and has never been prospectively assessed in large groups of consecutive patients. pN0 early-relapsing CRC can be explained by lymph node-ITC.

Aim : To assess the prevalence of ITCs in regional lymph nodes from 309 consecutive patients with pN0M0 (pathological (p)-tumour-node-metastasis (TNM) staging system) CRCs.

Patients and methods: ITCs were assessed by immunohistochemistry (MNF116 monoclonal antibody (1:100); Dako, Glostrup, Denmark) in two serial histological sections obtained from 5016 mesenteric lymph nodes from 309 patients with pN0 CRCs (mean number of lymph nodes per patient = 16.2; p-TNM stage 0, n = 25; p-TNM stage I, n = 123; and p-TNM stage II (A+B), n = 161). Tumour histology, vascular cancer invasion and pathological stage were also recorded.

Results: ITCs were detected in the regional lymph nodes of 156 of 309 (50.5%) patients with CRC, mostly in nodes located within 3 cm from the neoplasia. ITC status correlated with (a) tumour p-TNM stage (Pearson's χ^2: $p<0$; ordered logistic regression: odds ratio (OR) = 4.6; 95% confidence interval (CI) = 2.88 to 7.33; $p<0$) and (b) pT value (Pearson's χ^2: $p=0$; ordered logistic regression: OR = 4.9; 95% CI = 3.1 to 7.7; $p<0$). By multivariate analysis, including p-TNM stage, vascular invasion and ITC status, both stage (OR = 5.1; 95% CI = 2.9 to 8.9; $p<0$) and vascular invasion (OR = 4.2; 95% CI = 1.94 to 8.98; $p<0$) were found to be independent variables associated with ITC+ lymph nodes.

Conclusion: More than 50% of pN0-CRC patients have ITCs in the mesenteric lymph nodes. ITC status is significantly correlated with cancer stage and vascular cancer invasion. The clinicopathological effect of ITC remains to be prospectively evaluated.

In colorectal cancer (CRC) with no extranodal metastasis (M0), regional metastatic lymph nodes distinguish pathological (p)-tumour-node-metastasis (TNM) stages I and II (ie, pN0) from stage III (ie pN1/2) adenocarcinoma and discriminate patients requiring postsurgical adjuvant treatments.[1 2]

Although patients with p-TNM stage 0, I and II cancers are regarded as having localised disease, as many as 35% of patients with pN0 stage cancer develop extranodal metastases within 5 years of surgery.[3] The early identification of this subgroup of patients would allow postsurgical therapeutic measures, possibly resulting in a lower rate of cancer recurrence.

p-TNM stage I and II recurrent disease may result from pathological understaging of the tumour.[4 5] On the basis of this assumption, current guidelines require that no less than 12 lymph nodes should be histologically evaluated.[1 6]

In the spectrum of lymph node colonisation by cancer cells, three main situations occur: (a) metastases (metastatic implants with diameter >0.2 cm); (b) micrometastases (macroscopically undetectable metastases ranging between 0.02 and 0.2 cm in diameter); and (c) isolated tumour cells (ITCs, which are single or small nests of countable tumour cells, with diameter never >0.02 cm, only detectable by immunohistochemistry (IHC) or molecular biology methods).[1 7] The current nomenclature suggests that the presence of ITCs in lymph nodes should be reported as pN0(i+) or pN0(mol+), where "i" and "mol" indicate the methods used for ITC detection (IHC and molecular methods, respectively).[7] No information is available on interobserver agreement when ITCs are assessed by IHC, and the divergence in the prevalence of lymph node-ITC reported in the literature supports the claim that current histological criteria are bewildering or inconsistently applied.[8–18]

In patients with CRC, the prevalence and clinical effect of lymph node micrometastases and ITCs remain controversial.[3 4 8 9 11–16 19–21]

The relationship between lymph node-ITC and patient outcome is difficult to evaluate because (a) the interobserver consistency in the assessment of ITCs by IHC has never been tested; (b) available studies are based on small groups of retrospectively selected patients[5 8 9 11–16 18 21 22]; and (c) lymph node micrometastases and ITCs are considered together.[8 9 13–15 17]

This prospective study focuses on the prevalence of ITCs in the regional lymph nodes obtained from 309 consecutive patients with pN0M0 CRC. In all these patients, ITCs were assessed by IHC in two serial histological sections obtained from all lymph nodes.

PATIENTS AND METHODS

Patients

Between October 2002 and April 2004, 546 patients underwent radical surgical treatment for CRC at the Padova University School of Medicine and Teaching Hospital (Padova, Italy). The study was approved by the local human investigations committee (Committee of Ethics of Padova Teaching Hospital, Padova, Italy) and informed consent was obtained from all the patients concerned. The surgery was standardised according to the location of cancer, thus

Abbreviations: CRC, colorectal cancer; IHC, immunohistochemistry; ITCs, isolated tumour cells; p-TNM, pathological tumour-node-metastasis

minimising the variability in the surgical technique for lymphadenectomy.

Of the 546 patients, no lymph node metastases or micrometastases were detected by conventional histological examination (haematoxylin and eosin stain) in 309 patients (given no neoadjuvant treatment) who formed the study group. These patients included 187 men (60.5%) and 122 women (39.4%) with a mean age of 68.78 (SD 11.12; range 34–93) years. Table 1 shows their demographic data, pathological stage, cancer site and histological variables.

Vascular cancer invasion (defined as the presence of intravascular neoplastic cells covered by endothelium or associated with thrombus) was histologically shown in 82 of 309 (26.5%) patients. The prevalence of vascular invasion significantly increased in relation to (a) cancer pT value (Pearson's χ^2: p = 0; ordered logistic regression: odds ratio (OR) = 3.51; 95% confidence interval (CI) = 2.09 to 5.88) and (b) cancer p-TNM stage (p-TNM stage 0: 0; stage I: 22 (17.9%); p-TNM stage IIA: 55 (36.7%); p-TNM stage IIB: 5 (45%); Pearson's χ^2: p = 0; ordered logistic regression: OR = 3.48; 95% CI = 2.06 to 5.9; table 1).

Methods

Handling of gross surgical specimens and lymph node collection

All gross surgical specimens were fixed in 5–10% formalin for 12–24 h. Regional lymph nodes were defined as established by the sixth edition of TNM.[1]

After fixation, cancers were always extensively sampled, including peripheral areas where the neoplasia merged with the non-neoplastic adjacent intestinal wall.

In all patients, the peri-intestinal fat was dissected from the intestinal wall, distinguishing between fat tissue located >3 cm or <3 cm from the neoplasia. The fat tissue was sliced into gross sections (0.1–0.15 cm thick) and any lymph nodes encountered were collected for histological examination. When the macroscopic sectioning included large lymph nodes, the largest part of the node was collected. As a result, regional lymph nodes were divided in all cases into two groups—that is, (a) less and (b) more than 3 cm from the neoplastic lesion.

Histology and IHC

The lymph nodes were embedded in paraffin wax and histological sections (5-μm thick) were obtained and stained with haematoxylin and eosin. From each lymph node without metastasis (5016 lymph nodes in total), two additional histological sections (10 032 sections, in all) 75–100 μm apart were obtained and stained with monoclonal anti-cytokeratin MNF116 antibody (clone MNF116 mouse monoclonal antibody; working dilution 1:100, Dako).[23 24] All IHC reactions (including both negative and positive controls) were obtained automatically by using a standardised protocol implemented on the Ventana BenchMark IT immunostainer, under the supervision of specialists (CL, PB).

In ITC+ lymph nodes, isolated tumour cells were defined as phenotypically malignant (ie altered nucleus to cytoplasm ratio, atypical nuclei and prominent nucleoli), unequivocally MNF116-positive single cells dispersed in sinusoidal or extra-sinusoidal spaces (fig 1).[25 26] Clustered ITCs never consisted of more than 2–8 cells and never exceeded 0.02 cm in diameter. They also never included venous spaces or capillaries. As an occasional weak stain for cytokeratins has been reported in interfollicular stellate cells, only phenotypically malignant cells were considered to be consistent with ITCs.[27] The same pathologist (CM), with no knowledge of the cancer's p-TNM stage, examined all the immunostained sections. As for the ITC assessment, consistency for a pair of observers (CM;

Table 1 Pathological-tumour-node-metastasis stage, location of cancer and histology in 309 consecutive patients with pN0 colorectal cancer

p-TNM Stage	pT value	No of cases (%)	M:F	Mean age (range)	Cancer site					Histology				
					Ascending colon	Transverse colon	Descending colon	Rectum	NOS adenocarcinoma		Mucinous cancer	Vascular invasion (%)	Lymph nodes—total number (mean number per patient)
									Low grade	High grade			
Stage 0	is	25 (8.9)	19:6	68.8 (47–84)	9	3	11	2	25	0	0	0	305 (12.2)
Stage I	1	28 (9)	17:11	71.9 (56–87)	5	1	18	1	28	0	0	3 (10.7)	350 (12.5)
	2	95 (30.7)	58:37	70.2 (38–93)	22	2	42	27	85	6	4	19 (20)	1377 (14.5)
Stage IIA	3	150 (48.5)	87:63	67.1 (34–91)	50	15	72	13	129	13	8	55 (36.7)	2787 (18.6)
Stage IIB	4	11 (3.6)	6:5	64.3 (45–86)	6	1	1	3	7	1	3	5 (45.0)	197 (18.0)
Total		309	187:122	68.8 (34–93)	92	22	144	46	274	20	15	82 (26.5)	5016 (16.2)

M:F, male:female; p-TNM, pathological-tumour-node-metastasis.
The prevalence of vascular invasion is also reported. In five patients, the cancer site was not known (all five patients had p-TNM stage I cancer)

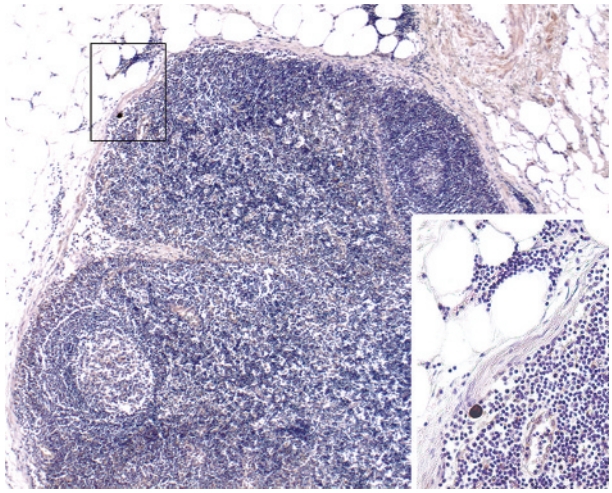

Figure 1 Isolated tumour cells in a lymph node: MNF116+ cells (undetectable on haematoxylin and eosin staining) are located in the peripheral sinus.

VMR) was calculated by using κ statistics in a series of 1500 unselected lymph nodes (fig 1).

Statistical analysis

Statistical analysis was carried out by using χ^2 for 2×2 tables and Pearson's χ^2 for 2×2 or 2×3 tables. A p value <0.05 was considered to be significant. Univariate logistic regression analysis was used as appropriate. Multivariate logistic regression analysis was used to calculate the strength of association between ITC status, cancer stage and vascular invasion. ORs and 95% CIs were also calculated. Stata software (Statistics Data Analysis, rel. 8.1; http//stata.com) was used for all calculations.

The κ coefficient for pairs of observers was interpreted in accordance with the Landis and Koch benchmarks (<0.4 = poor agreement; 0.41–0.8 = moderate or good agreement >0.8 = excellent agreement).[28]

RESULTS

A total of 5016 lymph nodes, ranging from 0.1 to 2.6 cm in diameter, were harvested from the 309 patients studied. Irrespective of cancer stage or site, a mean of 16.2 lymph nodes were obtained from each patient (SD = 12.7; median = 14; range = 1–107). No differences were seen in the number of lymph nodes obtained from the gross surgical specimens collected by the different surgery units participating in this study (Pearson's χ^2: p = 0.8, NS).

Interobserver agreement in the distinction between ITC+ and ITC− lymph nodes was ranked as excellent (κ coefficient = 0.86).[27]

Table 2 shows the ITC status according to the pT value and p-TNM stage of the cancer. ITCs were identified in 1 of 25 (4%) patients with pTis, 4 of 28 (14.3%) patients with pT1, 44 of 95 (60%) patients with pT2, 100 of 150 (72.7%) patients with pT3 and 7 of 11 (63.7%) patients with pT4 cancer. A significant correlation was detected between ITC status and pT value (Pearson's χ^2; p = 0; ordered logistic regression: OR = 4.94; 95% CI = 3.13 to 7.7; p<0). ITCs were identified in 1 of 25 (4%) patients with p-TNM stage 0, 48 of 123 (39.0%) patients with p-TNM stage I and 107 of 161 (66.4%) patients with p-TNM stage II (A+B). A significant association was shown between ITC status and cancer stage (Pearson's χ^2; p<0; ordered logistic regression: OR = 4.6; 95% CI = 2.88 to 7.33; p<0).

Among the 156 ITC+ patients, 113 (72.4%) patients had ITCs only in lymph nodes located within 3 cm from the neoplasia; 34 (21.8%) patients had MNF116+ cells in lymph nodes located both <3 cm and >3 cm from the cancer lesion; and 9 (5.8%) patients had ITCs only in lymph nodes located >3 cm from the cancer.

In all, we examined 5016 lymph nodes from the 309 patients considered and detected MNF116+ cells in one or both sections obtained from 496 (9.9%) lymph nodes. ITC+ lymph nodes were detected most often <3 cm from the neoplastic lesions (ITC+ lymph nodes <3 cm = 418/3084 (13.5%) *v* ITC+ lymph nodes >3 cm = 78/1932 (4.0%); Pearson's χ^2; p<0). A significant association was found between vascular invasion and the number of ITC+ lymph nodes (one-sided t-test for unequal variances and Welch's approximation; p = 0.01).

Because the univariate analysis identified p-TNM stage (0 *v* I *v* II) and vascular invasion as being significantly associated with ITC+ lymph nodes, these two variables were tested in a multivariate logistic regression model. As in the univariate analysis, both were confirmed as independent variables significantly associated with ITC status (with no statistical interaction). The OR associated with the p-TNM stage was 5.15 (95% CI = 2.98 to 8.92; p<0), whereas it was 4.18 (95% CI = 1.94 to 8.98; p<0) for vascular invasion.

To test the consistency of ITC detection in the same lymph node, two serial histological sections from each of the 5016 lymph nodes were considered (ie, 10.032 histology sections in all).

At the single patient level, the following situations were seen:

a. In 153 of 309 patients, neither of the two sections obtained from the same lymph node showed MNF116+ cells (ie ITC− status).

Table 2 Prevalence of isolated tumour cell-positive lymph nodes in 309 patients with pN0 colorectal cancer

		Number of ITC+Lymph nodes/total lymph node number					
P-TNM stage	pT value	No of ITC+/total (%)	Total no of lymph nodes (n)	Mean no of lymph nodes—no/no of patients	No of lymph nodes located <3 cm from cancer (%)	No of lymph nodes located >3 cm from cancer (%)	Total ITC+lymph nodes/total no of lymph nodes (%)
0	is	1/25 (4)	305	12.2	1/161 (0.62)	0/144 (0)	1/305 (0.3)
I	1	4/28 (14.3)	350	12.5	5/186 (2.68)	1/164 (0.60)	6/350 (1.7)
	2	44/95 (46.31)	1377	15	97/786 (12.34)	19/591 (3.21)	116/1377 (8.4)
IIA	3	100/150 (66.6)	2787	18.6	302/1730 (17.51)	54/1057 (5.10)	356/2787 (12.8)
IIB	4	7/11 (63.63)	197	18	13/103 (10)	4/94 (4.25)	17/197 (8.6)
Total		156/309 (50.48)	5016	16.2	418/3084 (13.58)	78/1932 (4)	496/5016 (9.9)

ITCs, isolated tumour cells; p-TNM, pathological-tumour-node-metastasis.
The table also shows the total number of lymph nodes examined in the whole series (per stage) and the number of lymph nodes in which ITCs were detected immunohistochemically. For each stage and pT value, the lymph nodes were also distinguished according to their distance from the neoplasia.

b. Of 309 patients, 156 were recorded as ITC+:

- in 85 of 156 (54.5%) patients, both sections showed MNF116+ cells (ie ITC+ concordant status);
- in 71 of 156 (45.5%) patients, MNF116+ cells were found in only one of the two sections (ie ITC+ discordant status).

Hence, the ITC status was concordant in the two histological sections in 238 of 309 (77%) patients (85 ITC+ patients and 153 ITC− patients).

At the single lymph node level, ITCs were found in 496 of 5016 (9.9%) lymph nodes(table 1):

a. In 4520 of 5016 (90.1%) lymph nodes, neither of the two sections showed MNF116+ cells (ie concordant ITC− lymph node sections).
b. In 496 of 5016 (9.9%) lymph nodes, MNF116+ cells were detected in one or both histological sections:

- in 210 of 5016 (4.2%) lymph nodes, both sections from the same lymph node showed MNF116+ cells (ie concordant ITC+ lymph node sections);
- in 286 of 5016 (5.7%) lymph nodes, only one section from the same lymph node showed MNF116+ cells (ie discordant ITC+ lymph node sections).

So, overall, a concordant ITC picture was seen in the two histological sections in 4730 of 5016 lymph nodes (4520 concordant ITC− lymph node sections, 210 concordant ITC+ lymph node sections; 94.3%).

DISCUSSION

The involvement of the lymph nodes is the most important prognostic variable in patients who undergo radical surgery for CRC, and lymph node metastases coincide with a high risk of recurrent disease.[20 22 24–29] Apart from the possible anatomical variability in the number of regional lymph nodes relating to the different segments of the large bowel,[4 5] the surgical treatment (extent of lymphadenectomy) and the handling of the surgical specimen (accuracy of lymph node dissection) are considered to be the major sources of inconsistency in the postsurgical retrieval of lymph nodes and subsequent assessment of metastatic nodal disease.

In 1999, the College of American Pathologists stated that ''12 to 15 negative lymph nodes predict for regional node negativity''.[6] A recent megatrial showed that ''the number of lymph nodes analyzed... is, itself, a prognostic variable on outcome''.[30] In 2005, a meta-analysis on the adequacy of pN staging in 116 995 CRCs unquestionably showed that ''most patients with CRC did not receive adequate lymph node evaluation''.[5] In this study, a mean of 16.2 lymph nodes per patient were harvested, which is one of the highest rates reported by applying manual nodal dissection.[10 13 14] As for the relationship between the number of lymph nodes harvested and location of cancer, this study confirms that considerably more lymph nodes were recovered from the peri-intestinal fat within 3 cm from the neoplasia than further away, irrespective of the cancer site.[5]

The growth of nodal metastases results from a multistep process, which includes the arrest, extravasation, implantation and proliferation of tumour cells.[6] Nodal micrometastases can be confidently considered to be the early step of any larger metastatic implant.[31] Nodal micrometastases differ from lymph node-ITCs in terms of biology and dimension. As regards dimension, only single cells or foci of MNF116+ cells (<0.02 cm in diameter) can be rightly defined as ITCs: this definition is relevant for unequivocal assessment and is crucial in any evaluation of the clinical effect of ITCs.[7] In most of the available literature, the definitions of ITCs and micrometastases are considered to be interchangeable, and the use of other definitions (eg mini-micrometastases) adds to the non-semantic confusion.[32]

This prospective study strictly applied the ITC definition established by the International Union Against Cancer,[7] also ruling out any association of clustered ITCs with angiogenic activity. Such strict histological criteria and the sensitivity of the immunostain enabled a consistent identification of lymph node-ITC, with an excellent κ-statistic value of interobserver agreement.[8 9 11 13 28 33 34]

On applying strict diagnostic criteria in a prospective series of patients with pN0M0 CRC, the prevalence of pN0(i+) cases was 50.5%, the highest percentage reported in the literature.[8 9 11 12 20 34 35] The only (retrospective) study with a comparable prevalence of lymph node-ITC (49%) considered micrometastases and ITCs together.[15] This was a Japanese trial, which also made a special attempt to assess the consistency of lymph node-ITC detection when multiple histological sections of the same lymph nodes were examined. The authors concluded that ITCs are randomly disseminated in the positive lymph nodes, which implies that positive cytokeratin cells may be missed even when lymph nodes are examined in multiple sections.[15] Our results contradict the above-mentioned observations for two main reasons: (a) ITCs markedly prevailed in the lymph nodes closest to the neoplastic focus (in contrast with the theory of a random distribution); (b) most of the pairs of serial sections obtained from the same lymph node were concordant in identifying the presence or absence of ITCs.

As for the distance between the cancer and the ITC+ lymph nodes, our findings are consistent with those obtained by Noura *et al*,[15] who showed that lymph node micrometastases (undetected by routine histological examination) are more often disclosed by multiple sections of lymph nodes proximal to the CRC. Taking into account the findings of Noura *et al* and those of our study, it seems that special attention should be paid in CRC p-TNM staging to retrieve the lymph nodes nearest to the cancer site.

Multivariate analysis showed a significant correlation between lymph node-ITC and both p-TNM stage and the presence of vascular invasion, which may provide information on the biological relevance of ITCs: although the clinical effect of lymph node-ITC can be established only by a long-term follow-up, our results lead us to hypothesise a specific clinicobiological risk associated with ITCs.[36]

Take-home messages

- A subgroup of pN0 colorectal cancer patients develop extranodal metastases within 5 years after surgery.
- The early identification of this patient subgroup would allow post-surgical therapeutic measures, possibly resulting in a lower rate of cancer recurrence.
- Presence of ITC in regional lymph nodes might help to explain pN0 early-relapsing CRCs.
- Lymph node-ITC presence significantly correlates with both pathological cancer stage and vascular invasion: ITC+ lymph nodes are most frequently detected within 3 cm of the neoplasia.
- Further studies are required to prospectively evaluate the clinico-biological relevance and the prognostic significance of ITC.

In conclusion, this prospective IHC study detected the highest prevalence of ITCs ever reported in regional lymph nodes from CRC (with an excellent interobserver consistency). ITCs were significantly associated with the highest pT cancer values and were seen considerably more often in lymph nodes within 3 cm from the neoplasia. The significant relationship between lymph node-ITC, cancer p-TNM stage and vascular invasion implies a clinicobiological relevance of ITCs and indicates that efforts should be made to prospectively evaluate the prognostic significance of this lesion.

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