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Predictability of the response to tyrosine kinase inhibitors via *in vitro* analysis of Bcr-Abl phosphorylation

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ABSTRACT

It would be of great value to predict the efficacy of tyrosine kinase inhibitors (TKIs) in the treatment of individual CML patients. We propose an immunoblot system for detecting the phosphorylation of Crkl, a major target of Bcr-Abl, in blood samples after *in vitro* incubation with TKIs. When the remaining phosphorylated Crkl after treatment with imatinib was evaluated as the "residual index (RI)", high values were found in accordance with imatinib resistance. Moreover, RI reflected the outcome of imatinib- as well as second generation TKIs with a high sensitivity and specificity. Therefore, this system should be useful in the selection of TKIs.

1. Introduction

The introduction of tyrosine kinase inhibitors (TKIs) targeting Bcr-Abl have dramatically improved the treatment of CML. Imatinib mesylate (Gleevec; Novartis Pharmaceuticals, East Hanover, NJ) was shown to induce high rates of cytogenetic and molecular responses, resulting in greatly prolonged survival in CML patients [1,2]. However, despite the remarkable improvement in survival and responsiveness with imatinib-treatment, a considerable proportion of the patients treated with imatinib have been reported to exhibit either primary or secondary resistance or intolerance [3–5]. Clinical resistance to imatinib can result from mutations in the Abl kinase domain at residues that directly contact imatinib or that influence imatinib binding [6]. As resistance can also arise in the absence of Bcr-Abl mutations, other mechanisms of resistance and disease progression may exist, including Bcr-Abl-independent signaling in CML cells [7]. To overcome the resistance and intolerance to imatinib, efforts have been made to develop second- and third-generation TKIs. Examples of such inhibitors include nilotinib (Tasigna, Novartis) [8], dasatinib (Sprycel, Bristol-Myers Squibb) [9] and other TKIs under clinical investigation such as bosutinib [10] and INNO-406 [11]. These TKIs are significantly more potent than imatinib and have exhibited efficacy against many types of imatinib-resistant Bcr-Abl mutants. Furthermore, they are also candidates for first-line therapy, as there is a need to improve the results achieved with imatinib [12–14]. In parallel with the entrance of new therapeutic compounds, an important question is which TKI is the most appropriate to each CML patient.

To establish a system with which we can predict the response of each patient to TKIs, we investigated in this study the phosphorylation of Crkl, a major target of Bcr-Abl, after *in vitro* incubation with or without TKIs in peripheral blood (PB) samples from patients either newly diagnosed or resistant to imatinib. It is demonstrated that this *in vitro* analysis system is highly reflective of the clinical response to TKIs of CML patients, and these data should prove useful in selecting TKIs in individual cases.

2. Patients, materials and methods

2.1. Patient blood samples

Thirty-one patients with CML in the chronic phase (CP) were included in this study (Table 1). The optimal response, response and resistance were defined in accordance with the European Leukemia Net (ELN) recommendations [15,16]. Briefly, an "optimal response" to imatinib means achieving a complete hematological response (CHR) at 3 months or complete cytogenetic response (CCyR) at

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6 months after the induction of imatinib, and resistance means failure to achieve such a response. On the other hand, in nilotinib- or dasatinib-treated patients, a "response" means a minor cytogenetic response (mCyR) at 3 months or partial cytogenetic response (PCyR) at 6 months after the induction of the second generation TKI, and resistance means failure to achieve this response.

Ten microliters of the PB samples were obtained from patients with informed consent at the beginning or before the initiation of imatinib, nilotinib or dasatinib. Half of each sample was used for examination of the Bcr-Abl sequence, which was performed by the SRL Co. (Tokyo, Japan), and the other half was used for immunoblot analysis.

Approvals for the study were obtained from the institutional review boards of all the participating facilities.

2.2. Reagents

Imatinib, methanesulfonate salt was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland), and nilotinib and dasatinib were purchased from LC laboratories (Boston, MA). The antibodies used in this study were as follows: anti-Lyn, anti-phospho-Crkl, anti-phospho-c-Abl from Cell Signaling Technology (Beverly, MA), anti-phospho-Lyn(Y396) from Epitomics (Burlingame, CA), anti-Crkl, anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA), and the secondary antibodies, anti-Rabbit IgG HRP and anti-Goat IgG HRP were from Promega (Madison, WI). Pervanadate was purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Cell line

A Bcr-Abl positive human cell line, K562, was used in the preliminary experiments in this study. K562 cells were maintained in RPMI1640 (nacalai tesque, Kyoto, Japan) supplemented with 10% fetus bovine serum (FBS) (EQUITECH-BIO, Kerrville, TX).

2.4. Immunoblot assays of patients' samples

Whole blood cell samples from patients were used within 3 h after blood had been drawn. Red cells were lysed with Whole Blood Lysing Reagents (Beckman Coulter, Brea, CA), and white blood cells were cultured with or without imatinib, nilotinib or dasatinib. After 5-h incubation, the cell lysates were collected and subjected to immunoblot assays. Gel electrophoresis and immunoblot assays were performed according to methods described previously [17,18]. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (PerkinElmer Life Sciences, Boston, MA).

2.5. Evaluation of phosphorylation intensity and determination of the "residual index (RI)"

The intensity of each blot of immunoreactive protein was quantified using ChemiDoc XRS+ with Image Lab Software (Bio Rad, Tokyo Japan). The RI values of each patient to TKIs were determined in accordance with the numerical expression, as indicated in Fig. 2A.

2.6. Statistical analysis

Analysis of variance was used to assess data reproducibility. The Mann–Whitney rank sum was used to define differences between groups.

3. Results

3.1. Immunoblot analysis of phosphorylated Crkl in CML patients

To assess the drug response of the CML patients, we performed immunoblot assays detecting phosphorylated Crkl, a direct target of Bcr-Abl kinase. To establish the experimental procedures, preliminary experiments were performed with K562, a CML blast crisis cell line, or blood sample from a newly diagnosed CML patient (Patient A), 98% of whose PB cells were Bcr-Abl-positive on fluorescence *in situ* hybridization (FISH). First, to determine the optimum incubation period for the TKIs, PB cells were incubated with or without TKIs for varying time periods. A two-hour incubation was not sufficient because imatinib did not completely suppress the phosphorylation of Crkl, while 24-h incubation was too long because the PB neutrophils appeared to die (Fig. 1A, left panel). A five-hour incubation completely eliminated the phosphorylation of Crkl without cell death. On the other hand, simultaneous treatment with a phosphatase inhibitor sustained the phosphorylation of Crkl even after treatment for 24 h (Fig. 1A, right panel). Thus, we decided to incubate cells for 5 h without phosphatase inhibitors. Next, to build an *in vitro* simulation model for the estimation of the activities of TKIs in the body, we fixed the concentrations of TKIs at the peak value of plasma concentrations in patients (Cmax) after administration of the recommended dose of TKIs. The Cmax of imatinib in CML patients after taking orally 400 mg of the drug is 3.0–4.8 μM, and that of nilotinib after taking 400 mg is 2.9–4.0 μM. In the case of

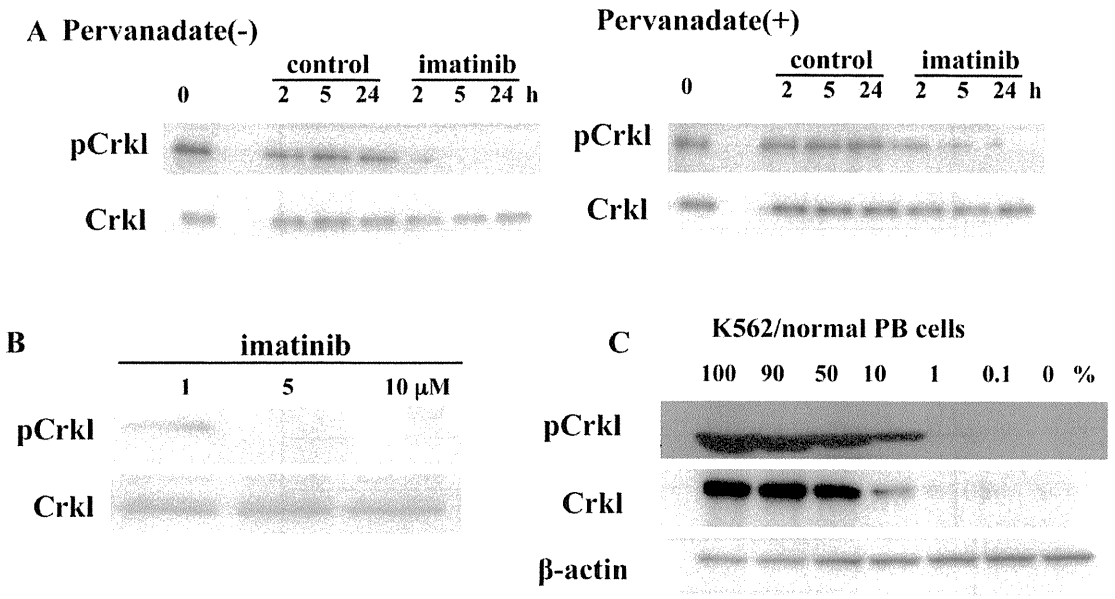

Fig. 1. Optimization of western blot after TKI-incubation. (A and B) Blood sample from Patient A was incubated with or without 5 μM imatinib supplemented with (right panel) or without (left panel) 10 μM of pervanadate for the indicated periods (A) or incubated with imatinib at the indicated concentrations for 5 h (B). The treated cells were lysed and subjective to immunoblot analysis using the indicated antibodies. (C) K562 cells were mixed into normal human PB cells at the indicated ratios. Then the samples were subjective to immunoblot analysis.

dasatinib, the Cmax after the ingestion of 100 mg dasatinib was 100nM [19–21]. In terms of pharmacokinetics, we fixed the concentrations of these TKIs (imatinib, nilotinib and dasatinib) at 5 μM, 5 μM, and 0.1 μM, respectively. As shown in Fig. 1B, 1 μM of imatinib did not eliminate the phosphorylation of Crkl in the examined sample of patient A who are newly diagnosed and well responded to imatinib, but 5 μM and 10 μM of imatinib did, indicating that 1 μM is too low concentration for estimation of clinical outcome. Finally, to estimate the sensitivity of this system, K562 cells were mixed with normal PB cells at variable ratios, as indicated. Fig. 1C shows that the phosphorylated Crkl at the lowest 1% was detectable in K562 cells. Thus, we analyzed patients having more than 10% Bcr-Abl-positive cells in PB by FISH.

3.2. Iimmunoblot analysis

To quantify the *in vitro* responsiveness to TKIs, we measured the density of each blot using a densitometric method. We then defined "residual index (RI)" for each TKI by the numerical expression as shown in Fig. 2A. Triplicate measurements were performed on 3 individual patients (Patient B, C and D). There were no significant variations among the RIs in each patient. Standard error for each sample set was less than 5% (4.6%, 1.2% and 3.4%, respectively) (Fig. 2B).

3.3. Responses to the TKIs in patients with various stages of CML

Fig. 3A represents typical results of the immunoblot analyses in 2 patients with newly diagnosed CML (Patient 1 and 2), and 2 patients who were receiving imatinib but were displaying resistance (Patient 16 and 17). Although all of these samples exhibited apparent phosphorylation of Crkl without TKIs, the phosphorylated Crkl disappeared from the samples of Patients 1 and 2 when incubated with imatinib, nilotinib or dasatinib. In the case of Patients 16 and 17, on the other hand, weak bands remained in the imatinib and/or nilotinib-incubated samples, but disappeared in the dasatinib-treated ones. Thus, this immunoblot analysis appeared to be useful in evaluating Crkl phosphorylation after *in vitro* TKI-incubation. All patients were divided into two groups: one being newly diagnosed and another receiving imatinib-therapy but showing resistance. The imatinib-RIs of the samples from the imatinib-resistant group (median RI: 34.2%) were much higher than those of the samples from newly diagnosed patients (median RI: 4.2%) (Fig. 3B).

3.4. Sequential examinations using the residual index

RI values were analyzed sequentially in the course of the different TKI-treatments in 2 imatinib-resistant patients (Patient 23 and 27).

Patient 23 (Fig. 4A): after six months of treatment with imatinib, the drug was changed to dasatinib because of a failure to achieve an optimal response (72% $Ph1^+$ in FISH). Six months after the start of dasatinib, $Ph1^+$ cells were disappeared. The samples were obtained twice: prior to the treatment with imatinib, and at the time of change to dasatinib. Immunoblot analysis showed that neither imatinib nor nilotinib eliminated the phosphorylation of Crkl at the initiation of treatment, but dasatinib did. Furthermore the RI values were under 10% only in the sample incubated with dasatinib.

Patient 27 (Fig. 4B): when the first sample was obtained, the percentage of $Ph1^+$ cells was 93% after 7-year treatment with imatinib.

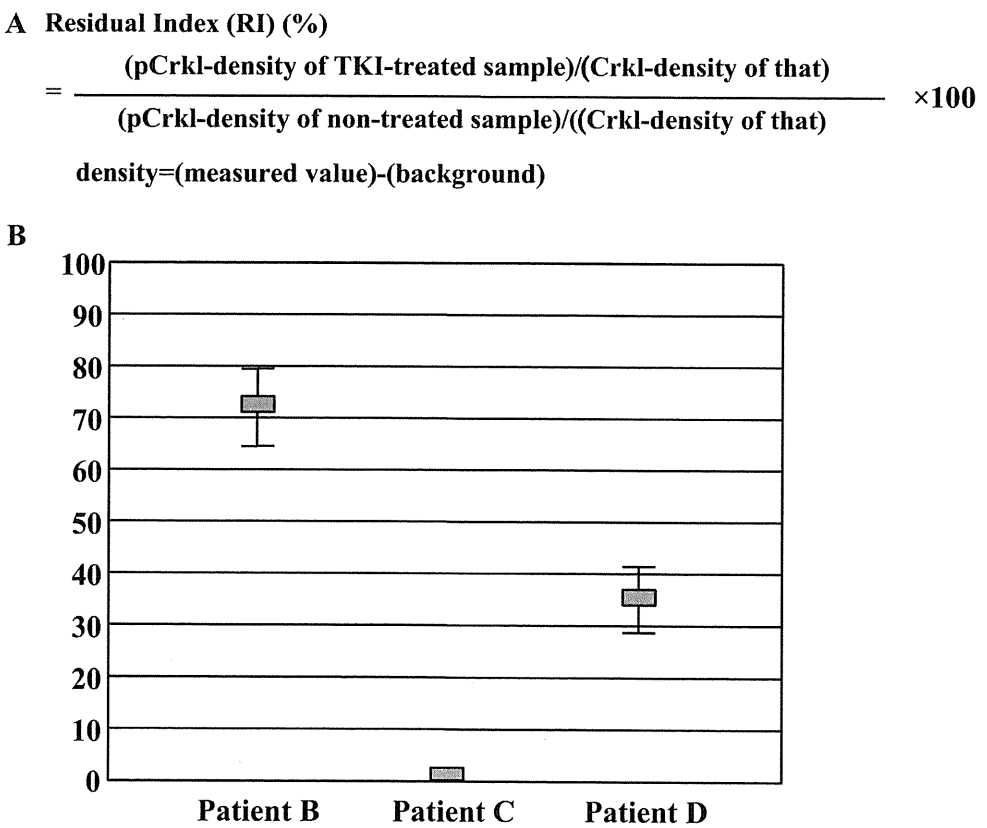

Fig. 2. "Residual index (RI)". (A) The numerical expression of RI. "Measured value" means the density of each blot measured by densitometric method. (B) The reproducibility of RIs for imatinib treatment. Means and standard errors, representing triplicate assays in 3 patients, are shown.

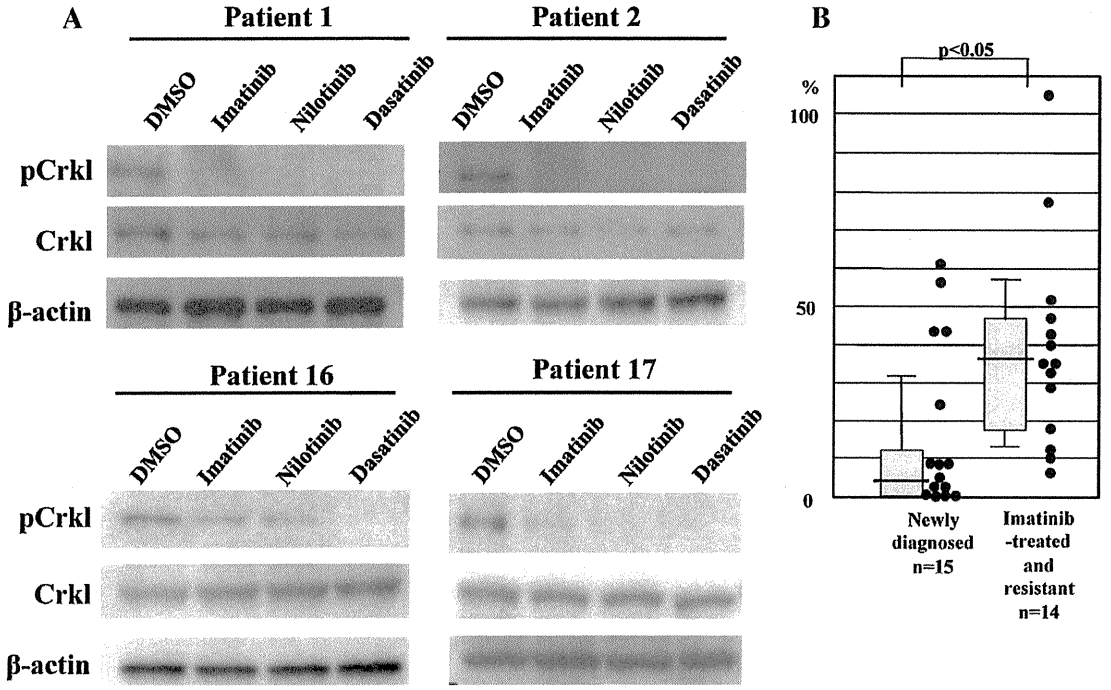

Fig. 3. Different RI values against imatinib between patients at diagnosis and patients showing imatinib-resistance. (A) Four typical data of immunoblots were represented. PB cells from newly diagnosed patients (Patient 1 and 2) or patients (Patient 16 and 17) who had been receiving imatinib-therapy but showed its resistance were incubated for 5 h *in vitro* with or without indicated TKIs. The concentration of imatinib, nilotinib, and dasatinib are 5 μM, 5 μM, and 0.1 μM, respectively. The incubated cells were lysed and subjected to immunoblot analysis using the indicated antibodies. (B) RIs against imatinib were calculated in 15 patients at diagnosis and 14 patients who had been receiving imatinib-therapy and showed its resistance. The distribution of RIs in each group was plotted. Representative box plots show values within the 25th to 75th percentile. Medians are indicated in crossbar. Fifth and 95th percentiles are shown by error bars. The statistical difference was $p < 0.05$.

Then the treatment was changed to dasatinib, which was stopped because of a strong pancytopenia. The patient was then treated with nilotinib, but the percentage of $Ph1^+$ cells again increased. The second sample was obtained at the time of the change from dasatinib to nilotinib. In both samples, the incubation with the three TKIs did not eliminate the phosphorylation of Crkl. Although the second sample exhibited a strong sensitivity only to dasatinib (RI = 4.1%), the remaining CML cells additionally displayed continuous Lyn-phosphorylation (Fig. 4B).

3.5. RIs in patients with Bcr-Abl point mutations

The most important issue in TKIs resistance is the acquisition of point mutations in Bcr-Abl. Bcr-Abl mutations were detected in 4 samples (Table 2). The RI values of Patient 28, with a threonine-to-isoleucine mutation at codon 315 (T315I), were higher than 10% in all the TKI-treated samples. In accordance with the *in vitro* results, the disease was refractory to both imatinib and dasatinib. A phenylalanine-to-leucine mutation at codon 317 (F317L) and a methionine-to-threonine at codon 351 (M351T) were detected in Patient 27. F317L is reported to confer high responsiveness to nilotinib, while M351T does the same to dasatinib. The RI values of this patient were over 10% in all of the samples treated with TKIs, which conformed the outcome of failing to achieve CHR after nilotinib or dasatinib treatment. Next, the RI value in the sample with the phenylalanine-to-valine mutation at codon 359 (F359V) (Patient 23) was less than 10% only in the dasatinib-treated sample, which does not conflict with the reported IC50 data. Finally, although the F317L mutation is reported to be highly sensitive to nilotinib, the RI value for nilotinib in Patient 19, who later proved to be resistant to nilotinib but responded to dasatinib, was higher than 10%, and lower than 10% for dasatinib. Therefore, RIs are likely to be highly correled with the favorability of Bcr-Abl mutations to TKIs, and in some cases, to predict the responsiveness with higher sensitivity than mutations.

3.6. Correlation of RI with patient outcome

To analyze whether the RIs correlate with the clinical response to TKIs, newly diagnosed patients ($n = 15$) were separated into two groups in accordance with the most recent outcome, imatinib-sensitive ($n = 13$), who achieved an optimal response after the sample collection, and imatinib-resistant ($n = 2$), who did not. The median RI of the patients in the sensitive group was 4.2% and that in the resistant group was 43.2% ($p < 0.05$) (Fig. 5, left panel). We also assessed the predictability of the response to nilotinib. Eight patients imatinib resistant had undergone nilotinib-therapy. Among them, 4 achieved optimal responses and the others failed. The median RI in the nilotinib-sensitive group was 3.5% in contrast to 31.2% in the resistant group (Fig. 5, middle panel). Although the sample size was too small to conduct statistical analysis, the RIs were clearly separated between dasatinib-sensitive and -resistant groups (Fig. 5, right panel).

When the cut-off value of RI was set at 10%, the specificities, sensitivities and predicted values were all 100% in terms of nilotinib and dasatinib responsiveness (Table 3). Also, in the evaluation of imatinib-treatment, the specificity and sensitiveness were more than 77%. Therefore, it is suggested that the RIs (cut-off value: 10%) are useful as a novel predictor for clinical utility of TKIs, especially in imatinib-resistant cases.

4. Discussion

Imatinib, the first approved TKI for CML, frequently induces durable cytogenetic remission and thus occupies an important position as the current standard of care. Now, second-generation

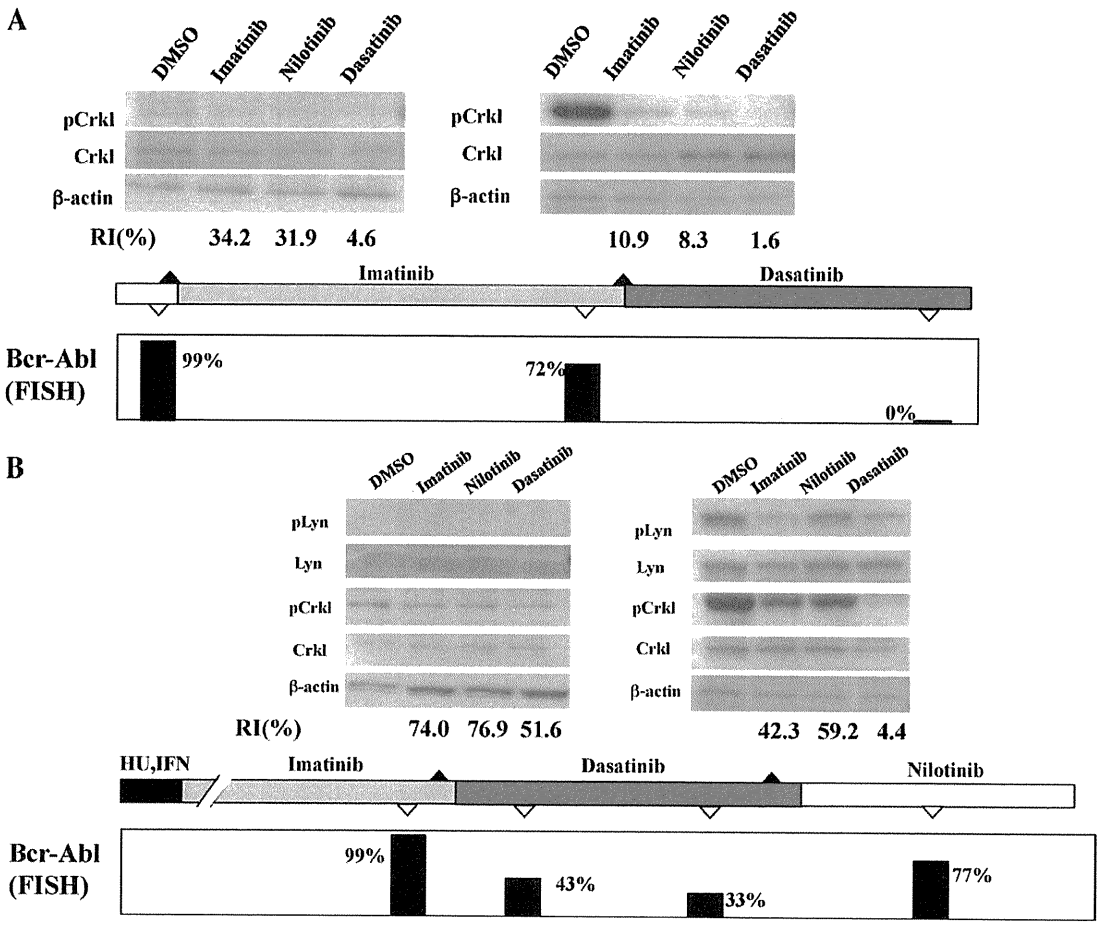

Fig. 4. Sequential examinations of RI values during clinical treatments in two patients. Immunoblots were sequentially analyzed during CML-treatment in two patients who showed resistance to TKIs. Data of immunoblots using the indicated antibodies are shown with their clinical course. FISH analyses are indicated by open triangles, and immunoblot analysis by closed triangles.

Table 1
Patient characteristics.

Characteristic	
No. of patients	31
Median age, y (range)	55 (20–89)
Sex (male/female)	14/17
Treatment before sample collection	
No	13
IFN	3
TKI	18
Bcr-Abl mutation	4
Median follow-up, months (range)	6 (3–14)

TKIs, such as nilotinib and dasatinib, have now been made available [12,13]. Although these TKIs are significantly more potent and show higher sensitivity against some imatinib-resistant mutations, there are no useful guidelines for the proper choice of second-generation TKIs in imatinib-resistant patients. Furthermore, second-generation TKIs have recently been recommended as first-line therapies based on the evidence that an earlier achievement of remission may provide a better clinical outcome or less disease progression. There is still a need for indicators pointing to the proper drug choice for individual patients. The *in vitro* responsiveness to TKIs in terms of cell proliferation has been demonstrated to be a predictor of clinical response. The IC50, a cell based screen for resistance determining the drug concentration that can induce 50% of growth suppression, is a potent predictor of the responsiveness to drugs. In patients with *de novo* CML, the $IC50^{imatinib}$ was reported to possess a high predictive value [22]. However, determination of the IC50 for each TKI requires so much effort and time that an application suitable for all patients may be quite a distant prospect. Furthermore, as the optimal concentration varies for each TKI, comparing the efficacy between different TKIs is difficult. Although the cellular IC50 s for the effect of TKIs on Bcr-Abl point mutations have been reported [23–26], this information

Table 2
Patients with BCR-ABL mutations, and their RI values.

Patient	Mutation	RIs			Clinical outcome
		Imatinib	Nilotinib	Dasatinib	
Patient 19	F317L	40.0	30.8	3.9	Imatinib and nilotinib resistant, and dasatinib respond
Patient 23	F359V	15.8	11.9	1.4	Imatinib resistant, and nilotinib and dasatinib intolerant
Patient 27	M351T/F317L	74.0	76.9	51.6	imatinib resistant, and nilotinib and dasatinib intolerant
Patient 28	T315I	104.2	88.0	93.0	Imatinib and dasatinib resistant

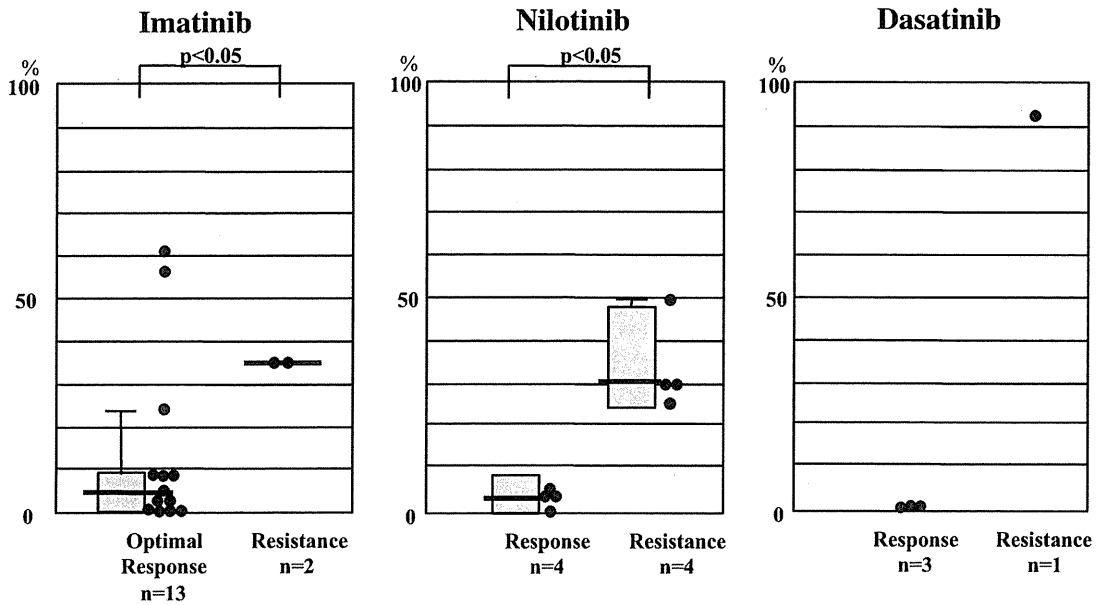

Fig. 5. RI values in patients grouped by clinical response to each TKI-therapy. Fifteen patients were newly diagnosed as CML, and their PB cells were obtained just before the beginning of imatinib-therapy. The patients were divided into two groups: "optimal response" in imatinib-treated patients means *de novo* CML patients who later proved to achieve optimal response, and "Resistance" means patients who later failed to achieve optimal response. Among 12 patients who had showed imatinib-resistance, 8 patients received nilotinib-therapy and 4 patients received dasatinib-therapy at a stretch of imatinib-therapy. Their PB cells were obtained just before the change of therapy. The patients were divided into two groups: that of responsive patients and of resistant patients to each TKI. Dot plots demonstrate the RI values of patients to each TKI. Representative box plots show values within the 25th to 75th percentile. Medians are indicated in crossbar. Fifth and 95th percentiles are shown by error bars.

Table 3
Sensitivity and specificity.

	Optimal response	Resistance	Predicted value
Newly diagnosed and Imatinib-treated patients ($n=15$)			
RI < 10	10	0	100%
RI ≥ 10	3	2	40%
Specificity/sensitivity	77%	100%	
Imatinib-resistant and Nilotinib-treated patient ($n=8$)			
RI<10	4	0	100%
RI ≥ 10	0	4	100%
Specificity/sensitivity	100%	100%	
Imatinib-resistant and Dasatinib-treated patients ($n=4$)			
RI < 10	3	0	100%
RI ≥ 10	0	1	100%
Specificity/sensitivity	100%	100%	
	Newly diagnosed and later achieved optimal response	Imatinib-treated and showed resistance to Imatinib	Predicted value
All included and evaluable patients ($n=27$)			
RI < 10	10	1	91%
RI ≥ 10	3	13	81%
Specificity/sensitivity	77%	93%	

is only useful when the mutated subclone is the predominant cell population.

In this study, we evaluated the effect of TKIs on Crkl phosphorylation as a "residual index". It is noteworthy that the samples from patients who had shown resistance to imatinib had much higher RIs than the samples from newly diagnosed patients. In the case of newly diagnosed patients, most samples responsive to imatinib *in vitro*, but two patients whose samples displayed markedly high RIs *in vitro* proved not to achieve an optimal response to the drug. Although substantial accordance was later detected in the immunoblot data between the responsiveness and resistance to imatinib, a few samples had markedly high RIs in patients who later achieved optimal responses to imatinib. These exceptional cases will have to be followed for a longer period. The data showed 100% of sensitivity and 77% of specificity when the RIs were separated at 10%. On the other hand, in imatinib-resistant patients, the results of the tests did reflect the patient outcome. Although the sample size was small, the immunoblot analysis was able to predict the clinical responsiveness to nilotinib or dasatinib treatment with 100% sensitivity and specificity. Thus, this system can be a useful tool for selecting TKIs, especially in imatinib-resistant patients. It may be inferred that the lower confidence in

the case of the untreated patients might due to a multiplicity of CML subclones.

CML patients develop imatinib resistance through either Bcr-Abl dependent or independent mechanisms. The most characterized and frequent mechanism is the acquisition of point mutations within the kinase domain of the Bcr-Abl gene, and some of the mutations such as T315I are potent predictors for outcome. However, even in those patients who have some mutations other than a few restricted mutations such as T315I and F317L, we cannot accurately predict the efficacy of TKIs. Furthermore, nearly half of the patients resistant to imatinib have no mutations in Bcr-Abl, which indicates that other mechanisms are also important for the acquisition of drug-resistance. Thus, we need other information for selecting TKIs. In this study, 4 patients carried point mutations in this region. Samples from 3 of them had RI values compatible with the predictive outcomes from the mutations. Notably, the RI values of the other sample contradicted the response of the mutation, but accorded with the actual response of the patient. From these points of view, the system described here can be utilized as another powerful predictor than IC50s for Bcr-Abl mutations.

The immunoblot system described here has the capacity to detect TKI-resistant subclones, including CML cells with Bcr-Abl mutations. In addition, our strategy seems to evaluate Bcr-Abl activity more directly than the cellular IC50 and require smaller population of TKI-resistant subclones than Bcr-Abl sequence analysis. Thus, when used together with the cellular IC50 values and Bcr-Abl sequence, this immunoblot system should help improve the treatment of patients with CML.

Conflict of interest

The authors state that they have no conflict of interest.

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Regimen Selection for First-line FOLFIRI and FOLFOX Based on *UGT1A1* Genotype and Physical Background is Feasible in Japanese Patients with Advanced Colorectal Cancer

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Objective: We examined the feasibility of regimen selection for first-line irinotecan, 5-fluorouracil and leucovorin or oxaliplatin, 5-fluorouracil and leucovorin in Japanese patients with advanced colorectal cancer based on *UDP-glucuronosyltransferase 1A1* genotype as well as physical status of patients related to diarrhea.

Methods: As first-line irinotecan, 5-fluorouracil and leucovorin is a little bit superior to oxaliplatin, 5-fluorouracil and leucovorin with respect to efficacy and toxicity, patients without risk factors of irinotecan-induced toxicity were first assigned to irinotecan, 5-fluorouracil and leucovorin. Patients with *UDP-glucuronosyltransferase 1A1*28/*28*, **6/*6*, **28/*6* or **28/*27* and those with ascites, peritoneal dissemination or diarrhea first received oxaliplatin, 5-fluorouracil and leucovorin to avoid the irinotecan-induced neutropenia and diarrhea, respectively. We retrospectively evaluated the feasibility of this strategy by assessing toxicity and total progression-free survival in first- and subsequent second-line therapies in all patients studied.

Results: In the first-line irinotecan, 5-fluorouracil and leucovorin ($n = 61$), Grade 4 neutropenia, febrile neutropenia and Grade 3 diarrhea occurred in 8.2, 3.3 and 3.3% of patients, respectively. In the first-line oxaliplatin, 5-fluorouracil and leucovorin ($n = 26$), Grade 4 neutropenia, febrile neutropenia, Grade 3 thrombocytopenia and Grade 3 neuropathy were observed in 11.5, 3.8, 3.8 and 7.7% of patients, respectively. In the second-line oxaliplatin, 5-fluorouracil and leucovorin ($n = 38$), Grade 3 diarrhea occurred in 2.6% of patients. In the second-line irinotecan monotherapy ($n = 11$), Grade 4 or febrile neutropenia occurred in 18% of patients and Grade 3 diarrhea in 9.1% of patients. In second-line S-1 ($n = 9$), Grade 3 anemia occurred in 2 patients. Median total progression-free survival in all 87 patients was 11.5 months.

Conclusions: Present regimen selection strategy would be feasible, since it causes less toxicity and similar efficacy comparing to previous studies. Determination of appropriate reduced dose in the second-line irinotecan monotherapy or other standard second-line therapy for patients with high-risk to irinotecan-induced toxicity might make this strategy more effective.

Key words: FOLFIRI – FOLFOX – physical condition – regimen selection – UGT1A1 genotyping

INTRODUCTION

Irinotecan is a camptothecin derivative that exerts cytotoxic effects by inhibiting topoisomerase I. This drug has been approved for the treatment of a wide variety of solid tumors, including colorectal cancer. However, patients and oncologists are deeply concerned about the dose-limiting toxic effects of irinotecan, such as myelosuppression and delayed-type diarrhea (1–3). Combined therapy with irinotecan, 5-fluorouracil (5-FU) and leucovorin (LV) (FOLFIRI) has been proven to be highly effective for the first-line treatment of patients with advanced colorectal cancer (4). The combination of oxaliplatin, 5-FU and LV (FOLFOX) is also a standard first-line regimen for advanced colorectal cancer (5). These regimens provide similar survival benefits, but have different toxicological profiles, depending mainly on the use of irinotecan or oxaliplatin. Furthermore, FOLFIRI followed by FOLFOX is associated with slightly, but not significantly longer survival than FOLFOX followed by FOLFIRI. In addition, the response rate of FOLFOX is superior to that of FOLFIRI when these regimens are used as the second-line therapy (5). Taking these lines of evidence into consideration, FOLFIRI is superior to FOLFOX as first-line treatment for patients with advanced colorectal cancer, if the patients do not have backgrounds, which are related to irinotecan-induced severe neutropenia or diarrhea.

Previously, physicians predicted the irinotecan-induced adverse events in FOLFIRI according to only physical conditions of patients with advanced colorectal cancer. Physicians tended not to use FOLFIRI as the first-line therapy for patients with ascites, peritoneal dissemination or diarrhea to avoid severe diarrhea induced by irinotecan. On the other hand, there have been no predictive markers of irinotecan-related severe neutropenia.

Several studies have linked *UDP-glucuronosyltransferase (UGT) 1A1*28* genotype to irinotecan-related neutropenia. Patients homozygous for *UGT1A1*28* have a significantly higher risk of severe neutropenia due to irinotecan than those who do not possess this genotype (6, 7), because *UGT1A1*28* decreases UGT1A1 protein expression and reduces glucuronidation capacity for SN-38. In Asians, a specific mutation, *UGT1A1*6* (8), has been proven to reduce the catalytic activity of UGT1A1 (9, 10). The *UGT1A1*28/*28*, **6/*6* and **6/*28* genotypes have been shown to be related not only to a lower ratio of the area under the plasma concentration–time curve of SN-38G to that of SN-38, but also to severe neutropenia in Asian populations (11–13). Compound heterozygotes of *UGT1A1*28* and *UGT1A1*27* seen in Japanese were also suggested to be related to severe neutropenia of irinotecan (6). Thus, the *UGT1A1* genotyping was established as predictive marker for irinotecan-induced severe neutropenia and was approved not only by the Food and Drug Administration in the USA but also by the Ministry of Health, Labour and Welfare of Japan.

Given that, we established a strategy for the regimen selection of FOLFIRI as the first-line therapy for patients with advanced colorectal cancer, aiming to avoid the irinotecan-induced severe toxicities that are related to the reduced dose intensity of irinotecan (Fig. 1). We considered the *UGT1A1* genetic testing in addition to the clinical physical status of patients to select FOLFIRI or FOLFOX regimen. Patients with *UGT1A1*28/*28*, **6/*6*, **28/*6* or **28/*27* first received FOLFOX to avoid the irinotecan-induced severe neutropenia. Patients who had the risk factor of irinotecan-induced severe diarrhea including ascites, peritoneal dissemination and diarrhea also first received FOLFOX, even though they possessed *UGT1A1*1/*1*, **1/*6* or **1/*28* genotypes. Patients with *UGT1A1*1/*1*, **1/*28* or **1/*6* and without the risk factor of irinotecan-induced severe diarrhea received first-line FOLFIRI.

To evaluate the feasibility of this regimen selection strategy for first-line FOLFIRI and FOLFOX, we retrospectively assessed toxicity and efficacy in first-line and subsequent second-line chemotherapies in all patients studied.

PATIENTS AND METHODS

PATIENTS

All patients with a histologically confirmed diagnosis of advanced colorectal cancer who had an Eastern Cooperative Oncology Group performance status of 0–2, adequate bone marrow, liver and renal functions and no history of chemotherapy for advanced disease were eligible. Patients with diarrhea of four times a day or more were excluded. Any previous adjuvant chemotherapy must have been completed at least 6 months before treatment. All patients signed

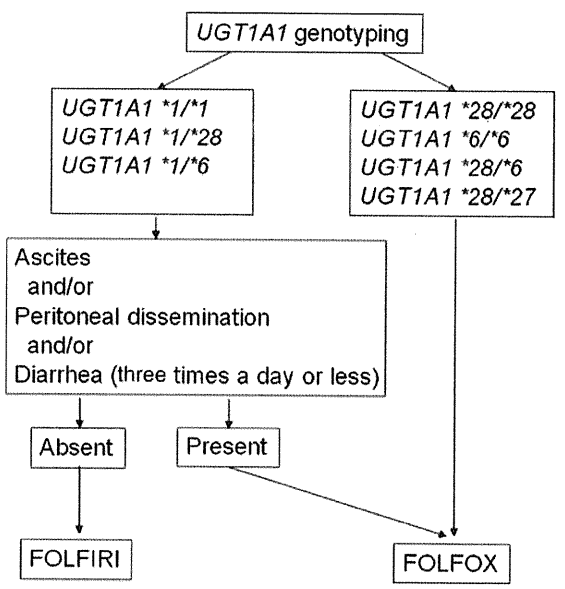

Figure 1. *UGT1A1* genotype-based strategy for the selection of FOLFIRI or FOLFOX as the first-line therapy in patients with advanced colorectal cancer.

written informed consent for their peripheral blood samples to be used for *UGT1A1* genotyping. The protocols of this retrospective study and *UGT1A1* genotyping were separately approved by the Institutional Review Board of Saitama Medical University.

Study Design

Patients with *UGT1A1*28/*28*, **6/*6*, **28/*6* or **28/*27* were considered high-risk group of irinotecan-induced severe neutropenia. Patients with ascites as judged by computed tomography or ultrasonography, hisotologically or cytologically confirmed peritoneal dissemination or diarrhea of three times a day or less were considered as high-risk group of irinotecan-induced severe diarrhea. These patients received FOLFOX as the first-line therapy. Other patients with *UGT1A1*1/*1*, **1/*28* or **1/*6* and without the risk factors for irinotecan-related diarrhea received FOLFIRI as the first-line therapy.

First-Line FOLFIRI and FOLFOX Treatments

The FOLFIRI regimen comprised a 2-h intravenous infusion of irinotecan (150 or 180 mg/m^2) and LV (200 mg/m^2) on Day 1, followed by an intravenous bolus injection of 5-FU (400 mg/m^2) and a 46-h intravenous infusion of 5-FU (2400 mg/m^2), repeated every 2 weeks. The FOLFOX regimen comprised a 2-h intravenous infusion of oxaliplatin (85 mg/m^2) and LV (200 mg/m^2), followed by an intravenous bolus injection of 5-FU (400 mg/m^2) and a 46-h intravenous infusion of 5-FU (2400 mg/m^2), repeated every 2 weeks.

Second-Line Treatments

FOLFOX was given by the same method as the first-line treatment. Irinotecan monotherapy regimen comprised a 1.5-h intravenous infusion of irinotecan (150 mg/m^2), repeated every 2 weeks. S-1 was given per oral twice daily for 28 consecutive days, followed by 2 weeks of rest. The dose of S-1 was fixed based on the patients' body surface area (BSA) according to the manufacturer's package insert as distributed in Japan. The dose was 80 mg/day for patients with a BSA of <1.25 m^2, 100 mg/day for those with a BSA of 1.25–1.5 m^2 and 120 mg/day for those with a BSA of >1.5 m^2.

Evaluation of Efficacy and Toxicity

Toxicity was assessed according to the National Cancer Institute common terminology criteria for adverse events, version 3.0 (http://ctep.cancer.gov/reporting/ctc_v30.html). Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (http://www.recist.com/index.html) for at least 2 months by computed tomography imaging or ultrasonography. Efficacy was evaluated on the basis of the overall response rate and progression-free survival (PFS). PFS was defined as the date of starting treatment with FOLFIRI, FOLFOX or second-line chemotherapies to the date of disease progression as defined by the RECIST criteria or the date of death from any cause. The same imaging method was used for baseline tumor measurements and tumor reassessments. Total PFS was defined as the summation of PFSs in first- and second-line chemotherapies observed in respective patients. When patients did not receive second-line chemotherapy, the total PFS was equal to the PFS in first-line chemotherapy.

UGT1A1 Genotyping

Genomic DNA was extracted from 200 μl of peripheral blood, which had been stored at −80°C until analysis, with the use of a QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany). Two polymorphisms [G71R (**6*) and P229Q (**27*)] were analyzed by the polymerase chain reaction-restriction fragment length polymorphism method, as described elsewhere (14). The TATA box polymorphism (**28*) was determined by the direct sequencing method, as described by Fujita *et al.* (14).

Statistical Analysis

Total PFS was calculated by the Kaplan–Meier method. The analysis was conducted using JMP version 6 software (SAS Institute, Inc., Cary, NC).

Assessment of Feasibility of the Present Regimen Selection

The feasibility of the selection strategy for first-line FOLFIRI or FOLFOX was assessed by toxicity and efficacy in all patients: (1) Frequencies of typical toxicity(ies) for first-line FOLFIRI and FOLFOX, and the second-line chemotherapies were equal to or less than those observed in representative previous studies; (2) Total PFS in first- and subsequent second-line chemotherapies observed in all patients studied was almost equal to that in representative previous studies.

RESULTS

Patient Characteristics

A total of 112 patients with advanced colorectal cancer received first-line chemotherapy from June 2003 through April 2008. Chemotherapeutic regimens given to all of the patients are shown in Table 1. First-line FOLFIRI was given to 61 patients and FOLFOX to 26. These 87 patients were studied. Patient characteristics are shown in Table 2. Six patients received first-line FOLFOX based on *UGT1A1* genotypes and 20 patients received FOLFOX according to their physical conditions to avoid irinotecan-induced toxicity.

Table 1. First-line chemotherapy performed for advanced colorectal cancer patients in our institute from June 2003 to April 2008

Regimen	Number of patients ($n = 112$)	%
FOLFIRI	61	54
FOLFOX	26	23
5-FU/LV	8	7
Irinotecan monotherapy	5	5
FOLFOXIRI	4	4
IFL	4	4
S-1	4	4

FOLFIRI, irinotecan plus 5-fluorouracil and leucovorin; FOLFOX, oxaliplatin plus 5-fluorouracil and leucovorin; 5-FU/LV, 5-fluorouracil plus leucovorin; FOLFOXIRI, oxaliplatin plus iriontecan plus 5-fluorouracil and leucovorin; IFL, irinotecan plus 5-fluorouracil and leucovorin.

Table 2. Patient characteristics

	FOLFIRI ($n = 61$)	FOLFOX ($n = 26$)
Gender, *n* (%)		
Male	38 (62)	12 (46)
Female	23 (38)	14 (54)
Age (years)		
Median (range)	59 (39–74)	62 (38–79)
ECOG PS, *n* (%)		
0	38 (62)	15 (58)
1	23 (38)	11 (42)
Total bilirubin level (mg/dl)		
Median (range)	0.5 (0.2–1.1)	0.6 (0.3–1.4)
Serum creatinine level (mg/dl)		
Median (range)	0.64 (0.39–1.27)	0.77 (0.41–1.54)
Primary tumor site, *n* (%)		
Colon	51 (84)	18 (69)
Rectum	10 (16)	8 (31)
UGT1A1 genotype, *n* (%)		
**1/*1*	43 (70)	9 (34)
**1/*6*	16 (26)	8 (30)
**1/*28*	2 (4)	3 (12)
**6/*6*	0 (0)	3 (12)
**28/*6*	0 (0)	1 (4)
**28/*27*	0 (0)	2 (8)
Patients assigned to FOLFOX		
UGT1A1 genotype		6 (23)
Peritoneal dissemination		15 (58)
Diarrhea		5 (19)

ECOG, Eastern Cooperative Oncology Group; PS, performance status. *UGT1A1, UDP-glucuronosyltransferase 1A1.*

Toxicity in First-line Treatments

The main adverse events associated with first-line FOLFIRI or FOLFOX are presented in Table 3. In FOLFIRI, Grade 4 neutropenia occurred in 5 (8.2%) patients. Febrile neutropenia and Grade 3 diarrhea were seen in 2 (3.3%) patients. In FOLFOX, Grade 4 neutropenia occurred in 3 (11.5%) patients. Febrile neutropenia and Grade 3 thrombocytopenia were observed in one patient (3.8%). Grade 3 neuropathy occurred in 2 (7.7%) patients. However, no other Grade 3 or 4 non-hematological adverse events occurred in FOLFOX. No patient who harbored *UGT1A1*6/*6*, **28/*6* or **28/*27* receiving FOLFOX had Grade 4 neutropenia or other toxic effects of Grade 3 or higher. The discontinuation of FOLFIRI or FOLFOX due to toxicity were 3 (4.9%) and 5 (19%) patients, respectively. There were no treatment-related deaths in both groups.

Efficacy in First-line Chemotherapies

The efficacy of first-line FOLFIRI or FOLFOX was evaluated on the basis of the overall response rate and PFS (Table 4). The overall response rates were 43% in FOLFIRI and 46% in FOLFOX (Table 3). Median PFS was 7.5 months in FOLFIRI and was 8.7 months in FOLFOX. The median number of FOLFIRI and FOLFOX treatments were 7.0 (range of 1–38) and 6.5 (range of 1–18), respectively.

Second-line Chemotherapies

Among the patients who received first-line FOLFIRI, 38 patients (62%) received second-line FOLFOX and 4 (7%) S-1. The remaining 19 (31%) did not receive any second-line chemotherapies (10 others including surgery or radiotherapy and 9 best supportive care). In second-line FOLFOX, no Grade 4 or febrile neutropenia was observed. Grade 3

Table 3. Toxicity in patients treated with first-line FOLFIRI or FOLFOX

		FOLFIRI ($n = 61$)				FOLFOX ($n = 26$)			
Toxicity	Grade	1	2	3	4	1	2	3	4
		n	*n*	*n*	*n*	*n*	*n*	*n*	*n*
Neutropenia		4	14	12	5	0	5	9	3
Febrileneutropenia		0	0	2	0	0	0	1	0
Anemia		37	7	2	0	4	10	0	0
Thrombocytopenia		6	0	0	0	12	1	1	0
Nausea		31	14	3	0	15	1	0	0
Vomiting		19	6	3	0	6	1	0	0
Diarrhea		8	6	2	0	4	1	0	0
Neuropathy		3	0	0	0	19	3	2	0
Hypersensitivity		0	0	0	0	2	1	0	0

Table 4. Response rate and progression-free survival in patients treated with first-line FOLFIRI or FOLFOX

	FOLFIRI ($n = 61$)	FOLFOX ($n = 26$)
	n (%)	*n* (%)
Response		
CR	0 (0)	0 (0)
PR	26 (43)	12 (46)
SD	20 (33)	12 (46)
PD	10 (16)	1 (4)
NE	5 (8)	1 (4)
Overall response rate		
% of patients	43	46
Progression-free survival		
Median (months)	7.5	8.7
Range	0.9–20.0	1.5–28.3

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

anemia and diarrhea occurred in respective one patient (2.6%). However, no other Grade 3 or 4 adverse events occurred. The overall response rate and median PFS in the second-line FOLFOX were 13% and 5.4 months, respectively. In second-line S-1, Grade 3 anemia occurred in one patient. No other Grade 3 or 4 adverse events occurred. The overall response rate and median PFS in second-line S-1 were 0% and 0.8 months, respectively.

In patients treated with first-line FOLFOX, 11 patients (42%) received second-line irinotecan monotherapy, 5 (19%) S-1 and 10 (38%) best supportive care. In second-line irinotecan monotherapy group, Grade 4 or febrile neutropenia was observed in respective 2 patients (18%). Grade 3 anemia and Grade 3 thrombocytopenia occurred in 2 (18%) and 1 patient (9.1%), respectively. Grade 3 diarrhea occurred in 1 patient (9.1%). The overall response rate and median PFS in the second-line irinotecan monotherapy were 0% and 2.0 months, respectively. In the second-line S-1, Grade 3 anemia occurred in one patient. No other Grade 3 or 4 adverse events were observed. The overall response rate and median PFS in second-line S-1 were 0% and 1.5 months, respectively.

Among six patients with *UGT1A1*6/*6*, **28/*6* or **28/*27* who received first-line FOLFOX, three received second-line irinotecan monotherapy, one was given S-1 and others received best supportive care. Irinotecan therapy was started with the standard dose of 150 mg/m^2 in Japan, because there has been no information regarding the optimal reduced dose of irinotecan for patients possessing these *UGT1A1* genotypes. Among three patients given second-line irinotecan monotherapy, two patients experienced respective Grade 3 or 4 neutropenia and one patient Grade 3 diarrhea. The irinotecan doses in these patients for the next courses were reduced by the physicians in charge.

Total PFS in First- and Second-line Therapies in All Patients Examined

The median total PFS in all 87 patients studied was 11.5 months (Fig. 2).

DISCUSSION

This is the first study to select the first-line FOLFIRI or FOLFOX regimen by considering *UGT1A1* genetic testing in addition to physical conditions in patients with advanced colorectal cancer. The feasibility of this strategy was evaluated as follows:

1. The toxicities observed during the all first- and second-line chemotherapies were compared with those observed in representative studies.

In patients treated with first-line FOLFIRI, the frequency of Grade 4 neutropenia was slightly lower than that previously reported (9%) (4, 5, 15). The frequencies of febrile neutropenia and Grade 3 diarrhea were lower than those reported previously (febrile neutropenia, 7% and Grade 3–4 diarrhea, 14%) (4, 5, 15). The patient selection for FOLFIRI adopted in the present strategy appears to be effective to reduce the irinotecan-induced toxicities. In the first-line FOLFOX, the frequencies of Grade 4 neutropenia, Grade 3 thrombocytopenia and Grade 3 neuropathy were lower than those reported previously (Grade 4 neutropenia, 13%; Grade 3 thrombocytopenia, 5% and Grade 3 neuropathy, 34%) (4, 5, 15). Patients who were assigned to FOLFOX because of ascites, peritoneal dissemination and diarrhea did not suffer from Grade 3 or higher gastrointestinal adverse events such as nausea, vomiting and diarrhea, which were relatively often observed in FOLFIRI. Furthermore, no patient who harbored *UGT1A1*6/*6*, **28/*6* or **28/*27* receiving

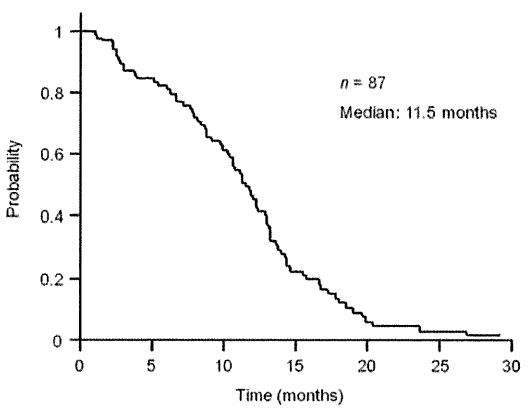

Figure 2. Kaplan–Meier analysis of total progression-free survival in all 87 patients.

FOLFOX had Grade 4 neutropenia or other toxic effects of Grade 3 or higher.

In second-line FOLFOX, the frequencies of Grade 3 or 4 neutropenia, Grade 3 thrombocytopenia and Grade 3 neuropathy were lower than those reported previously (Grade 4 neutropenia, 17%; Grade 3 thrombocytopenia, 1% and Grade 3 neuropathy, 20%) (5). The frequency of toxicities seen in the second-line irinotecan monotherapy was compared with that in the second-line FOLFIRI (5), since (i) there have been few studies of second-line irinotecan monotherapy with large number of patients and (ii) we can evaluate the toxicity more severely in second-line irinotecan monotherapy, because FOLFIRI is stronger than irinotecan monotherapy in terms of toxicity. The frequencies of Grade 3 or 4 neutropenia and febrile neutropenia seen in second-line irinotecan monotherapy were higher than those previously reported (Grade 3 or 4 neutropenia, 21% and febrile neutropenia, 1%) (5), while the frequency of Grade 3 diarrhea was similar to that reported previously (8%) (5). Because patients who had the risk factor for irinotecan-induced severe toxicity received second-line irinotecan monotherapy, frequencies of severe neutropenia and febrile neutropenia were higher than that previously reported. In second-line S-1, the frequency of Grade 3 anemia was similar to that reported in previous study (16). The frequencies of non-hematological toxicities such as nausea, diarrhea and mucositis were lower than those reported previously (16).

Collectively, the present regimen selection strategy appears to be feasible in terms of toxicities, except for the patients with risk for irinotecan-induced toxicity who received the second-line irinotecan monotherapy. Appropriate reduced dose should be determined and other chemotherapies without irinotecan should be developed for these patients.

2. The median total PFS in all 87 patients evaluated was 11.5 months (Fig. 2). We compared this clinical outcome during first- and second-line treatments with duration of disease control (DDC) used in OPTIMOX studies, which collected the data until second-line therapy (17, 18), since the definition of DDC is almost equal to that of our total PFS. In OPTIMOX studies, DDC was defined as PFS in first-line FOLFOX and maintenance with simplified 5-FU and LV regimen plus PFS of FOLFOX reintroduction (17, 18). The median total PFS of 11.5 months in our study was almost similar to that reported in OPTIMOX studies (10.6–13.1 months) (17, 18).

Taking these considerations into account, the regimen selection of the first-line FOLFIRI or FOLFOX therapy based on the *UGT1A1* genotyping in addition to patient physical conditions that are related to irinotecan-induced toxicity might be feasible, since it causes less toxicity and similar efficacy comparing to previous studies. Determination of appropriate reduced dose in second-line irinotecan monotherapy or other standard second-line therapy for patients with high risk to irinotecan-induced toxicity might make this strategy more effective.

Previous studies of first-line FOLFIRI therapy in patients with advanced colorectal cancer have reported the response rate of 31–56% and PFS of 8.5 months (4, 5, 15). First-line FOLFOX therapy showed the similar efficacy as FOLFIRI (response rate, 34–54% and PFS, 8.0 months) (5, 15, 19). In our study, the response rate and PFS in patients assigned to FOLFIRI or FOLFOX were comparable to those reported previously. It should be noted that the response rate and PFS [50% and 8.6 months (range of 2.5–15.2)] seen in patients who received FOLFOX because of harboring *UGT1A1*6/*6*, **28/*6* or **28/*27* genotype were not statistically significantly different from those observed in patients assigned to FOLFIRI.

To further confirm the present results, the prospective study involving larger numbers of patients should be planned to confirm our data, even though many patients are now treated with FOLFIRI or FOLFOX combined with monoclonal antibodies such as bevacizumab or cetuximab as the first-line therapy for advanced colorectal cancer in Japan (20–23).

At present, there has been no evidence whether or not the present strategy is applicable when FOLFIRI or FOLFOX are used in combination with bevacizumab or cetuximab. Further studies are necessary to confirm this point.

If the optimal reduced dose(s) of irinotecan can be determined for patients who have a high risk of irinotecan-induced neutropenia because of *UGT1A1*28/*28*, **6/*6*, **28/*6* or **28/*27* genotype, *UGT1A1* genotyping should become essential not only for regimen selection but also for dose decision-making.

In summary, our results demonstrate that the selection of first-line FOLFIRI or FOLFOX in patients with advanced colorectal cancer based on *UGT1A1* genotyping in addition to patient physical condition would be feasible, since it causes less toxicity and similar efficacy comparing to previous studies. Determination of appropriate reduced dose in second-line irinotecan monotherapy or other standard second-line therapy for patients with high risk to irinotecan-induced toxicity might make this strategy more effective. Severe irinotecan-induced neutropenia in first-line FOLFIRI was avoided in patients with *UGT1A1*28/*28*, **6/*6*, **28/*6* or **28/*27* by assigning these patients to first-line FOLFOX. This strategy of regimen selection for first-line FOLFIRI and FOLFOX might be feasible.

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Conflict of interest statement

None declared.

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Original Article

Comparison Between Intravesical and Oral Administration of 5-Aminolevulinic Acid in the Clinical Benefit of Photodynamic Diagnosis for Nonmuscle Invasive Bladder Cancer

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BACKGROUND: This study was undertaken to evaluate the clinical value of photodynamic diagnosis (PDD) with intravesical and oral instillation of 5-aminolevulinic acid (ALA) (ALA-PDD), and transurethral resection of bladder tumor (TURBT) guided by ALA-PDD (PDD-TURBT) for nonmuscle invasive bladder cancer. **METHODS:** Of all 210 cases, 75 underwent PDD with intravesically applied ALA, and 135 cases underwent PDD with orally applied ALA. Diagnostic accuracy was evaluated by comparing the level on images of ALA-induced fluorescence with the pathological result. PDD-TURBT was performed in 99 completely resectable cases corresponding to 210 ALA-PDD cases. To evaluate the abilities of PDD-TURBT, survival analysis regarding intravesical recurrence was retrospectively compared with the historical control cases that underwent conventional TURBT. **RESULTS:** The diagnostic accuracy and capability of ALA-PDD were significantly superior to those of conventional endoscopic examination. Moreover, 72.1% of flat lesions, including dysplasia and carcinoma in situ, could be detected only by ALA-PDD. The recurrence-free survival rate in the cases that underwent PDD-TURBT was significantly higher than that of conventional TURBT. Moreover, multivariate analysis revealed that the only independent factor contributing to improving prognosis was PDD-TURBT (hazard ratio, 0.578; $P = .012$). Regardless of the ALA administration route, there was no significant difference in diagnostic accuracy, ability of PDD, or recurrence-free survival. All procedures were well tolerated by all patients without any severe adverse events. **CONCLUSIONS:** This multicenter study is likely to be biased, because it is limited by the retrospective analysis. This study suggests that regardless of the ALA administration route, ALA-PDD and PDD-TURBT are remarkably helpful in detection and intraoperative navigation programs. ***Cancer* 2012;118:1062-74.** © *2011 American Cancer Society.*

KEYWORDS: photodynamic diagnosis, 5-aminolevulinic acid, nonmuscle invasive bladder cancer, administration route, intravesical recurrence.

INTRODUCTION

Bladder cancer is the second most common genitourinary neoplasm, with more than 60,000 and 120,000 new cases diagnosed each year in the United States and Europe, respectively.[1,2] In Japan, about 16,000 new cases are diagnosed and 50,000 endoscopic surgeries are performed each year.[3] The standard therapy for nonmuscle invasive cancer, accounting for approximately 70% bladder cancer, is transurethral resection of bladder tumor (TURBT).[4] TURBT enables a high quality of life, with preservation of the bladder and a good prognosis. However, TURBT results in frequent residual tumor, resulting in frequent subsequent intravesical recurrence in the early postoperative period. The high recurrence rate is attributed to residual lesions, such as minute lesions, flat lesions, and concomitant flat lesions with raised lesions.

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In particular, flat tumors, such as carcinoma in situ (CIS) and dysplasia, are difficult to detect accurately by cystoscopy; thus, it is no exaggeration to say that they are endoscopically invisible lesions.

5-Aminolevulinic acid (ALA) has received much attention as a new-generation photo-sensitive substance for photodynamic diagnosis (PDD) in recent years. ALA is an endogenous natural amino acid, and a common precursor of chlorophyll in plants and bilirubin in animals. The administered photosensitive substance, ALA, is incorporated by cells and synthesized into a fluorescent substance, protoporphyrin IX. In various cancer cells, this protoporphyrin IX biosynthesis pathway is promoted, whereas the protoporphyrin IX-metabolizing pathway is inhibited, resulting in the excess accumulation of protoporphyrin IX in cancer cells,[5,6] and the tumor selectivity is 17:1 in the urothelium, which is particularly high.[7] Because protoporphyrin IX exhibits photoactivity, when protoporphyrin IX is excited by irradiation with a specific wavelength of light, mainly visible blue light (375-445 nm), it emits red fluorescence. Cancer cells can be accurately identified by detecting this fluorescence.[8] This is the mechanism of PDD using ALA. This means that PDD mediated by ALA (ALA-PDD) is the most advanced photodynamic technology based on the fundamental biological profile of cancer cells, providing good visualization and precise detection of the lesions, leading to improved surgical curability and subsequent prognosis in various cancers, including bladder cancer.

Recently, orally applied ALA was approved as an optical imaging agent to enhance intraoperative detection of malignant glioma in Europe.[9] Moreover, the hexyl ester derivative of 5-ALA (hexaminolevulinate), which was applied intravesically, was approved as an optical imaging agent to enhance intraoperative detection of bladder cancer, in particular CIS in Europe and the United States.[9] Since then, excellent results have been reported for with hexaminolevulinate in the diagnostic accuracy[10-17] and TURBT guided by PDD with hexaminolevulinate (PDD-TURBT) in the prognosis[18-22] of nonmuscle invasive bladder cancer. Several prospective, randomized, multicenter studies have recently shown the contribution of PDD-TURBT to the improvement in outcome,[23,24] but there are still some points to discuss.[25] Moreover, it was demonstrated in the retrospective series that, although PDD with ALA and hexaminolevulinate applied intravesically was demonstrated to be significantly superior to white light cystoscopy, there were no significant differences between ALA and hexaminolevulinate in clinical outcome such as residual tumor and recurrence-free survival.[26] Thus, in this study, we retrospectively evaluated the value of ALA-PDD and also PDD-TURBT, and whether the differences depending on the ALA administration route affect the diagnostic accuracy, ability, and recurrence-free survival in nonmuscle invasive bladder cancer.

MATERIALS AND METHODS

Patients

PDD with intravesical instillation of ALA was approved by the ethics committees of Kochi Medical School in September 2004, and PDD with oral instillation of ALA was approved in January 2007.

In this study, all patients who were candidates for transurethral biopsy of the bladder or TURBT were enrolled after providing written informed consent in the Department of Urology of Kochi Medical School Hospital, Kochi National Hospital and Chikamori Hospital between September 2004 and August 2010. All patients were informed about the potential efficacy and also adverse events of ALA-PDD, for example, bladder irritability, such as urinary frequency and urgency, and systemic response, such as skin photosensitivity, transient elevation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), nausea, and vomiting in conformity with the Common Terminology Criteria for Adverse Events version 3.0.[27]

ALA-PDD was performed in 210 patients, of whom 172 were men and 38 were women, with a median age of 70.6 (range, 44-90) years; 98 were primary cases, and 112 were recurrent cases of bladder cancer. There was no statistically significant difference in these background factors between 75 cases of intravesical administration of ALA and 135 cases of oral administration of ALA (Table 1).

PDD-TURBT was also performed in 99 completely resectable cases corresponding to 210 PDD cases of bladder cancer, of whom 80 were men and 19 were women, with a median age of 69.8 (range, 46-90) years; 58 were primary cases, and 41 were recurrent cases of nonmuscle invasive bladder cancer. To evaluate the abilities of PDD-TURBT, survival analysis regarding vesical recurrence was retrospectively examined compared with historical control cases that had undergone conventional TURBT under white light guidance. There was no significant difference in any variables of patient characteristics between fluorescence TURBT and conventional TURBT (Table 2).

The patient characteristics of 32 cases of intravesical administration of ALA and 67 cases of oral administration

Table 1. Patient Characteristics in Photodynamic Diagnosis

Variable	All Cases	ALA Administration		
		Intravesical	Oral	*P*
Patients	210	75	135	
Examination period	September 2004-August 2010	September 2004-August 2010	January 2007-August 2010	
Age, y				.894[a]
Mean	70.6	71.2	70.3	
Range	44-90	44-88	45-90	
Sex				.979[a]
Men	172	62	110	
Women	38	13	25	
Past history				.852[a]
Primary case	149	55	94	
Recurrent case	61	20	41	
Prior therapy				.061[a]
TURBT	65	18	47	
TURBT+BCG	27	2	25	
Tumor stage				.154[b]
Normal	30	19	11	
pT				
is	47	13	34	
a (+CIS)	101 (29)	26 (5)	75 (24)	
1 (+CIS)	65 (18)	27 (8)	28 (10)	

Abbreviations: ALA, 5-aminolevulinic acid; BCG, Bacillus Calmette-Guerin; CIS, carcinoma in situ; TURBT, transurethral resection of bladder tumor.
Patient characteristics, including number of patients, examination period, age, sex, past history, prior therapy and tumor stage, are shown in all 210 cases. There was no statistical significance in these background factors between 75 cases of intravesical administration of ALA and 135 cases of oral administration of ALA.
[a] Fisher exact test (2 × 2).
[b] Chi-square test.

of ALA are shown in Table 3. The presence of concomitant CIS and high tumor grade in fluorescence TURBT with oral administration of ALA was statistically greater than in fluorescence TURBT with intravesical administration of ALA.

Administration of ALA

For PDD, we used ALA as a photosensitizer. ALA hydrochloride (Cosmo Bio Co., Tokyo, Japan) was dissolved in 50 mL of 5% glucose solution, and 8.4% sodium hydrogen carbonate ($NaHCO_3$) solution was added to adjust to pH 7.8 to 8.0. In 210 patients, 75 cases underwent PDD with intravesical instillation of 1.5 g ALA 1.5 hours before endoscopic examination, and 135 cases underwent PDD with oral instillation of 1.0 g ALA 3.0 hours before endoscopic examination.

PDD system

For ALA-PDD, a D-LIGHT System (Karl Storz GmbH & Co., Tuttlingen, Germany), including D-Light C, CCU Tricam SLII/3CCD CH Tricam-P PDD, and a HOPKINSII PDD telescope (30°), was used. The light source, D-Light C (300 W xenon arc lamp), is equipped with a band-pass filter that is designed to transmit blue light (excitation wavelength, 375-445 nm) (for excitation of fluorescence). The video camera system, CCU Tricam SLII/3CCD CH Tricam-P PDD, is equipped with a long-pass filter that is designed to cutoff blue light (for observation of fluorescence; fluorescence emission wavelength, 600-740 nm). This PDD system has the advantage that it can instantly switch between blue light mode for fluorescent observation and white light mode for conventional observation.

Examination Procedure

Under conventional white light and fluorescence light guidance, tumor locations were recorded and cold cup biopsies were taken. If cases were endoscopically completely resectable, tumorous lesions under white light guidance and lesions with fluorescent excitation under

Table 2. Patient Characteristics in Fluorescence TURBT and Conventional TURBT

Variable[32]	Fluorescence TURBT	Conventional TURBT	*P*
Patients	99	99	
Examination period	September 2004-August 2010	May 1982-July 2008	
Age, y			.399[a]
Mean	69.8	71.7	
Range	46-90	36-92	
Sex			.100[a]
Men	80	69	
Women	19	30	
Tumors, No.			.420[b]
Single	46	52	
2-7	48	45	
≥8	5	2	
Tumor size			.510[a]
<3 cm	89	85	
≥3 cm	10	14	
Prior recurrence			.160[b]
Primary	58	58	
≤1 rec/y	32	24	
>1 rec/y	9	17	
T category			.100[a]
Ta	69	57	
T1	30	42	
Concomitant CIS			.260[a]
No	78	85	
Yes	21	14	
Tumor grade			.400[b]
G1	5	10	
G2	59	55	
G3	35	34	
Prior therapy			.820[a]
TURBT	26	25	
TURBT+BCG	15	16	
Adjuvant therapy			.120[a]
None	79	88	
Intravesical BCG	20	11	

Abbreviations: BCG, Bacillus Calmette-Guerin; CIS, carcinoma in situ; rec, recurrence; TURBT, transurethral resection of bladder tumor.

Patient characteristics including number of patients, examination period, age, sex, past history, prior therapy, adjuvant therapy, and the factors based on the European Organization for Research and Treatment of Cancer risk tables[32] are shown for 99 cases of fluorescence TURBT and 99 cases of conventional TURBT. There was no significant difference in any variables of patient characteristics between fluorescence TURBT and conventional TURBT.

[a] Fisher exact test (2 × 2).

[b] Chi-square test.

blue light (fluorescence) guidance were resected sequentially. First, biopsy using a cold cup was performed. After conventional systematic biopsy, specimens of the vesical mucosa emitting right fluorescence or with an abnormality under the white light source were collected from 8 vesical regions (neck of the urinary bladder, triangular region, posterior, left, and right walls, apex, anterior wall, and prostatic region of the urethra). When a tumor occupied these regions, the tumor tissue was collected. The specimens were categorized and recorded by fluorescence intensity-based evaluation using the blue light mode and macroscopic malignancy evaluation using the conventional white light mode. In the evaluation using blue light mode, the samples were evaluated by roughly dividing them into 3 categories following the semiquantitative macroscopic diagnostic method of red fluorescence

Table 3. Patient Characteristics in Fluorescence TURBT With Intravesically Applied ALA and Orally Applied ALA

Variable[32]	Intravesical ALA	Oral ALA	*P*
Patients	32	67	
Examination period	October 2004-December 2007	March 2007-August 2010	
Age, y			.896[a]
Mean	73.3	68.1	
Range	46-87	49-90	
Sex			.370[a]
Male	27	53	
Female	5	14	
Tumors, No.			.454[b]
Single	13	34	
2-7	18	29	
≥8	1	4	
Tumor size			.412[a]
<3 cm	28	61	
≥3 cm	4	6	
Prior recurrence			.160[b]
Primary	19	39	
≤1 rec/y	11	21	
>1 rec/y	2	7	
T category			.199[a]
Ta	10	49	
T1	12	18	
Concomitant CIS			.038[a]
No	29	49	
Yes	3	18	
Tumor grade			.005[b]
G1	4	1	
G2	19	40	
G3	9	26	
Prior therapy			.566[a]
TURBT	8	18	
TURBT+BCG	5	10	
Adjuvant therapy			.146[a]
None	28	51	
Intravesical BCG	4	16	

Abbreviations: ALA, 5-aminolevulinic acid; BCG, Bacillus Calmette-Guerin; CIS, carcinoma in situ; rec, recurrence; TURBT, transurethral resection of bladder tumor.
Patient characteristics including number of patients, examination period, age, sex, past history, prior therapy, adjuvant therapy, and the factors based on the European Organization for Research and Treatment of Cancer risk tables[32] are shown for 32 cases of intravesical administration of ALA and 67 cases of oral administration of ALA. The presence of concomitant CIS and high tumor grade in fluorescence TURBT with oral administration of ALA is statistically greater than that in fluorescence TURBT with intravesical administration of ALA.
[a] Fisher exact test (2 × 2).
[b] Chi-square test.

emission used in a clinical study on brain tumors performed by Miyoshi et al[28]: none (no fluorescence emission), weak (weak fluorescence emission), and strong (strong fluorescence emission), based on the red fluorescence intensity. In the evaluation using the conventional white light mode, samples were evaluated by roughly dividing them into 3 categories based on comprehensive macroscopic malignancy in consideration of important features such as the mucosal properties and concentration of blood vessels: none (no abnormal finding), weak (mild abnormality with difficulty in judging benignity or malignancy), and strong (marked abnormality with a high