SPANDIDOS PUBLICATIONS

Sensitivity to CPT-11 and platinum derivatives of stage III/Dukes' C colorectal cancer with occult neoplastic cells in lymph node sinuses

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Received January 8, 2007; Accepted February 8, 2007

Abstract. The sensitivity of LN metastases to anticancer drugs such as CPT-11 and platinum agents was investigated by assessing Topo-1 and Bax/ERCC-1 expression in patients who had stage III/Dukes' C colorectal cancer with ONCs. In the recurrence group (RG) (n=21), immunohistochemical expression of Topo-1 was high in 8 patients (38.1%), and low in 13 patients (61.9%), while the non-recurrence group (N-RG) (n=12) showed high expression in 1 patient (8.3%) and low expression in 11 patients (91.7%) (not significant; N.S.). Regarding the immunohistochemical expression of Bax/ERCC-1, high Bax/low ERCC-1 expression was observed in 6 patients (28.6%) from the RG and other patterns of expression were seen in 15 patients (71.4%), while high Bax/low ERCC-1 expression level was observed in 3 patients (25.0%) from the N-RG and other patterns were found in 9 patients (75.0%) (N.S.). PCR analysis of Topo-1 expression in the RG (n=13) revealed high expression in 10 patients (76.9%) and low expression in 3 patients (23.1%), while the N-RG (n=3) showed high expression in 3 patients (100%) and low expression in none (N.S.). With respect to ERCC-1, PCR analysis of the RG (n=13) revealed high expression in 6 patients (46.2%) and low expression in 7 patients (53.8%), while the N-RG (n=3) showed high expression in 2 patients (66.7%) and low expression in 1 patient (33.3%) (N.S.). These results suggest that tumor sensitivity to CPT-11 and platinum derivatives is similar in stage III colorectal cancer patients with ONCs.

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Abbreviations: ONCs, occult neoplastic cells; LN, lymph node; 5-FU, 5-fluorouracil; LV, leucovorin; CPT-11, irinotecan; Topoisomerase-1 inhibitor, Topo-1; L-OHP, oxaliplatin; CDDP, cisplatin; Bax, bcl-2 associated X; ERCC-1, excision repair cross-complementation group 1; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; RG, recurrence group; N-RG, non-recurrence group

Key words: colorectal cancer, occult neoplastic cells, CPT-11, Topoisomerase-1 inhibitor, platinum agents, Bax, ERCC-1, isolated tumor cells

Introduction

Tumor metastasis/recurrence in the liver and lungs after curative resection of colorectal cancer is presumed to be related to the survival and proliferation in distant organs of residual cancer cells that circulate during the perioperative period and escape the host's defenses (1-4). There have been many studies suggesting a close relationship between the metastasis/recurrence of cancer and the detection of cytokeratin positive occult neoplastic cells (ONCs) floating in lymph node (LN) sinuses distant from the primary tumor (5-8). ONCs can be counted by staining this small number of malignant cells that become trapped in the microcirculation of the LNs, using a simple immunostaining method (patent pending since 2002, Japan) (2,3). Free cancer cells are mentioned as isolated tumor cells (ITCs) in the breast cancer section of the sixth version of the TNM classification. At the St. Gallen meeting in 2005, ITCs were also defined as cells/clusters with a diameter of <0.2 mm that had not penetrated the walls of vessels or lymphatic sinuses (9) (Viale G, S12, 9th International Conference on Primary Therapy of Early Breast Cancer, St. Gallen, 2005). Larger clusters of floating cancer cells are also observed sometimes, but the significance of such cell clusters remains unclear (9). Solitary ONCs floating in LN sinuses far from the primary tumor can be detected by cytokeratin immunstaining, but ONCs are also defined to include clusters of ≤10 cells, while malignant micro-aggregates contain >10 ONCs (10). ONC clusters have the potential to

cause metastasis/recurrence in any organ, and should be differentiated from micrometastases (0.2-2 mm) anchored in LNs or from ITCs (<0.2 mm), since ONCs seem to be more malignant occult systemic metastases (10-12).

When postoperative adjuvant chemotherapy is performed for stage III colorectal cancer with LN metastasis, the combination of irinotecan hydrochloride (CPT-11), a Topoisomerase-1 inhibitor (Topo-1) with a novel mechanism of action, and 5-fluorouracil + leucovorin (5-FU/LV) and/or oxaliplatin (L-OHP; a new-generation platinum analogue) have come into widespread use (13,14). The FOLFOX regimen with L-OHP has been reported to be significantly superior to the FOLFIRI regimen in terms of the survival of patients with metastatic colorectal cancer, and it has been rapidly adopted in the US and Europe (15,16). Platinum agents such as cisplatin (CDDP) generally inhibit DNA synthesis by forming DNA-platinum adducts. Similar to 5-FU, their efficacy is greatly affected by the sensitivity or resistance of the tumor. Pharmacological efficacy has been reported to be related to: 1) an increase of DNA excision repair due to the up-regulation of nuclear excision repair genes such as the excision repair cross-complementation group-1 (ERCC-1) gene; 2) increased expression of anti-apoptotic genes such as those of the Bcl-2 family coupled with the P53 tumor suppressor gene; and 3) increased removal of drugs from tumor cells by the CDDP/glutathione (GSH: γ-glutamyl cysteinyl glycine) complex and ATP-binding cassette transporter (ABC transporter) (17-21). Up-regulation of ERCC-1 gene expression is reported to be related to the acquisition of tumor resistance to platinum drugs such as CDDP and L-OHP (17,19,20). It is also reported that resistance to apoptosis due to decreased expression of the Bcl-2-associated X (Bax) gene has a marked influence on the acquisition of resistance to platinum drugs (18). These studies suggest that tumors with high Bax/low ERCC-1 expression may be more sensitive to platinum derivatives.

For assessment of tumor drug sensitivity, immunohistochemical staining of the primary lesion and a molecular biological approach involving the PCR have been tried. However, there have been no studies about immunohistochemical/molecular biological investigation of Topo-1 and Bax/ERCC-1 in the metastatic LNs of patients with stage III colorectal cancer who are positive for ONCs. In the present study, the sensitivity of LN metastases to anticancer drugs such as CPT-11 and platinum agents was investigated by assessing Topo-1 and Bax/ERCC-1 expression in patients who had stage III/Dukes' C colorectal cancer with ONCs, and drug sensitivity was compared between CPT-11 and platinum agents.

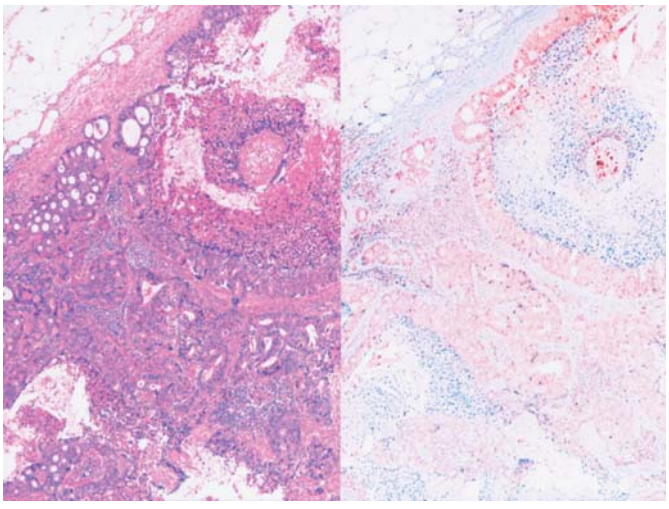

Figure 1. Immunohistochemical staining for topoisomerase-1 (Topo-1) (high expression; +++) in the metastatic lymph node of a patient with recurrence of stage III/Dukes' C colorectal cancer and positive occult neoplastic cells (left: H&E stain, x200; right: Topo-1, x200).

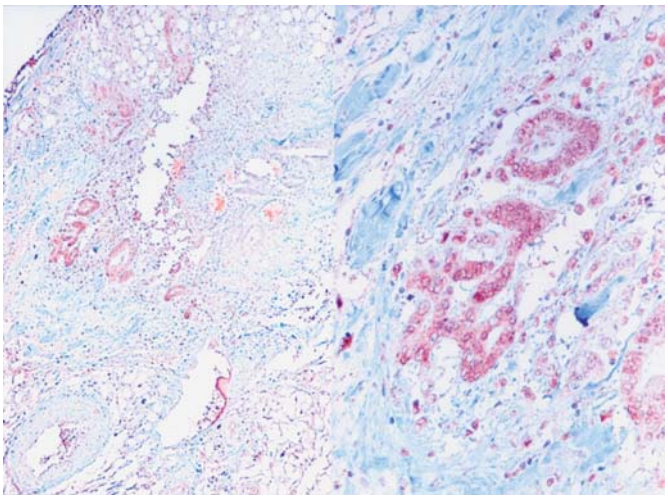

Figure 2. Immunohistochemical staining for Bcl-2-associated X (Bax) and excision repair cross-complementation group 1 (ERCC-1) (high expression; +++) in the metastatic lymph node of a patient with recurrence of stage III/Dukes' C colorectal cancer and positive occult neoplastic cells (left: Bax, x50; right: ERCC-1, x200).

Materials and methods

A total of 105 patients with stage III/Dukes' C LN-positive primary colorectal cancer underwent curative resection during the 13 years from 1987 to 2001 and met the following criteria: 1) complete medical records were available and the presence/absence or recurrence and survival could be confirmed; and 2) at least 10 LNs without metastasis on routine H&E staining had been harvested. Of the 105 patients, 33 were registered in the database of the Occult Neoplastic Cells Research and Study Group (President: H. Makuuchi, Tokai University School of Medicine; Group Leader: M. Mukai, Tokai University Hachioji Hospital). In these 33 patients, the presence of ONCs was confirmed by cytokeratin immunohistochemical staining (AE1/AE3; Fuji Chemical Industries, Ltd., Japan) (22-25).

Immunohistochemical staining. The 33 patients were divided into a recurrence group (RG) (n=21) who showed recurrence/metastasis within five years and a non-recurrence group (N-RG) (n=12) without recurrence after at least five years. Immunohistochemical staining of LNs for Topo-1 and Bax/ERCC-1 was done by the routine indirect immunoperoxidase method using a monoclonal anti-Topo-1 antibody as the primary antibody (Topo-1; 1D6, Dako, Denmark), as well as poly-

xpression of Bax and ERCC-1 in the metastatic lymph nodes of 33 patients with stage III/Dukes' C colorectal cancer
ve occult neoplastic cells (ONCs) in their lymph node sinuses.[a]

Metastatic LNs Total 33 cases	Immunohistochemical staining			
	Bax		ERCC-1	
	High-level	Low-level	High-level	Low-level
Recurrence (n=21)	57.1% (12/21)	42.9% (9/21)	42.9% (9/21)	57.1% (12/21)
Non-recurrence (n=12)	50.0% (6/12)	50.0% (6/12)	33.3% (4/12)	66.7% (8/12)

[a]The recurrence group comprised 21 patients and the non-recurrence group had 12 patients. Expression was classified as low (-, +) or high level (++, +++). Bax, Bcl-2 associated X; ERCC-1, excision repair cross-complementation group 1; LNs, lymph nodes.

clonal anti-Bax and anti-ERCC-1 antibodies (Bax; PC66T, Calbiochem, Germany, ERCC-1; SPM243, Spring Bioscience, USA). Minor modifications were also applied, such as retrieval of antigenicity (26-29). Tumor cell counts were calculated by examination at a high power and were assigned to the following four grades according to the rate of tumor positivity: 0% (-), <5% (+), (5% to <30% (++), and (30% (+++). (-) and (+) were defined as 'low level', while (++) and (+++) were 'high level' (Figs. 1 and 2) (22-25). To determine whether or not tumors were sensitive to CPT-11 and platinum agents, the expression of Topo-1, Bax and ERCC-1 was investigated and the rate of patients with high Topo-1 levels or with high Bax/low ERCC-1 levels was determined.

Real-time PCR. Real-time PCR for Topo-1 and ERCC-1 was conducted by the Danenberg tumor profile method using paraffin blocks from the 33 LN-positive cancer patients, who were divided into recurrence and non-recurrence groups. To categorize the expression of Topo-1 and ERCC-1 as high or low, cut-off values were established (30,31).

Laser-caputured microdissection. A representative formalin-fixed, paraffin-embedded, tumor specimen that was harvested before S1 treatment was selected by a pathologist after examination of the H&E-stained slides. Then sections cut at a thickness of 10 μm and were stained with nuclear fast red to enable visualization of the histology for laser capture micro-dissection (PALM Microlaser Technologies AG, Bernried, Germany), which was done to ensure that only tumor cells were studied (32-34).

RNA isolation, cDNA synthesis, and reverse transcriptase-PCR. Isolation of RNA from formalin-fixed, paraffin-embedded specimens was done according to the method of Response Genetics, Inc. (Los Angeles, CA; US patent no. 6,248,535). Then, cDNA was prepared from each RNA sample as described previously. Quantitation of cDNA for Topo-1/ERCC-1 and an internal standard gene (ß-actin) was done using a fluorescence-based real-time detection method (ABI-PRISM 7900 sequence detection system (TaqMan), Applied Biosystems, Foster City, CA), as described previously (35,36).

Statistical analysis. The χ^2 test was used to examine the significance of differences between the recurrence and non-recurrence groups. P-values of <0.05 were considered to indicate a significant difference and SPSS 13.0 J software (SPSS Japan, Inc., Tokyo, Japan) was used for all analyses.

Results

Immunohistochemistry for Bax/ERCC-1. In the RG (n=21), high expression of Bax was observed in 12 patients (57.1%) and low expression was found in 9 patients (42.9%). In the N-RG (n=12), high Bax expression was observed in 6 patients (50%) and low expression was noted in the other 6 patients (50%) (not significant; N.S.) (Table I). With respect to ERCC-1, the RG (n=21) showed high ERCC-1 expression in 9 patients (42.9%) and low expression in 12 patients (57.1%), while the N-RG (n=12) showed high expression in 4 patients (33.3%) and low expression in 8 patients (66.7%) (N.S.) (Table I). There were no significant differences in the expression of Bax/ERCC-1 between the two groups (Table I).

Immunohistochemistry for Topo-1 and Bax/ERCC-1. In the RG (n=21), high expression of Topo-1 was observed in 8 patients (38.1%) and low expression was seen in 13 patients (61.9%), while the N-RG (n=12) showed high expression in 1 patient (8.3%) and low expression in 11 patients (91.7%) (N.S.) (Table II). In the RG (n=21), high Bax expression combined with low ERCC-1 expression (high Bax/low ERCC-1 expression) was observed in 6 patients (28.6%) and other patterns were seen in 15 patients (71.4%), while the N-RG (n=12) showed high Bax/low ERCC-1 expression in 3 patients (25.0%) and other patterns in 9 patients (75.0%) (N.S.) (Table II). There were no significant differences in the expression of Topo-1 and Bax/ERCC-1 between the two groups (Table II).

RT-PCR for Topo-1 and ERCC-1. When RT-PCR of metastatic lymph node tissue was done in the 33 patients, Topo-1 and ERCC-1 were detected in 16 patients (48.5%; 13 from the RG and 3 from the N-RG). Expression was within the range of 0.59-4.90 (median: 1.50), therefore the cut-off value was set at 1.0 to separate low from high expression. For Topo-1, the RG (n=13) showed high expression in 10 patients (76.9%) and low expression in 3 patients (23.1%), while the N-RG group (n=3) showed high expression in all 3 patients (100.0%) and none of them had low expression (N.S.)

Table II. Level of Topo-1 expression and the combination of high Bax/low ERCC-1 expression in the metastatic lymph nodes of 33 patients with stage III/Dukes' C colorectal cancer and positive occult neoplastic cells (ONCs) in their lymph node sinuses.[a]

Metastatic LNs Total 33 cases	Immunohistochemical staining			
	Topo-1		Bax and ERCC-1	
	High-level	Low-level	High Bax/low ERCC-1	Others
Recurrence (n=21)	38.1% (8/21)	61.9% (13/21)	28.6% (6/21)	71.4% (15/21)
Non-recurrence (n=12)	8.3% (1/12)	91.7% (11/12)	25.0% (3/12)	75.0% (9/12)

[a]The recurrence group comprised 21 patients and the non-recurrence group had 12. Expression was classified as low (-, +) or high (++, +++). Topo-1, Topoisomerase-1 inhibitor; Bax, Bcl-2 associated X; ERCC-1, excision repair cross-complementation group 1; LNs, lymph nodes.

Table III. Expression of Topo-1 and ERCC-1 in the metastatic lymph nodes of 33 patients with stage III/Dukes' C colorectal cancer and positive occult neoplastic cells (ONCs) in their lymph node sinuses.[a]

Metastatic LNs Total 33 cases (Undetected, 17 cases)	Real-time PCR			
	Topo-1		ERCC-1	
	High-level	Low-level	High-level	Low-level
Recurrence (n=13/21)	76.9% (10/13)	23.1% (3/13)	46.2% (6/13)	53.8% (7/13)
Non-recurrence (n=3/12)	100.0% (3/3)	0.0% (0/3)	66.7% (2/3)	33.3% (1/3)

[a]The recurrence group included 21 patients and the non-recurrence group had 12. Expression was classified as low (<1.0) or high (≥1.0). Topo-1, Topoisomerase-1 inhibitor; ERCC-1, excision repair cross-complementation group 1; LNs, lymph nodes.

(Table III). In the case of ERCC-1, the RG (n=13) showed high expression in 6 patients (46.2%) and low expression in 7 patients (53.8%), while the N-RG (n=3) showed high expression in 2 patients (66.7%) and low expression in 1 patient (33.3%) (N.S.) (Table III). There were no significant differences in the expression of Topo-1 and ERCC-1 between the two groups (Table III).

Discussion

There have been many immunohistochemical and molecular biological investigations of resistance to CPT-11 and platinum agents using primary tumor tissues, but there has been no previous investigation of the expression of Topo-1, Bax, and ERCC-1 in metastatic LNs. We considered that tumor drug sensitivity should be investigated for ONCs which exist in organs/tissues other than the primary lesion and might cause metastasis. However, we thought that it would be difficult to prepare serial sections of LNs with a diameter of a few millimeters to one centimeter for routine H&E staining and then detect Topo-1 and Bax/ERCC-1 expression using the same ONCs, therefore we used metastatic LNs instead. Topo-1 and Bax/ERCC-1 were easily detected because the cancer cells were well stained. In particular, the nucleus and cytoplasm were prominently stained, but the cell membranes, interstitial cell, non-cancerous regions, and normal epithelial cells were weakly stained or unstained. Both CPT-11 and platinum derivatives are DNA synthesis inhibitors, hence they were well stained in the nucleus of cancer cells and excessively stained in the cytoplasm. This was presumed to be related to mitochondrial DNA and RNA transmission. For further investigation of the localization and distribution of Topo-1 and Bax/ERCC-1, immune electron microscopy may be necessary.

There have been a number of molecular biological investigations of Topo-1 and ERCC-1 in primary tumor tissues, but no previous investigation has assessed metastatic LNs. Laser capture microdissection using paraffin blocks requires 10-μm thick sections, and similar to immunohistochemical investigation, we thought that it would be difficult to determine staining using the same ONCs, therefore we studied metastatic LNs instead. The expression of Topo-1 and ERCC-1 was detected by PCR in the metastatic LNs of 16 out of 33 patients (48.5%). These 16 patients included three in the non-recurrence group, which suggests that it is difficult to detect the expression of these enzymes in tiny metastatic lesions. Topo-1 expression ranged from 0.87 to 4.90 (median: 2.31; mean: 2.26), while ERCC-1 expression ranged from 0.59 to 3.00 (median: 2.25; mean: 2.09). In the present small group of patients, the cut-off value was set at 1.0 for separating low from high expression. However, we need to accumulate data on more subjects and establish a more accurate cut-off level.

form effective postoperative adjuvant chemotherapy revention of metastasis/recurrence after curative resection of a primary tumor, it is necessary to eliminate residual cancer cells that can circulate through various organs/tissues during the perioperative period. Active systemic consolidation chemotherapy provided during the early period after surgery is expected to be most effective. According to a study on the sensitivity of tumors to 5-FU + LV therapy, there were not many patients with sensitive tumors, including those with high TS/low DPD expression, at the time when ONC clusters circulated causing metastasis. This suggests that 5-FU is not sufficiently powerful (22-24). To solve this problem, combination with CPT-11 and/or L-OHP, which are more widely used in the US and Europe than in Japan, may be useful as modulators and an additive or synergistic effect can be expected. Although the dosage/administration schedule is closely related to the outcome, sensitivity to the anticancer drugs used for combination therapy is presumed to be a key factor in achieving better results. There were no differences in the expression of Bax/ERCC-1 between the recurrence and non-recurrence groups, therefore we investigated the numbers of patients with high Bax/low ERCC-1 expression or high Topo-1 expression in the recurrence group. High Topo-1 expression was observed in 38.1% of the patients, while high Bax/low ERCC-1 expression was seen in 28.6%. The number of patients with high Topo-1 expression was larger in the recurrence group than the non-recurrence group (8.3%) (p=0.06). On PCR analysis, high Topo-1 expression was observed in 76.9% and low ERCC-1 expression was seen in 53.8%, but only a small number of patients was studied and no significant differences were observed. However, both immunohistochemical and PCR findings suggested that it may be better to use CPT-11 as the first-line drug for post-operative adjuvant therapy in patients with stage III colorectal cancer. The cost of L-OHP is covered by government health insurance in Japan if it is used for the treatment of distant metastasis/recurrence in organs such as the liver or lungs, hence it may be reasonable to try L-OHP if adjuvant therapy with CPT-11 is not sufficiently effective.

Improvement of the survival rates after recurrence is considered to be most important for improving the outcome for patients (37,38). For the treatment of stage III/Dukes' C LN-positive colorectal cancer, it appears to be important to detect patients with a high risk of recurrence and provide appropriate treatment at the stage of occult metastasis soon after curative resection and before overt recurrence. To achieve better efficacy, it also appears to be preferable to use 5-FU in combination with other more active drugs. However, further investigation of the dosage/administration is necessary.

Acknowledgements

This study was supported by grants from the Occult Neoplastic Cells Research and Study Group (#2006-5048; Tokai University Hachioji Hospital, Hachioji, Tokyo, Japan) and the Research and Study Program of Tokai University Educational System General Research Organization (#2005-41; Tokai University Hospital, Isehara, Kanagawa, Japan).

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