Sensitivity to CPT-11 and platinum derivatives of stage I/II node-negative breast, lung, and gastric cancer with occult neoplastic cells in lymph node sinuses

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Abstract. Tumor sensitivity to anticancer drugs such as CPT-11 and platinum derivatives was investigated by assessing Topo-1 and Bax/ERCC-1 expression in patients with stage I/II breast, lung, and gastric cancer who were positive for ONCs, and tumor sensitivity was compared between CPT-11 and platinum derivatives. In the recurrence group (RG) (n=5), immunohistochemistry revealed high expression of Topo-1 in 3 patients (60%) and low expression in 2 patients (40%), while the non-recurrence group (N-RG) (n=17) showed high Topo-1 expression in 3 patients (17.6%) and low expression in 14 patients (82.4%) (not significant; N.S.). High Bax expression combined with low ERCC-1 expression was observed in 2 patients (40%) from the RG and other patterns of expression were seen in 3 patients (60%), while high Bax/low ERCC-1 expression was observed in 3 patients (17.6%) from the N-RG and other patterns were found in 14 patients (82.4%) (N.S.). PCR analysis of Topo-1 expression in the RG (n=4) revealed high expression in 4 patients (100%), while the N-RG (n=5) showed high expression in 3 patients (60%) and low expression in 2 patients (40%) (N.S.). With respect to ERCC-1, PCR analysis of the RG (n=4) also revealed high expression in 4 patients (100%), while the N-RG (n=5) again showed high expression in 3 patients (60%) and low expression in 2 patients (40%) (N.S.). There were significant differences between the expression of high Topo-1 and low ERCC-1 in the RG ($p<0.01$). These results suggest that tumor sensitivity to CPT-11 may be higher than that for platinum derivatives in patients with node-negative stage I/II breast, lung, or gastric cancer who are positive for 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; ITCs, isolated tumor cells; RG, recurrence group; N-RG, non-recurrence group

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

Introduction

Tumor metastasis/recurrence affecting the liver and lungs after curative resection of colorectal cancer is presumed to be related to the survival and proliferation of residual cancer cells that escape the host's defenses during the perioperative period (1-4). There have been many studies suggesting a close relationship between metastasis/recurrence of cancer and the detection of cytokeratin-positive occult neoplastic cells (ONCs) in lymph node (LN) sinuses distant from the primary tumor (5-8). ONCs can be counted by staining these malignant cells trapped in the LNs using a simple immuno-staining method (patent pending since 2002 in 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 (9). 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 (Viale G, S12, 9th International Conference on Primary Therapy of Early Breast Cancer, St. Gallen, 2005). Larger clusters of floating cancer cells are sometimes observed, but the significance of such cell clusters remains unclear. ONCs floating in the sinuses of LNs distant from the primary tumor can be detected by cytokeratin immunostaining, and are defined as including

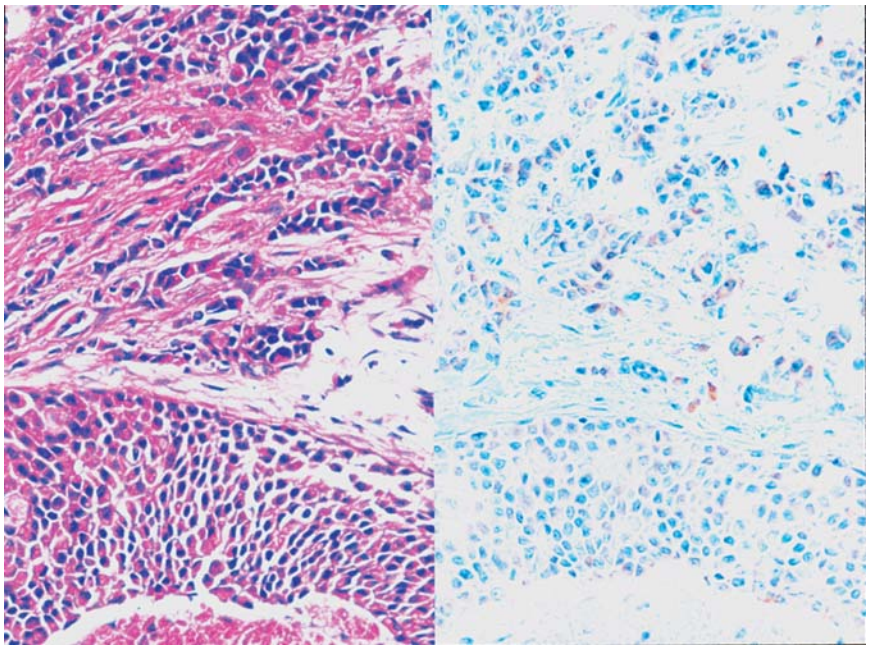

Figure 1. Immunohistochemical staining for Topoisomerase-1 (Topo-1) (high expression, +++) in the primary tumor of a patient with recurrence of stage II/ breast cancer who was positive for occult neoplastic cells (left: H&E stain, x200; right: Topo-1, x200).

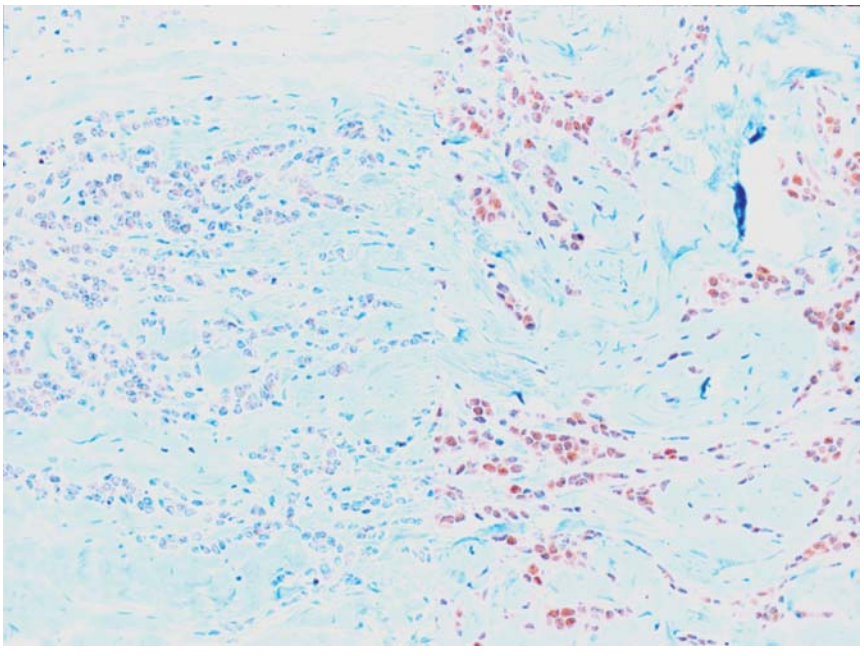

Figure 2. Immunohistochemical staining for Bcl-2-associated X (Bax) and excision repair cross-complementation group 1 (ERCC-1) (high expression, +++) in the primary tumor of a patient with recurrence of stage II/breast cancer who was positive for occult neoplastic cells (left: Bax, x200; right: ERCC-1, x200).

clusters of ≤10 cells, while malignant micro-aggregates contain >10 cells (2,3,10). ONC clusters have the potential to cause metastasis/recurrence in any organ, and thus should be distinguished from micrometastases (from 0.2 mm to ≤2 mm in size) anchored in LNs or from ITCs (≤0.2 mm in size), since ONCs seem to be have a higher malignant potential (10-12).

When stage I or II cancer of the breast, lung, or stomach without lymph node (LN) metastasis is treated by curative resection, life-threatening metastasis/recurrence in the liver or lungs has been reported to occur in 5-15% of patients (13-15). In Japan, oral preparations of 5-fluorouracil (5-FU) derivatives are widely used to treat various types of stage I/II cancer, including breast, lung, and gastric cancer without LN metastasis. In particular, these preparations are considered to be first-line postoperative adjuvant chemotherapy for stage II colorectal cancer or gastric cancer. 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),

Table I. Expression of Bax and ERCC-1 in the primary tumors of 22 patients with stage I/II breast, lung, or gastric cancer and occult neoplastic cells (ONCs) in their lymph node sinuses.[a]

Primary tumor Total 22 cases	Immunohistochemical staining [high-level, (+++) (++); low-level, (+) (-)]			
	Bax		ERCC-1	
	High-level	Low-level	High-level	Low-level
Recurrence (n=5)	80.0% (4/5)	20.0% (1/5)	40.0% (2/5)	60.0% (3/5)
Non-recurrence (n=17)	47.1% (8/17)	52.9% (9/17)	64.7% (11/17)	35.3% (6/17)

[a]The recurrence group comprised 5 patients and the non-recurrence group included 17 patients. Expression was classified as low (-, +) or high (++, +++). Bax, Bcl-2 associated X; ERCC-1, excision repair cross-complementation group 1.

and 5-fluorouracil + leucovorin (5-FU/LV) and/or oxaliplatin (L-OHP; a new-generation platinum derivative) has come into widespread use in the US and Europe (16-18). Platinum derivatives 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 target tumor. The efficacy of these drugs of chemotherapy 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 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 up-regulation of the p53 tumor suppressor gene, and 3) more rapid removal of drugs from tumor cells by the CDDP/glutathione (GSH: γ-glutamyl cysteinyl glycine) complex and ATP-binding cassette transporter (ABC transporter) (19-23). Up-regulation of ERCC-1 gene expression is reported to be related to the acquisition of resistance to platinum derivatives such as CDDP and L-OHP (19,21,22). It has also been 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 derivatives (20). These studies suggest that tumors with high Bax/low ERCC-1 expression may be more sensitive to these drugs.

For assessment of tumor drug sensitivity, both immunostaining and the polymerase chain reaction (PCR) have been tried. However, there have been no immunohistochemical or PCR studies on Topo-1 and Bax/ERCC-1 expression in the primary tumors of patients with stage I/II breast, lung, or gastric cancer who are positive for ONCs. In the present study, tumor sensitivity to anticancer drugs such as CPT-11 and platinum derivatives was investigated by assessing Topo-1 and Bax/ERCC-1 expression in patients with stage I/II breast, lung, and gastric cancer who were positive for ONCs, and tumor sensitivity was compared between CPT-11 and platinum derivatives.

Materials and methods

A total of 124 patients with stage I/II node-negative primary breast, lung, or gastric cancer underwent curative resection during the 13 years from 1987 to 2001 and met the following criteria: 1) complete medical records were available so that recurrence and survival could be confirmed, and 2) at least 10 LNs without metastasis on routine H&E staining had been harvested. Of the 124 patients, 22 (breast 8, lung 3, stomach 11) 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 22 patients, the presence of ONCs was confirmed by cytokeratin immunostaining (AE1/AE3; Fuji Chemical Industries, Ltd., Japan) (24-27).

Immunohistochemical staining. The 22 patients were divided into a recurrence group (RG) (n=5) who showed recurrence/metastasis within five years and a non-recurrence group (N-RG) (n=17) without recurrence after at least five years. Immunohistochemical staining of primary tumors for Topo-1 and Bax/ERCC-1 was done by the routine indirect immunoperoxidase method using a monoclonal anti-Topo-1 antibody (Topo-1; 1D6, Dako, Denmark), or polyclonal anti-Bax and anti-ERCC-1 antibodies (Bax; PC66T, Calbiochem, Germany, ERCC-1; SPM243, Spring Bioscience, USA) as the primary antibodies. Minor modifications were made to the standard method, such as retrieval of antigenicity (28-31). Tumor cells were counted by examination at a high power and tumor staining was classified into the following four grades according to the positive tumor cell rate: 0% (-), <5% (+), ≥5% to <30% (++), and ≥30% (+++). Both (-) and (+) were defined as 'low expression', while (++) and (+++) indicated 'high expression' (Figs. 1 and 2) (24-27). 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 percentage of patients with high Topo-1 expression or with high Bax and low ERCC-1 expression was determined.

Real-time PCR. Real-time PCR for Topo-1 and ERCC-1 was performed by the Danenberg tumor profile method using paraffin blocks obtained from the 22 patients with ONCs. To categorize the expression of Topo-1 and ERCC-1 as high or low, cut-off values were employed (32,33). These data were compared with the expression of high Topo-1 and low ERCC-1 between each group.

Laser capture microdissection. A representative formalin-fixed, paraffin-embedded, tumor specimen that had been

Table II. Topo-1 expression and combined high Bax/low ERCC-1 expression in the primary tumors of 22 patients with stage I/II breast, lung, or gastric cancer and occult neoplastic cells (ONCs) in their lymph node sinuses.[a]

Primary tumor Total 22 cases	Immunohistochemical staining [high-level, (+++) (++); low-level, (+) (-)]			
	Topo-1		Bax and ERCC-1	
	High-level	Low-level	High Bax/low ERCC-1	Others
Recurrence (n=5)	60.0% (3/5)	40.0% (2/5)	40.0% (2/5)	60.0% (3/5)
Non-recurrence (n=17)	17.6% (3/17)	82.4% (14/17)	17.6% (3/17)	82.4% (14/17)

[a]The recurrence group comprised 5 patients and the non-recurrence group included 17 patients. 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.

Table III. Expression of Topo-1 and ERCC-1 in the primary tumors of 22 patients with stage I/II breast, lung, or gastric cancer and occult neoplastic cells (ONCs) in their lymph node sinuses.[a]

Primary tumor Total 22 cases (undetected, 13 cases)	Real-time PCR (low-level <1.0 ≤high-level)			
	Topo-1		ERCC-1	
	High-level	Low-level	High-level	Low-level
Recurrence (n=4/9)	100.0% (4/4)[b]	0.0% (0/4)	100.0% (4/4)	0.0% (0/4)[b]
Non-recurrence (n=5/9)	60.0% (3/5)	40.0% (2/5)	60.0% (3/5)	40.0% (2/5)

[a]The recurrence group included 5 patients and the non-recurrence group had 17 patients. Expression was classified as low (<1.0) or high (≥1.0). Topo-1, Topoisomerase-1 inhibitor; ERCC-1, excision repair cross-complementation group 1; [b]p<0.01.

harvested before treatment was selected by a pathologist after examination of the H&E-stained slides. Then sections were cut at a thickness of 10 μm and were stained with nuclear fast red to enable visualization of the histology for laser capture microdissection (Palm Microlaser Technologies AG, Bernried, Germany), in order to ensure that only tumor cells were studied (34-36).

Reverse transcriptase-PCR. Isolation of RNA from formalin-fixed, paraffin-embedded tumor 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 levels 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 (37,38).

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

Results

Immunohistochemistry for Bax/ERCC-1. In the RG (n=5), high expression of Bax was observed in 4 patients (80%) and low expression was found in 1 patient (20%). In the N-RG (n=17), high Bax expression was observed in 8 patients (47.1%) and low expression was noted in the other 9 patients (52.9%) (not significant; N.S.) (Table I). There was high ERCC-1 expression in 2/5 patients (40%) from the RG and low expression in 3/5 patients (60%), while high ERCC-1 expression was seen in 11/17 patients (64.7%) from the N-RG and low expression was noted in 6/17 patients (35.3%) (N.S.) (Table I). There were no significant differences in the expression of Bax and ERCC-1 between the two groups (Table I).

Immunohistochemistry for Topo-1 and Bax/ERCC-1. In the RG (n=5), high expression of Topo-1 was observed in 3 patients (60%) and low expression was seen in 2 patients (40%), while the N-RG (n=17) showed high expression in 3 patients (17.6%) and low expression in 14 patients (82.4%) (N.S.) (Table II). In the RG (n=5), high Bax expression was combined with low ERCC-1 expression (high Bax/low ERCC-1 expression) in 2 patients (40%) and other patterns of expression were seen in 3 patients (60%), while high Bax/low ERCC-1 expression was seen in 3/17 patients (17.6%) from the N-RG and other

patterns of expression were noted in 14 patients (82.4%) (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 primary tumor tissue specimens was done in the 22 patients, Topo-1 and ERCC-1 were detected in 9 patients (40.9%), comprising 4 from the RG and 5 from the N-RG (Table III). Expression was within the range of 0.55-4.99 (median: 1.34), therefore the cut-off value to separate low expression from high expression was set at 1.0. Using this cut-off value, all 4 patients from the RG showed high Topo-1 expression (100%), as did 3/5 patients from the N-RG (60%), and low Topo-1 expression was seen in 2 patients from the N-RG (40%) (N.S.) (Table III). High ERCC-1 expression was also found in all 4 patients from the RG (100%) and 3/5 patients from the N-RG (60%), while low ERCC-1 expression was noted in 2 patients from the N-RG (40%) (N.S.) (Table III). There were significant differences between the expression of high Topo-1 and low ERCC-1 in the RG ($p<0.01$) (Table III).

Discussion

There have already been many immunohistochemical and molecular biological investigations of resistance to CPT-11 and platinum derivatives using primary tumor tissues, but there are no previous studies of the expression of Topo-1, Bax, and ERCC-1 in the primary tumors of patients with ONCs. Although it would have been ideal to assess the drug sensitivity of ONCs, which exist in distant organs/tissues and might cause metastasis, it would have been difficult to prepare serial sections of LNs for routine H&E staining and then detect Topo-1 or Bax/ERCC-1 expression in the same cells on adjacent sections, hence we studied the primary tumors instead. Topo-1 and Bax/ERCC-1 were easily detected in the cancer cells. In particular, the nucleus and cytoplasm of tumor cells were prominently stained, but the cell membranes, interstitial regions, non-cancerous regions, and normal epithelial cells were weakly stained or unstained. Both CPT-11 and platinum derivatives are DNA synthesis inhibitors, hence their enzymes were well stained in the nucleus of the cancer cells and were overexpressed in the cytoplasm. For further investigation of the localization and distribution of Topo-1 and Bax/ERCC-1, immuno-electron microscopy may be necessary.

There is no previous report on Topo-1 and ERCC-1 expression in the primary tumor of patients with ONCs. 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 the staining of the same ONCs, hence we studied the primary tumors instead. The expression of Topo-1 and ERCC-1 was detected by PCR in the primary tumors of 9 out of 22 patients (40.9%). Topo-1 expression ranged from 0.84 to 2.94 (median: 1.49, mean: 1.34), while ERCC-1 expression ranged from 0.55 to 4.99 (median: 1.34, mean: 1.79). In the present small group of patients, the cut-off value was set at 1.0 to separate low from high expression, but we need to accumulate data from a larger group of subjects to establish a more accurate cut-off level.

Effective postoperative adjuvant chemotherapy for the prevention of metastasis/recurrence after curative resection of the primary tumor should eliminate residual cancer cells that can circulate through various organs/tissues during the perioperative period. Active systemic consolidation chemotherapy during the early period after surgery is expected to achieve this objective. According to previous studies on the sensitivity of tumors to 5-FU + LV, not many patients had sensitive tumors, including those with high TS/low DPD expression, at the time when ONC clusters were circulating to cause metastasis. This suggests that 5-FU is not sufficiently powerful (24-27). To solve this problem, combining 5-FU with CPT-11 and/or platinum derivatives such as CDDP/L-OHP, which are more widely used in the US and Europe than in Japan, may be useful and an additive or synergistic effect can be expected. Although the dosage/administration schedule is closely related to the outcome, tumor sensitivity to the anti-cancer drugs used for combination therapy is presumed to be a key factor in achieving better results. There were no significant differences of high Bax/low ERCC-1 expression between the recurrence group and the non-recurrence group in the present study, 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 60% of the recurrence group, while high Bax/low ERCC-1 expression was seen in 40% by immunohistochemistry (N.S.). On PCR analysis, high Topo-1 expression was observed in 100% of the recurrence group, while low ERCC-1 expression was observed in none ($p=0.0047$), but only a few patients were studied. Both of the immunohistochemical and PCR findings suggested that it may be better to use 5-FU with CPT-11 for first-line combination therapy in the post-operative adjuvant setting when treating patients with stage I/II breast, lung, or gastric cancer who have ONCs and a higher risk of metastasis/recurrence. Also, for the treatment of distant metastasis/recurrence in organs such as the liver or lungs and for pleural dissemination, it may be reasonable to try CPT-11 + CDDP or other drugs as the new second-line combination without 5-FU, if postoperative adjuvant 5-FU-based therapy is not sufficiently effective.

Improvement of overall survival after recurrence is considered to be very important when treating cancer (39,40). For patients with ONC-positive stage I/II breast, lung, or gastric cancer, it appears to be important to identify those with a high risk of recurrence/metastasis soon after curative resection and provide appropriate treatment to eliminate occult metastases during the period before overt recurrence. To achieve sufficient efficacy, it also appears to be preferable to use both standard 5-FU analogues and active combination therapy. It is possible that the standard multi-drug adjuvant chemotherapy for patients with stage II/III cancer will also be effective for those with stage I/II cancer and ONCs. However, it will be necessary to investigate more patients in order to determine whether intra-venous drugs are necessary for stage I/II breast, lung, or gastric cancer when patients have ONCs in their LN sinuses.

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