特集

胃癌腹膜転移治療の最前線

胃癌腹膜転移に対する腹腔内化学療法奏効後の手術

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胃癌腹膜転移に対する腹腔内化学療法奏効後の手術

Surgery for gastric cancer with peritoneal metastasis after response to intraperitoneal chemotherapy

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【ポイント】
◆ パクリタキセル腹腔内投与を併用した化学療法により，胃癌腹膜転移の強力な制御が可能である．
◆ 化学療法により腹水細胞診が陰性化し，腹膜播種が消失または著明に縮小した場合に手術適応と判断した．
◆ 腹膜転移100例中60例に手術を施行し，生存期間中央値は34.5か月であり，重篤な術後合併症を認めなかった．

臨外　68(6)：675～679，2013

はじめに

高い奏効率を示す抗癌剤の出現により，根治切除不能なStage Ⅳ胃癌に対する新たな治療戦略の可能性が広がった．腹膜播種を伴う胃癌に対しては，タキサン系抗癌剤（パクリタキセル：PTX，ドセタキセル：DTX）腹腔内投与の有用性が報告され，化学療法の奏効後に手術を施行する集学的治療の試みが行われている．本稿では，当院における腹腔内投与併用化学療法および奏効後手術の成績を報告する．

S-1＋PTX経静脈・腹腔内併用療法

PTX腹腔内投与と全身化学療法の併用療法は，卵巣癌腹膜転移において有用性が確認され[1]，欧米では推奨治療の一つとみなされている．胃癌腹膜転移に対しては，わが国よりPTXおよびDTX腹腔内投与併用化学療法の治療効果が報告されてきた[2～6]．当院では2006年にS-1＋PTX療法（OGSG 0105レジメン）と腹腔内投与を併用する「S-1＋PTX経静脈・腹腔内併用療法」を考案し，臨床試験を施行した[5,6]．腹腔内投与による全身性の有害事象が軽微であることに着目し，全身化学療法の効果を維持しながら腹腔内投与の上乗せ効果を得ることを狙い，OGSG 0105レジメンの用量を減量せずにPTX腹腔内投与を追加した．

第Ⅰ相試験では，21日間を1コースとして，標準投与量のS-1（14日間内服，7日間休薬）と週1回（第1，8日目）50 mg/m^{2}のPTX経静脈投与にPTX腹腔内投与を併用し，用量を20 mg/m^{2}から10 mg/m^{2}ずつ増量した．その結果，白血球・好中球減少および下痢を用量制限毒性としてPTX腹腔内投与の最大耐用量を30 mg/m^2，推奨投与量を20 mg/m^{2}に決定した（**図1**）．

第Ⅱ相試験にはP1またはCY1の40例が登録された．患者背景は，年齢中央値62（範囲29～86）歳，ECOG-PS 0/1/2 23/15/2例であった．播種の程度は，P1/P0CY1 34/6例，胃癌取扱い規約第12版分類P1/P2/P3 9/5/20例であり，腹水貯留21例，水腎症9例，腸管狭窄6例，卵巣転移6例と腹膜播種が進行した症例が多く含まれていた．生存解析時における投与回数は中央値7（範囲1～23）コースであり，中止理由は腫瘍増悪15例，有害事象5例であった．原発巣を有する27例中16例では，腹水細胞診陰性化および腹膜播種の消失または著明な縮小が確認され（**図2**），胃切除を施行した．

Kaplan-Meier法により算出した1年全生存率は

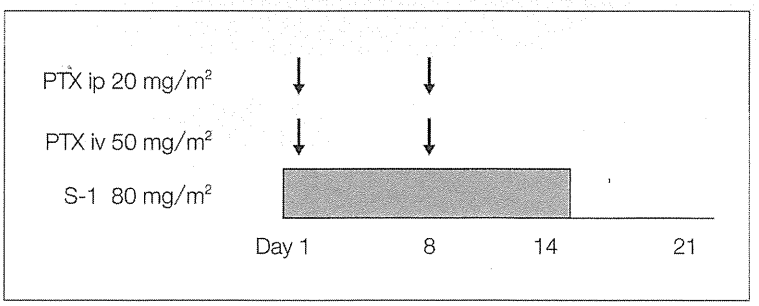

図1　S-1＋パクリタキセル経静脈・腹腔内併用療法

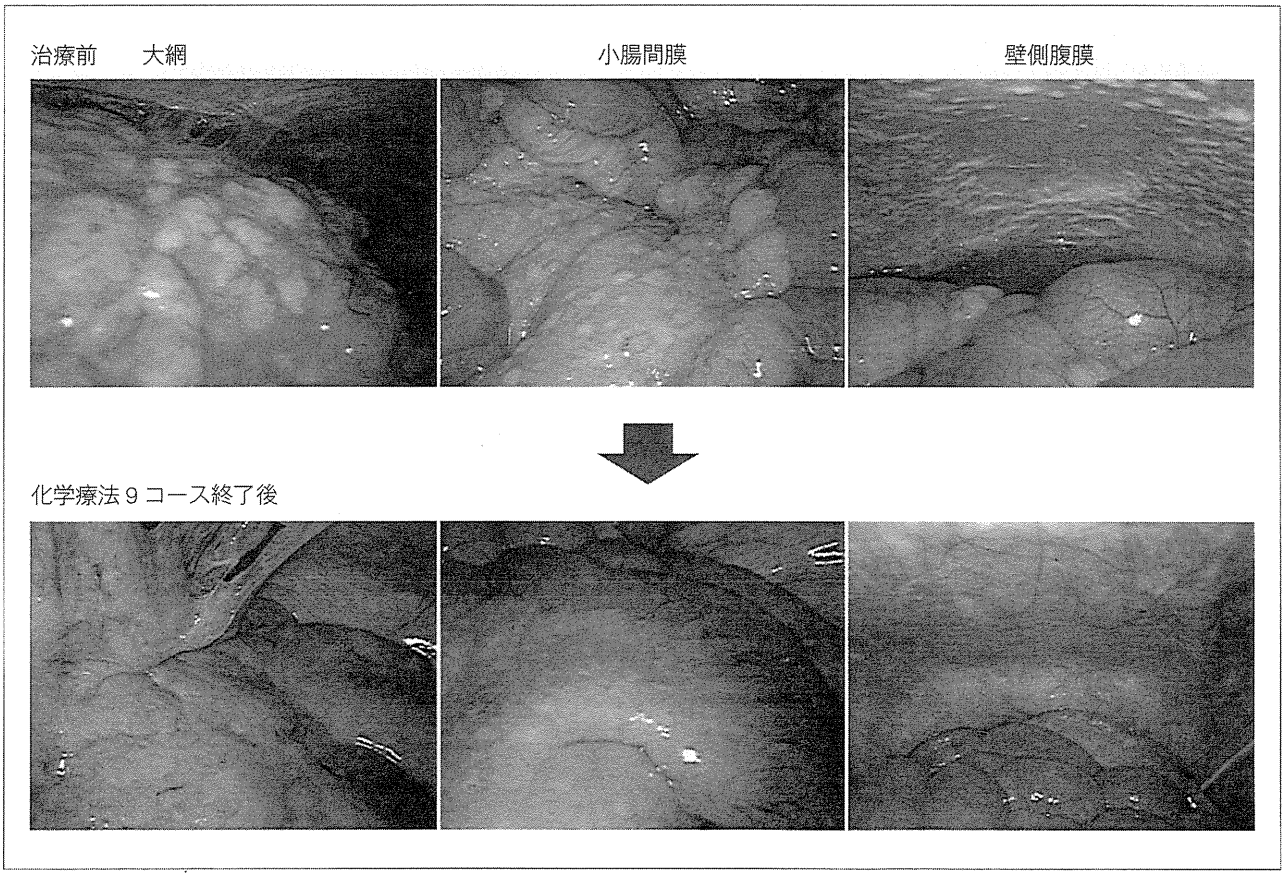

図2　腹膜播種に対する治療効果

78%（95%信頼区間，65～90%），MSTは23.6か月であった（**図3**）．標的病変を有する18例におけるRECISTガイドラインv.1.0に基づく最良総合効果はPR 10例，SD 6例，PD 2例であり，奏効率は56%であった．癌性腹水に対する効果としては，腹水量の減少を21例中13例（62%），腹水細胞診陰性化を28例中24例（86%）に認めた．主な有害事象（Grade3以上）は，白血球減少（18%），好中球減少（38%），ヘモグロビン減少（10%），悪心・嘔吐（8%）であり，治療関連死は認めなかった．

化学療法奏効例に対する手術

従来，根治切除不可能な遠隔転移を伴う進行胃癌は手術適応外と考えられてきた．原発巣切除により腫瘍を減量しても，遠隔転移を化学療法で制御することができず，手術侵襲による全身状態の低下により，かえって転移巣が進行するという臨床経験をしてきたためである．しかし，当科では，腹腔内投与併用化学療法により腹膜播種が長期間にわたり強力に制御されることを経験し，化学療法奏効後の原発巣切除は生存期間の延長につながる可能性があると考えるに至った．

根治切除不可能なP1またはCY1胃癌に対して

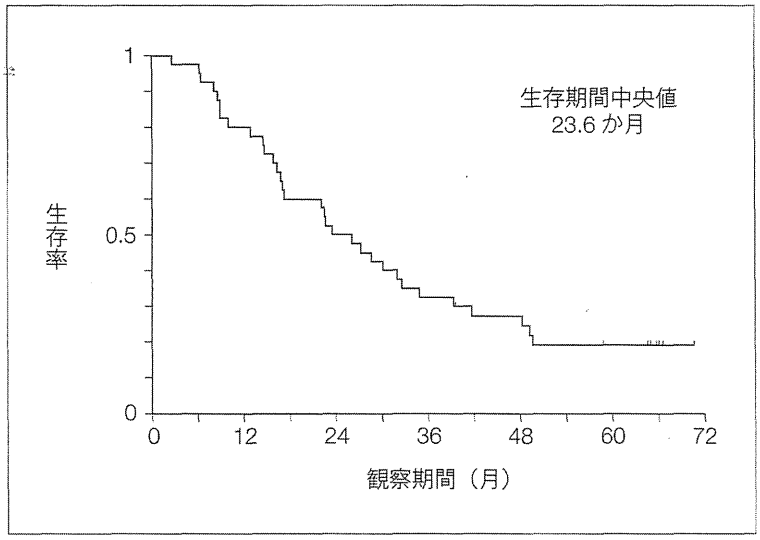

図3　第Ⅱ相試験　全生存期間（*n*＝40）

表1　患者背景

年齢	中央値（範囲）	57（28～86）
性別	男性/女性	30/30
ECOG PS	0/1	46/14
組織型	分化型/未分化型/混合	5/48/7
転移	P0CY1/P1	6/54
	規約第12版 P1/P2/P3	5/16/33
	腹水貯留33，水腎症5，腸管狭窄7，卵巣転移10	

PTX腹腔内投与併用化学療法を反復し，肉眼的治癒が望める状態にまで奏効した症例を手術適応と判断した．その具体的条件は，腹腔ポートを用いた腹水（または腹腔洗浄）細胞診が陰性化し，画像診断上明らかな非治癒因子を認めないこととした．これらの条件を満たした症例では，播種の程度と化学療法の奏効度に応じた治療期間の後に二次審査腹腔鏡を実施し，腹膜播種の消失または明らかな縮小が確認された場合に，手術の実施を決定した．そして開腹所見により，定型手術または膵体尾部，脾，結腸，小腸，付属器などの合併切除で切除可能な場合に，胃切除を施行した．腫瘍の浸潤により膵頭十二指腸切除，開胸や広範囲の腹膜切除など高度の侵襲を伴う術式が必要となる場合は切除の適応外と判断した．リンパ節郭清は，初期には治癒をめざしてD2郭清を原則としていたが，症例経験に伴い術後再発のリスクが高いことが明らかとなり，予防的郭清のための脾摘は行わない方針に変更した．

術後はできるだけ早期にPTX経静脈・腹腔内投与を再開し，経口摂取が安定した段階でS-1を標準投与量より減量して再開した．その後，明らかな腫瘍増悪や重篤有害事象がみられない限りは治療を継続し，術後2～3年間無増悪で経過した場合に，3種の治療を段階的に減量または中止した．

2006～2011年に腹腔内投与併用化学療法を施行したP1またはCY1の初発胃癌100例中60例に手術を施行した（**表1**）．胃癌取扱い規約第12版分類P3の症例が過半数を占め，腹水貯留や腸管狭窄などを伴う高度播種症例が多く含まれていた．

術前に施行した化学療法は中央値4（範囲1～16）コースであった（**表2**）．手術術式は胃全摘が9割を占め，脾臓，結腸，付属器などの合併切除が行われた．リンパ節郭清はD2 28例，D1＋ 32例であった．腫瘍遺残R0が42例（70%）で達成され，組織学的にはgrade 1b以上の奏効が26例（43%）で確認された．術後，縫合不全および膵液瘻を各2例に合併したが，保存的に軽快し，手術関連死は認めなかった．

切除症例60例のMSTは34.5か月であり，播種の程度別ではP0CY1および規約第12版分類P1（計11

表 2　結果

術前化学療法						
	コース数	1～2	3	4～6	7～9	10～16
	症例数	6	21	11	11	11
手術術式	胃全摘 54，幽門側胃切除 6					
合併切除臓器	脾臓 19，膵臓 4，結腸 13，小腸 2，付属器 8					
リンパ節郭清	D1＋　32，D2　28					
腫瘍遺残	R0　　42（70%）					
	R1　　8（13%）					
	R2　　10（17%）					
組織学的効果	Grade 1a　34（57%）					
	Grade 1b　12（20%）					
	Grade 2　　13（22%）					
	Grade 3　　1（ 2%）					
術後合併症	縫合不全 2，膵液瘻 2，手術関連死 0					

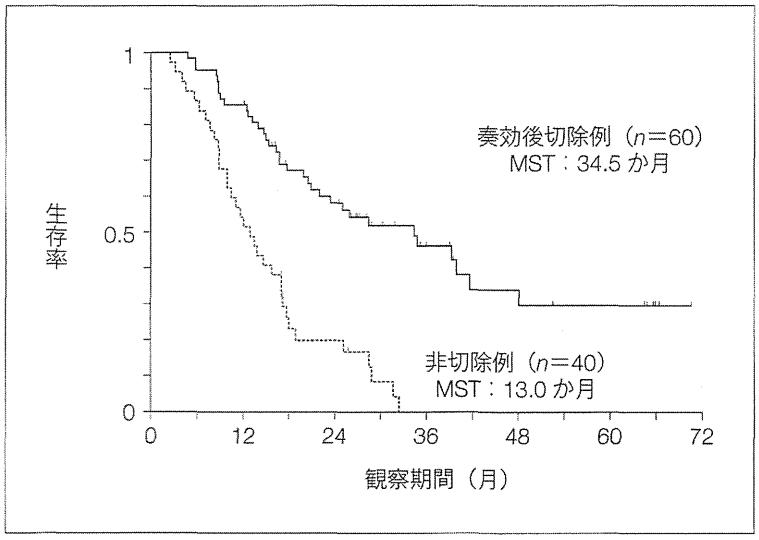

図 4　全生存期間

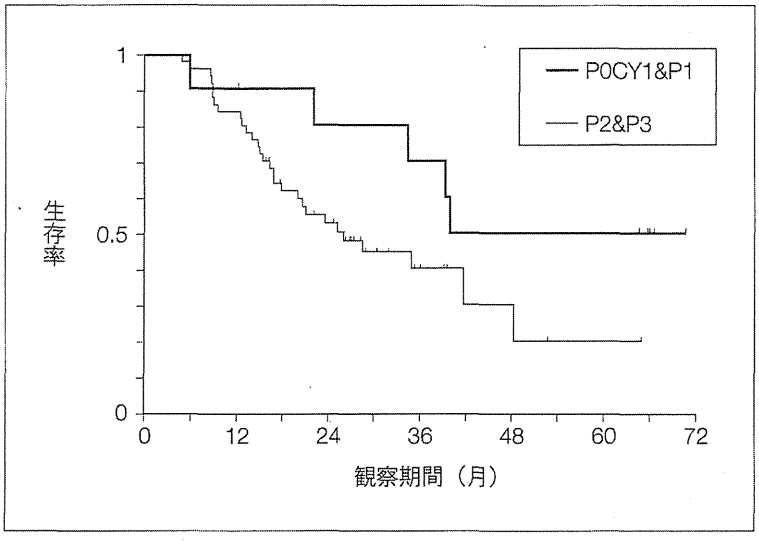

図 5　全生存期間

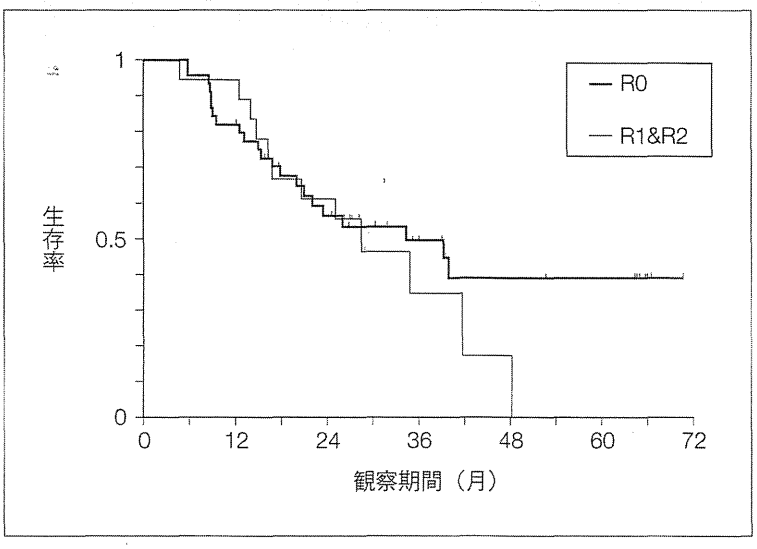

図6　全生存期間

例）の2年生存率81%に対して，P2・P3（計49例）では55%であった（**図4，5**）．一方，十分な奏効が得られず切除に至らなかった40例のMSTは13.0か月であった．また，腫瘍遺残の程度別では，R0症例の5年生存率39%に対して，結果としてR1またはR2となった症例では0%であったが，3年目までの生存曲線は重なっており，全生存期間に統計学的有意差は認めなかった（**図6**）．化学療法開始時を起点とした無再発・無増悪生存期間中央値は19.6か月であった．再発または増悪の初発部位は腹膜が24例と最も多かったが，22例では腹膜以外（リンパ節8例，肝3例，骨3例，卵巣2例，胸膜2例，副腎2例，髄膜2例）であり，非常に多様であった．

切除例と非切除例の統計学的な比較により手術の有用性を検討することは，両群の背景が異なり，化学療法の奏効度も異なるため，困難である．しかし，手術を施行せず化学療法のみで3年以上生存した腹膜播種症例の経験や報告はないことを考慮すると，手術の貢献度は大きいと考えている．また，腫瘍遺残がR1またはR2の場合でもR0の症例と遜色ない成績が得られていることより，化学療法が奏効している状況下では，腫瘍が多少遺残する手術も許容されうると考えている．今後，さらに症例を集積し，手術の適応，タイミングや術式について検討していきたい．

おわりに

腹膜播種を伴う胃癌に対して，S-1＋PTX経静脈・腹腔内併用療法と胃切除による集学的治療は安全かつ有効と考えられた．現在，先進医療として，本療法とS-1＋CDDP併用療法を比較する多施設共同の第Ⅲ相試験（PHOENIX-GC試験）を実施中である．本試験によりPTX腹腔内投与の有用性が証明され，保険収載につながることが期待される．

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Original Article

Flow Cytometric Quantification of Intraperitoneal Free Tumor Cells in Patients with Peritoneal Metastasis

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Background: Peritoneal metastasis (PM) is the most life-threatening type of metastasis in abdominal malignancy. To improve the diagnostic accuracy of cytologic detection (CY) of free tumor cells (FTC) in the peritoneal cavity, we tried to quantify the FTC to leukocyte ratio using flow cytometry in patients with peritoneal metastasis.

Methods: Cells were recovered from ascites or peritoneal lavages from 106 patients who underwent abdominal surgery and additional 89 samples which were obtained from peritoneal catheter or access port in patients with PM (+) gastric cancer. The cells were immunostained with monoclonal antibodies to CD45 and to CD326 (EpCAM). Using flow cytometry, CD326 (+) and CD45 (+) cells were classified as either tumor cells (T) or leukocytes (L) and the T/L ratio (TLR) was calculated.

Results: In 106 samples obtained by laparotomy, Median (*M*) of the TLR of PM (+) patients was 1.39% (0–807.87%) which was significantly higher than PM (−) patients (*M*=0%, 0–2.14%, $P<0.001$). In PM (+) patients, 86 CY (+) samples showed higher TLR than 61 CY (−) samples (*M*=2.81%, 0.02–1868.44% vs. *M*=0%, 0–3.45%, $p<0.0001$). In all of the 24 patients who were monitored for TLR before and after intraperitoneal (IP) chemotherapy, the TLR was reduced which was more dramatic than the results of the change in cytology.

Conclusions: TLR measured with FACS is an excellent reflection of the tumor spread in the peritoneal cavity and could be a reliable diagnostic biomarker to determine the severity of PM as well as effectiveness of IP chemotherapy.

Key words: peritoneal metastasis; intraperitoneal free tumor cell; peritoneal cytology; flowcytometry; CD326

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Peritoneal metastasis is one of the most frequent types of recurrent abdominal malignancy, especially as a result of gastric and ovarian cancer. It is most likely that peritoneal recurrence is caused by intraperitoneal free tumor cells (PFC), which have been exfoliated from the serosal surface of primary tumors (1,2). In fact, the detection of free tumor cells (FTC) by peritoneal cytology (CY) at the time of surgery has been reported to be one of the most reliable prognostic factors for peritoneal recurrence in gastric (3–6), colorectal (4,7), pancreatic (8), and gynecologic (9) malignancies. However, cytological diagnosis of peritoneal fluids is qualitative and

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largely dependent on institutions, as well as pathologists, which may result in confusion in the clinical evaluation of outcome of CY (+) patients (10,11).

More recently, molecular detection of tumor cell-specific mRNA, such as carcinoembryonic antigen (CEA) or cytokeratin (CK) 19 or 20 has been introduced, using real-time PCR, which improves sensitivity in detecting free intraperitoneal cancer cells (12–15). However, amplified mRNA can be derived from dead cells and CEA and CK can be expressed and released from hematopoietic cells in an inflammatory context (16), thus the clinical significance of false positive cases remains to be addressed. In this study, we report on our development of a new method to quantify the accurate volume of FTC in the abdominal cavity, using flow cytometry. We evaluate the clinical value of this method in patients with peritoneal metastasis.

MATERIALS AND METHODS

Patients

Ascites or peritoneal lavages were recovered from 106 patients who underwent abdominal surgery for gastric ($n = 90$) or colorectal cancer ($n = 16$) in the Department of Surgical Oncology between August 2008 and May 2013. All patients underwent open abdominal surgery and peritoneal washing was performed using 200 ml of normal saline. 100ml samples were obtained by lavage before operative manipulation. In cases of the presence of ascites, 20 ml of fluid from ascites was obtained soon after laparotomy. During the same period, 89 samples were obtained from intraperitoneal catheter or a subcutaneous intraperitoneal access port in patients who received intraperitoneal (IP) chemotherapy for peritoneal metastasis of gastric cancer. Pathologists evaluated peritoneal cytology for all samples. Informed consent was obtained in writing from all patients. This study was carried out in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Tokyo.

MAbs

PE-conjugated mAb to CD326 (EpCAM) was purchased from Miltenyi Biotec (Auburn, CA). FITC-conjugated mAb to CD45, Fc-blocker and 7AA, as well as control mouse IgG1, were all purchase from Becton-Dickinson (San-Jose, CA).

Cell Processing and Flow Cytometry

After the centrifugation of ascites or peritoneal lavages at 1,500 rpm for 10min, the pellets were resuspended in PBS+0.02% EDTA and overlayed on Ficoll-Hypaque solution (Pharmacia Biotech, Piscataway, NJ). After centrifugation at 3,000 rpm for 10 min, the intermediate layer was taken and washed twice with PBS+0.02% EDTA. During the procedure, most of the cell clusters were dissociated to form single cell suspensions. The cells ($1x10^6$) were suspended in 100μl of PBS+0.02% EDTA, incubated with 10 μl of Fc-blocker for 20 min and then immunostained with FITC-conjugated CD45, PE-conjugated CD326 and 7AA for 30 min in 4°C as per the manufacturers' recommendation. After washing, 10^4 cells were acquired in the 7AA-negative area and analyzed for the expression of CD45 and CD326 with FACS-Caliber (Becton-Dickinson, San-Jose, CA). In some cases, the samples were observed with fluorescent microscopy Biozero (Keyence, Tokyo Japan).

Statistics

In the comparison of the tumor cell/leukocyte ratio (TLR), *P*-value was calculated using Wilcoxon's nonparametric analysis with JUMP software.

RESULTS

Calculation of Tumor Cells/Leukocytes Ratio (TLR)

Figure 1 is representative of the FACS profiles of the cells recovered from ascites in a patient with peritoneal metastasis (Case 1, upper panel) and peritoneal lavage from a patient without peritoneal metastasis (Case 2, lower panel). Figures 1A–1E, 1K–1O show the results of staining with control mAbs, while Figures 1F–1J, 1P–1T show staining with FITC-conjugated anti-CD45mAb, PE-conjugated anti-CD326 mAb and 7AAD. First, 7AAD-positive areas were determined to be dead cells in Figures 1D, 1I, 1N, and 1S and excluded from the analysis. Then, in the 7AAD-negative region (R1), the FL-1 (FITC) and FL-2 (PE) intensities were plotted against SCC (Figs. 1B, 1G, 1L, and 1Q and Figs. 1C, 1H, 1M, and 1R). In Figures 1B and 1C and Figures 1L and 1M, negative areas were determined for CD45 and CD326, respectively. Usually, the threshold for fluorescein intensity increased as the SCC increased and the positive areas for CD45 (R2) and CD326 (R3) show the "sox like" shape. In Figures 1G and 1H, case 1, the number of CD45 (+) leukocytes (L) and CD326 (+) tumor cells (T) were calculated as the dot number located in the gated areas R1+R2 and R1+R3, respectively, and the tumor cell leukocyte ratio (TLR) was calculated as the relative frequency of FTC in the abdominal cavity. To make the calculation more accurate, the number of the cells located in the positive region in control IgG staining (Figs. 1B and 1C) was subtracted from those values to delete the cells with nonspecific binding. Thus, the TLR was calculated as the following formula.

$$\mathrm{TLR}\,(\%) = (\mathrm{PE-conjugated\ CD326(+)\ cells} - \mathrm{PE\text{-}conjugated\ mIgG\ (+)\ cells}) / (\mathrm{FITC-conjugated\ CD45\ (+)\ cells} - \mathrm{FITC-conjugated\ mIgG\ (+)\ cells}) \times 100$$

Then, the TLR in case 1 was calculated as (315-1)/(9157-18) x100 = 3.40 (%). Similarly, TLR in case 2 was calculated as (2-2)/(9348-71) x100 = 0(%), suggesting that no tumor cells were present in case 2.

FACS profiles of two additional cases with peritoneal metastasis (Cases 3 and 4) were expressed in Figure 2.

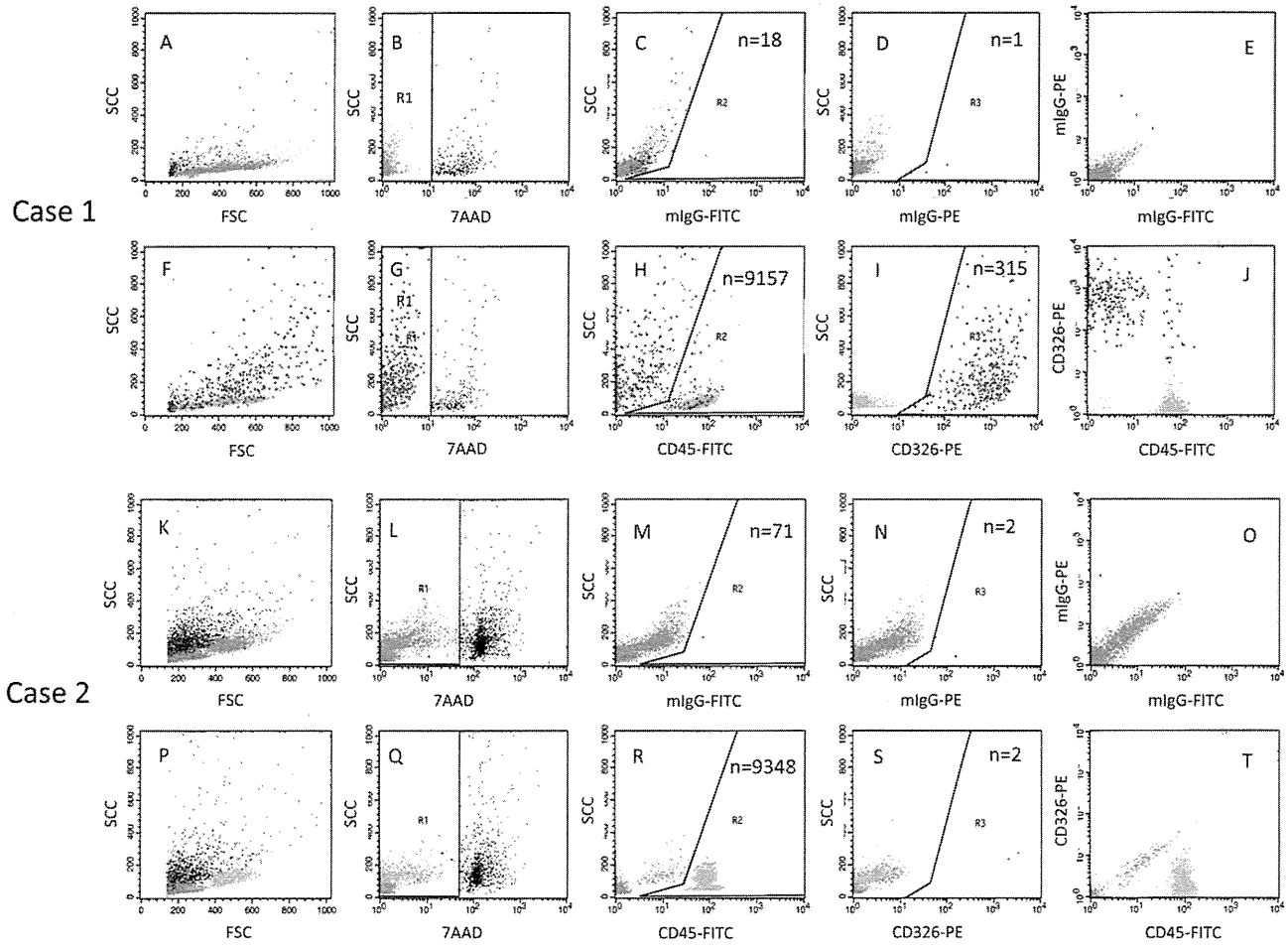

FIG. 1. FACS profiles of the patients with (Case 1, upper panels) or without (Case 2, lower panels) peritoneal metastasis. **A–E** and **K–O** show the staining with control mAbs, while **F–J** and **P–T** show the staining with FITC-conjugated anti-CD45 and PE-conjugated CD326. Figures A, F, K, and P show dot plots of FSC/SCC and E, J, O, and T show the FL1/FL2 profile. In figures B, G, L and Q of the FL3/SCC profile, 7AA negative cells were analyzed as living cells. Figures C, H, M and R show FL1/SCC and D, I, N and S show the FL2/SCC profile. CD45 and CD326-positive areas were defined as R2 and R3, respectively, and counted dot numbers were expressed. All the figures were expressed as multi-color plotting and green and red dots show CD45 (+) and CD326 (+) cells, respectively.

Although both cases were diagnosed as cytologically positive (CY+), TLR of case 3 was calculated as 1.84%, while that of case 4 was 190.15%. This indicates that the frequency of intraperitoneal tumor cells varied widely among patients with positive peritoneal cytology.

As shown in Figures 1F and 2D, CD326 (+) tumor cells (red spots) were generally distributed at relatively higher area in the FSC and SCC profile, as compared with CD45 (+) leukocytes (green spots). In addition, the expression of CD326 and CD45 were mutually exclusive in most cases (Figs. 1J, 2B, and 2E).

Observation with Fluorescent Microscopy

Figures 2C and 2F show the merged images of the same samples of Case 3 and 4, respectively, observed with immunofluoresence microscopy. These figures show that most of the cells in these samples consisted of single cells, which were stained with either FITC labeled anti-CD45 mAb or PE-labeled anti-CD326 mAb. The ratio of red tumor cells to green leukocytes was mostly consistent with TLR calculated with FACS analysis in both samples. The microscopic observation also indicates that tumor cells are relatively larger than leukocytes, which is consistent with the FSC/SCC profile of FACS analysis.

TLR of Peritoneal Fluid in Operative Patients

Figure 3A shows the TLR of the patients with or without peritoneal metastasis obtained at laparotomy. In general, samples derived from patients without peritoneal metastasis (PM-) contained few CD326-reactive cells and median (*M*) of TLR of the PM(-) 48 cases was 0% (0–2.14%). Indeed, the TLR of 30 of the 48 cases was 0% and less than 0.1% in the other 10. In contrast, the TLR of the samples recovered from the 58 patients with peritoneal metastasis (PM+) showed significantly higher

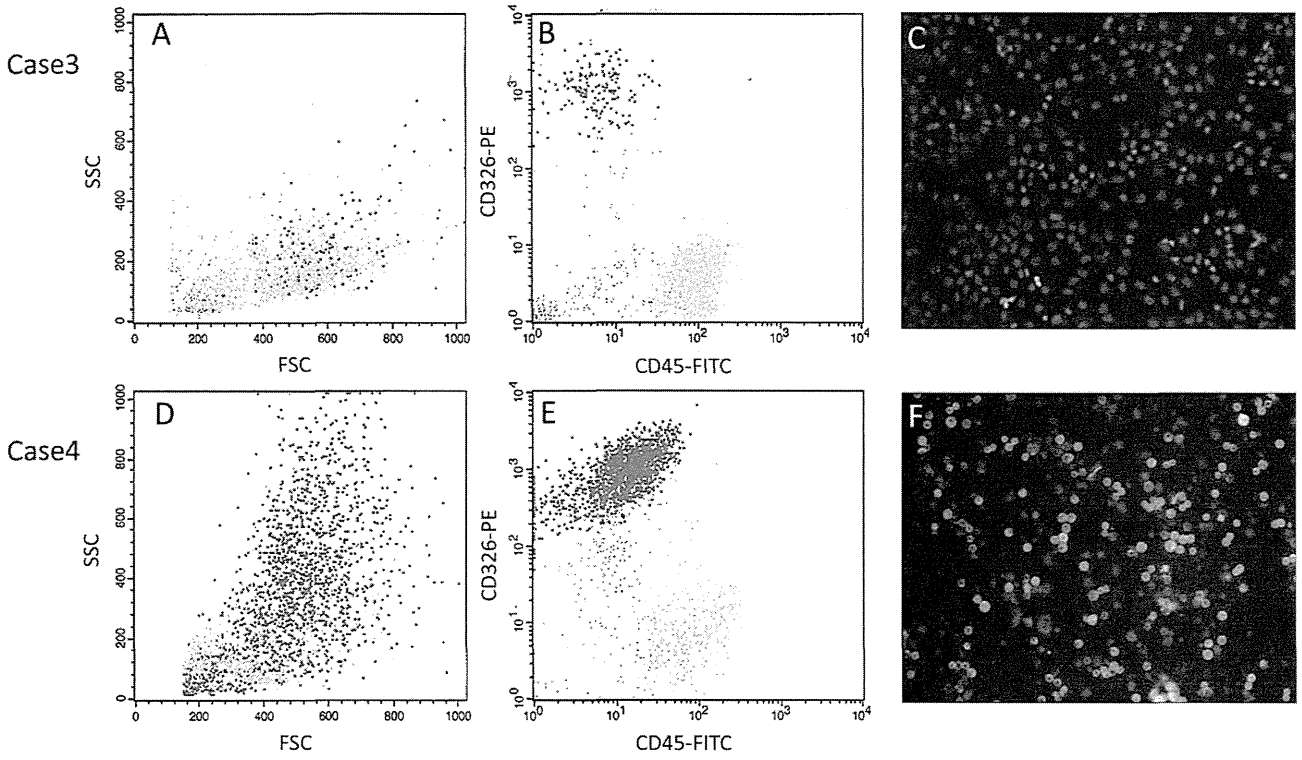

FIG. 2. FACS profiles of 2 additional cases (**A**, **B** and **D**, **E**) with peritoneal metastasis and positive cytology. Each figure was expressed in the same fashion as Figure 1. (**C**), (**F**): Samples of Case 3 (**C**) and Case 4 (**F**) were placed on a plastic plate and observed with Fluorescent microscopy. Green and red fluorescence were detected under the corresponding wavelengths and the two images were merged.

TLR (M=1.39%, 0-807.87%) ($P < 0.001$). However, TLRs were highly variable among the samples from PM (+) patients. In the 58 patients, 7 cases contained extremely high levels of CD326 (+) cells with TLR over 100%, indicating that tumor cells are more predominant than leukocytes in the abdominal cavity. On the contrary, TLRs were less than 0.1% in 7 cases.

TLR of CY (+) and CY (−) Samples in Patients with Peritoneal Metastasis

Figure 3B shows the TLR of the samples derived from the patients with peritoneal metastasis, including 58 samples obtained at laparotomy and 89 samples obtained from a peritoneal catheter or access port. TLR and CY showed the good correlation (Table 1). All of the 86 CY (+) samples contained a significant number of CD326-reactive cells (M =2.81%, 0.02~1868.44%). In contrast, 61 CY (−) samples showed significantly lower TLR (M=0%, 0~3.45%, P <0.001). Their TLR was 0% in 36 cases and less than 0.1% in the remaining 6, which was mostly similar to TLR in samples obtained from the PM(-) patients at laparotomy.

TLR Before and After Intraperitoneal Chemotherapy

In 24 patients, the TLR was measured before and after IP chemotherapy. Cytology was initially positive in 21 and negative in 3 cases. As shown in Figure 4, TLR was reduced by IP chemotherapy in all cases including the 3 CY(-) cases. Among the 21 CY(+) cases, TLR was reduced to 0% in 10 cases after chemotherapy, which was accompanied with negative cytology after chemotherapy. In 11 other cases, however, TLR after IP chemotherapy was calculated to be between 0.016%~35.4% and 4 of the 11 samples were diagnosed as CY(+), even after chemotherapy. Even in those cases, however, we confirmed that the peritoneal lesions were partially reduced by laparoscopic findings, the change of TLR might be more sensitive than conventional cytology in terms of the response to chemotherapy.

DISCUSSION

Cytologic detection of FTC from a peritoneal lavage is now recognized as the most important determinant in the prediction of the development of peritoneal recurrence in patients with many types of abdominal malignancies (5,6) (3,4,7) (8) (9). However, conventional examination with Papanicoloaou staining is reported to lack the sensitivity, and thus immunostaining methods using specific mAbs to tumor cell-associated antigens have been used to increase sensitivity (17-19). In this study, we used the immunostaining method and tried to quantify the relative frequencies of FTC in the abdominal cavity using flow cytometry.

We used pan-leukocyte markers CD45 and CD326 (EpCAM), which are widely overexpressed in a variety