



Occult neoplastic cells in the lymph node sinuses and recurrence/metastasis of stage III/Dukes' C colorectal cancer

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Abstract. Lymph nodes from patients with colorectal cancer were immunohistochemically stained for cytokeratin to investigate the relationship between the presence of occult neoplastic cells (ONCs) and recurrence/metastasis. A total of 80 patients with stage III/Dukes' C colorectal cancer were divided into 16 patients who developed recurrence/metastasis (recurrence group) and 64 patients without recurrence (non-recurrence group). ONCs were compared between the two groups with respect to i) single cells (≥ 3 floating ONCs), ii) clusters of cells (1 or more floating aggregates of 2-20 ONCs) and iii) single cells + clusters. When single cells were detected, the sensitivity for recurrence was 87.5% (14/16, $p=0.002$), the positive predictive value (PPV) was 32.6% (14/43), the specificity was 54.7% (35/64) and the negative predictive value (NPV) was 94.6% (35/37). For clusters, the sensitivity was 87.5% (14/16, $p<0.001$), PPV was 41.2% (14/34), specificity was 68.8% (44/64) and NPV 95.7% (44/46). With single cells + clusters, the values were 87.5% (14/16, $p<0.001$), 48.3% (14/29), 76.6% (49/64) and 96.1% (49/51), respectively. These results suggest that the detection of single cells + clusters is a sensitive indicator of a high risk of recurrence/metastasis, while ONCs are useful for identifying the low-risk group of patients with stage III colorectal cancer.

Introduction

The 5-year survival rate of Japanese patients who have stage II/Dukes' B colorectal cancer without lymph node metastasis and receive curative resection is approximately 80% (colon, $84.5\pm 2.8\%$; rectum, $79.8\pm 4.0\%$), whereas the 5-year survival rate is considerably lower at approximately 60% (colon, $74.0\pm 3.5\%$; rectum, $64.7\pm 4.3\%$) for patients with stage III/Dukes' C colorectal cancer and lymph node metastasis who undergo curative resection (1-4). Thus, recurrence/metastasis develops in 30-40% of stage III/Dukes' C patients after curative resection and can be fatal (2,4). According to the pathological concept of breast cancer, positive lymph node metastasis indicates systemic disease with the potential for metastasis to other organs and the presence or absence of lymph node metastasis is considered to be one of the most important clinical markers (5,6). Hematogenous metastasis to the liver or the lungs in patients with lymph node metastasis who undergo curative resection is presumed to occur when cancer cells circulating in the blood during the perioperative period, escape the immune system enter the microcirculation of the liver or the lungs, and find appropriate microenvironment for growth and proliferation (7-10). Various reports have been published about the close relationship between recurrence/metastasis of cancer and detection of occult neoplastic cells (ONCs) positive for cytokeratin immunohistochemical staining and floating in the sinuses of lymph nodes distant from the primary tumors (11-15). ONCs can be semi-quantified by a relatively simple immunostaining method and represent floating tumor cells target trapped in the microcirculation of the lymph nodes, which are part of the immune mechanism (7,8). Although the detection rate of ONCs is low in patients with recurrence of stage II/Dukes' B colorectal cancer at approximately 20-30%, the detection rate is 70-80% or higher in patients with recurrence of stage III/Dukes' C colorectal cancer, and a relationship of these cells with recurrence/metastasis has been strongly suggested (9,10). ONCs can be classified into single cells, clusters (2-10 ONCs forming a mass <0.2 mm in diameter) and aggregates (>10 ONCs). It has been reported that ONCs should be seen as more malignant occult systemic metastases by distinguishing isolated tumor cells (≤ 0.2 mm) from micrometastases (0.2 mm to ≤ 2 mm) among small metastatic foci in the lymph nodes (16-18).

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Abbreviations: ONCs, occult neoplastic cells; PPV, positive predictive value; NPV, negative predictive value; FP, false positive; FN, false negative

Key words: colorectal cancer, occult neoplastic cells, high-risk group, recurrence/metastasis, stage III/Dukes' C, isolated tumor cells

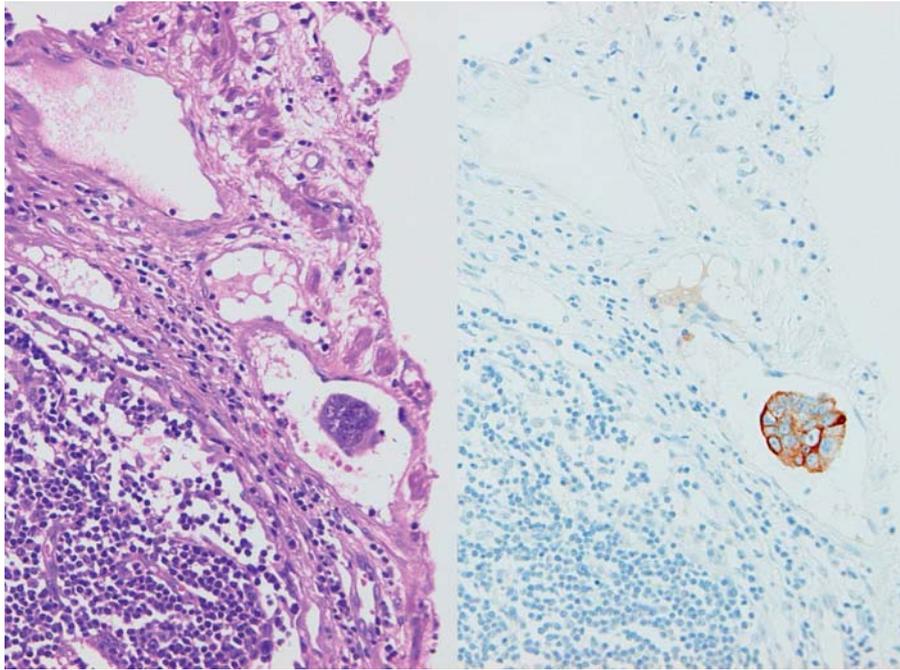


Figure 1. Floating occult neoplastic cells (ONCs) in lymph node sinuses detected by cytokeratin immunohistochemistry and classified as clusters.

Cancer cells remaining in the microcirculation could be eradicated by early postoperative adjuvant chemotherapy, although tumor susceptibility to anticancer agents and the optimal dosage/administration schedule are factors that need to be considered. If it is possible to identify a high-risk group among patients with stage III/Dukes' C colorectal cancer who are more likely to develop recurrence/metastasis, the survival rate could be improved by providing potent adjuvant chemotherapy for these patients in the early postoperative period. Also, identifying a low-risk group of patients who are not likely to develop recurrence/metastasis would contribute to reducing the psychological burden on patients and to devising appropriate follow-up schedules.

Cytokeratin is an epithelial marker that is useful for detecting micrometastasis to lymph nodes because more than 99% of normal lymph nodes are not stained, and AE1/AE3 and CAM 5.2 are well known anti-cytokeratin antibodies (19-23). Because cancer cells can be examined to assess their nuclear structure and cytoplasm, histological and immunohistochemical studies are superior to tests such as PCR in terms of assessing the viability and proliferative capacity of each cell (19,20). A detailed clinicopathological examination of the clinical course and subclassification of ONCs into single cells or clusters in patients with stage III colorectal cancer has not been reported so far. Accordingly, the purpose of this study was to investigate the presence of various types of ONCs by cytokeratin immunostaining of lymph nodes in the surgically resected specimens of patients with stage III/Dukes' C colorectal cancer.

Patients and methods

Among 88 patients with stage III/Dukes' C colorectal cancer for whom complete medical records were available and whose survival was followed between January 2005 and

January 2010, 80 patients were enrolled in this study from whom >15 lymph nodes were retrieved (17). Of these patients, recurrence/metastasis occurred within 3 years in 16 patients (recurrence group) (20.0%), whereas 64 patients had no recurrence (non-recurrence group) (80.0%). The dissected lymph node specimens obtained from these two groups were immunohistochemically stained for cytokeratin to compare the presence of ONCs with the clinical course.

The routine indirect immunoperoxidase method was used for cytokeratin staining of the lymph nodes (19,20). Thin sections (3 μ m) were prepared from the largest cut surface of each formalin-fixed and paraffin-embedded lymph node. After deparaffinization, the sections were immunostained by an autoanalyzer (BenchMark[®] XT; Roche Diagnostics K.K., Tokyo, Japan). After enzymatic treatment with protease 1 (Roche Diagnostics K.K.) (0.5 units/ml) for 4 min at 37°C to activate the antigen, monoclonal anti-cytokeratin antibodies (AE1, AE3 or PCK26; Roche Diagnostics K.K.) were used as the primary antibody and an iView DAB Detection kit (Roche Diagnostics K.K.) was used as autoimmunostaining reagents. Dehydration and mounting were performed after nuclear staining with hematoxylin.

H&E staining and cytokeratin immunostaining were done for serial sections of each lymph node to detect positive cells. After excluding cancer cells and/or cancer nests associated with fibrosis in the lymph nodes, immunostained cells floating in the lymph node sinuses were identified (7-10). The lymph nodes with histological metastatic foci ≥ 2 mm in size and lymph nodes with notable fibrosis around the metastatic foci without floating tumor cells were excluded. Separately identifiable tumor cells were defined as single cells, while 2-20 tumor cells forming a small aggregate (≤ 0.2 mm) were defined as a cluster. Then ONCs were classified as i) single cells (≥ 3 ONCs), ii) clusters of cells (1 or more floating aggregates of 2-20 ONCs), iii) single cells + clusters (Fig. 1). Cells satis-

SPANDIDOS PUBLICATIONS Detection of occult neoplastic cells (ONCs) in lymph nodes (single cells) in the two groups.

Total 80 cases (efficiency 61.3%)	Recurrence group (n=16)	Non-recurrence group (n=64)
Single cells (+) 43 cases (PPV 32.6%)	14 cases ^a (sensitivity 87.5%)	29 cases (FP rate 45.3%)
Single cells (-) 37 cases (NPV 94.6%)	2 cases (FN rate 12.5%)	35 cases (specificity 54.7%)

^ap=0.002, hazard ratio 0.713 (95% CI, 0.571-0.890). PPV, positive predictive value; NPV, negative predictive value; FP, false positive; FN, false negative.

Table II. Detection of occult neoplastic cells (ONCs) in lymph node sinuses (clusters) in the two groups.

Total 80 cases (efficiency 72.5%)	Recurrence group (n=16)	Non-recurrence group (n=64)
Clusters (+) 34 cases (PPV 41.2%)	14 cases ^a (sensitivity 87.5%)	20 cases (FP rate 31.2%)
Clusters (-) 46 cases (NPV 95.7%)	2 cases (FN rate 12.5%)	44 cases (specificity 68.8%)

^ap<0.001, hazard ratio 0.615 (95% CI, 0.461-0.820). PPV, positive predictive value; NPV, negative predictive value; FP, false positive; FN, false negative.

fying all of the above conditions were judged to be positive and other cells were classified as negative. Then the sensitivity, false positive (FP) rate, specificity, false negative (FN) rate, positive predictive value (PPV), negative predictive value

Table III. Detection of occult neoplastic cells (ONCs) in lymph node sinuses classified (single cells + clusters) in the two groups.

Total 80 cases (efficiency 78.8%)	Recurrence group (n=16)	Non-recurrence group (n=64)
Single cells + Clusters (+) 29 cases (PPV 48.3%)	14 cases ^a (sensitivity 87.5%)	15 cases (FP rate 23.4%)
Single cells + Clusters (-) 51 cases (NPV 96.1%)	2 cases (FN rate 12.5%)	49 cases (specificity 76.6%)

^ap<0.001, hazard ratio 0.538 (95% CI, 0.377-0.769). PPV, positive predictive value; NPV, negative predictive value; FP, false positive; FN, false negative.

(NPV) and efficiency were calculated for each type of ONC. Histopathological diagnosis was performed by Y.S., who had no knowledge of the clinical background of the recurrence group or the non-recurrence group, while M.M. and K.K. performed data collection and analysis.

Statistical analysis. The χ^2 test was used to compare the recurrence group with the non-recurrence group and risk ratios (95% CI) were calculated. A p-value <0.05 was considered to indicate significance in all analyses. SPSS statistics software version 17 (SPSS Japan Inc., Tokyo, Japan) was employed.

Results

Single cells (≥ 3 ONCs). When single cells were detected, the sensitivity for predicting the recurrence/metastasis was 87.5% (14/16, p=0.002, hazard ratio 0.713; 95% CI, 0.571-0.890), the FP rate was 45.3% (29/64), the specificity was 54.7% (35/64 cases) and the FN rate was 12.5% (2/16). In addition, the PPV was 32.6% (14/43), NPV was 94.6% (35/37) and efficiency was 61.3% (Table I).

Clusters (1 or more floating aggregates of 2-20 ONCs). For clusters, the sensitivity was 87.5% (14/16, p<0.001, hazard ratio 0.615; 95% CI, 0.461-0.820), the FP rate was 31.2% (20/64 cases), the specificity was 68.8% (44/64), the FN rate was 12.5% (2/16), PPV was 41.2% (14/34), NPV was 95.7% (44/46) and efficiency was 72.5% (Table II).

Single cells and clusters. For single cells + clusters, the sensitivity was 87.5% (14/16, p<0.001, hazard ratio 0.538; 95% CI, 0.377-0.769), the FP rate was 23.4% (15/64), the specificity was 76.6% (49/64), and the FN rate was 12.5% (2/16), while PPV was 48.3% (14/29), NPV was 96.1% (49/51) and efficiency was 78.8% (Table III).

Discussion

It has been reported that D2 lymph node dissection during resection of primary colorectal cancer contributes to the survival of patients with stage II/Dukes' B colorectal cancer, but not patients with stage III/Dukes' C colorectal cancer (24). The original purpose of lymph node dissection during surgery is the complete en bloc removal of metastatic nodes and it also

contributes to the standardization of the method for determining true node negativity by collecting a large number of nodes including those without metastasis from specific sites of resection (25). Identification of at least 12 lymph nodes is also recommended in the NCI guideline for diagnosing true node negativity and the factor that is important for the prognosis is considered to be the number of metastatic foci observed in lymph nodes retrieved by D2 resection (25,26).

Postoperative adjuvant chemotherapy is recommended in the Japanese guideline for the high-risk group of patients among those with stage III/Dukes' C colorectal cancer and lymph node metastasis, but a detailed description of the criteria is not provided other than lymph node metastasis affecting ≥ 4 nodes (TNM; N2), infiltration of other organs, budding at the site of deepest infiltration of the primary tumor and the presence or absence of vascular involvement (2,4). Approximately 30-40% of patients with systemic disease and lymph node metastasis are presumed to belong to the high-risk group for recurrence, while the remaining 60-70% belong to the low risk group. It is presumed that numerous single cells are dispersed into the portal circulation when compared with patients who have stage II/N0-localized tumors and the level of non-specific host immunity was also reported to be much lower in stage III patients than stage II patients (27-31). It was considered to be the result of persistent tumor immunity leading to eradication of single cells. Also, the number of clusters is presumed to be increased compared with that in patients who have stage II/N0-localized tumors, and more potent postoperative adjuvant chemotherapy would be required for stage III patients with a high risk of recurrence (31,32). Aside from various factors such as the host immunity and tumor susceptibility to anticancer agents, the results of this study suggested that the presence of single cells + clusters of ONCs was related to a high risk of recurrence based on its high sensitivity, while ONCs are a useful negative indicator for identifying the low-risk group because the absence of ONCs showed a high NPV. Clinical indicators with a high sensitivity/high PPV and a high specificity/high NPV should be investigated in the future to more accurately identify the high-risk and low-risk groups for recurrence/metastasis, respectively.

The relationship between single cells in the lymph node and recurrence/metastasis has not been clarified so far, possibly because ONCs with lower viability become trapped in the lymph node sinuses and these cells are not involved in recurrence or metastasis (7,8). Single cells are assumed to be eradicated by the host immune system however numerous they are and thus may not cause recurrence or metastasis, whereas clusters of several to a dozen ONCs may not be eliminated by the immune system and may proliferate to form micrometastases. We therefore investigated the presence of ONC clusters consisting of 2-20 tumor cells for which viability can be easily judged in addition to the conventional assessment of single cells. Clusters are assumed to be either spheres ≤ 0.2 mm in diameter or structures like a bunch of grapes, but it is not known at present whether or not there is an interstitial component connecting the individual cancer cell or whether the clusters contain cancer stem cells with resistance to chemotherapy agents that transmit important information for tumor survival (18,32). However, it was considered easier

compared with single cells to judge the proliferative capacity, based on the nuclear structure, cytoplasmic morphology and staining. The number of clusters was increased to 5-6 or in stage III patients more compared with 1-3 in patients with stage II/Dukes' B colorectal cancer (data not shown). Since a significant difference was observed in the presence of clusters in this study compared with the presence of single cells, it seems that single cells can be considered as an indicator of systemic dispersion whereas clusters indicate distant metastasis/recurrence. A certain number of ONC clusters would be required for tumor cells to survive the host immune defenses in the microcirculation and grow to produce distant metastasis/recurrence. More detailed clinicopathological investigations, including the number of clusters and susceptibility to anticancer agents, should be performed in the future in a larger number of patients.

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