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.

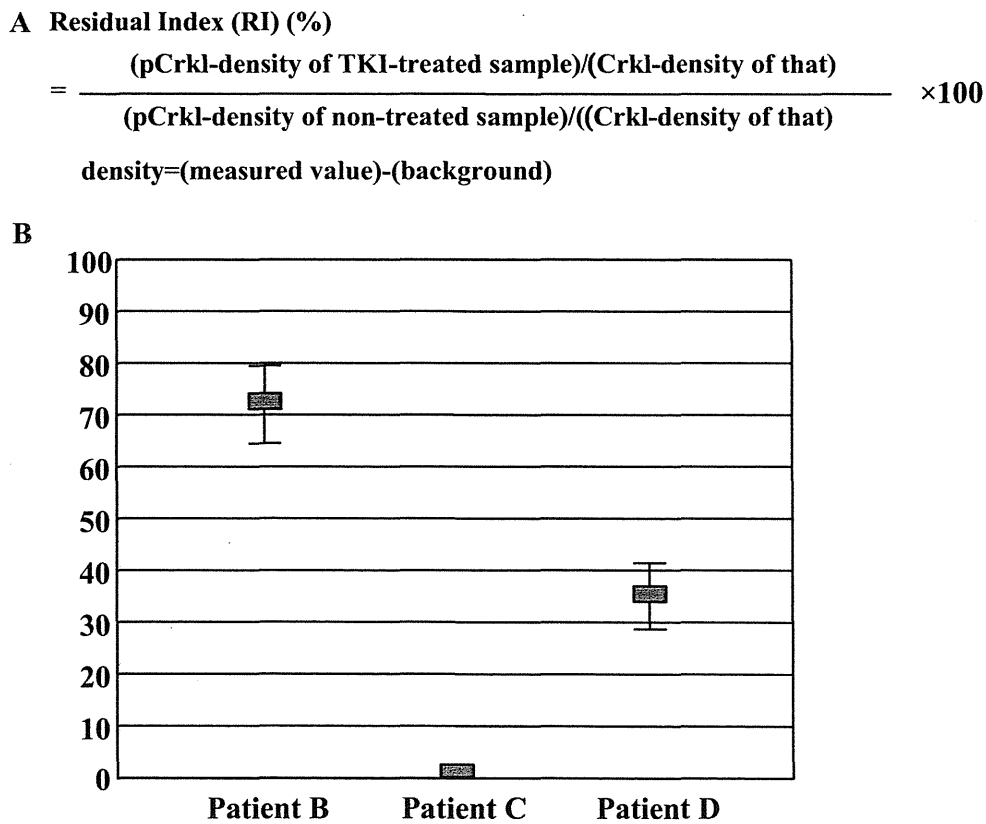

Fig. 2. "Residual index (RI)". (A) The numerical expression of RI. "Measured value" means the density of each blot measured by densitometric method. (B) The reproducibility of RIs for imatinib treatment. Means and standard errors, representing triplicate assays in 3 patients, are shown.

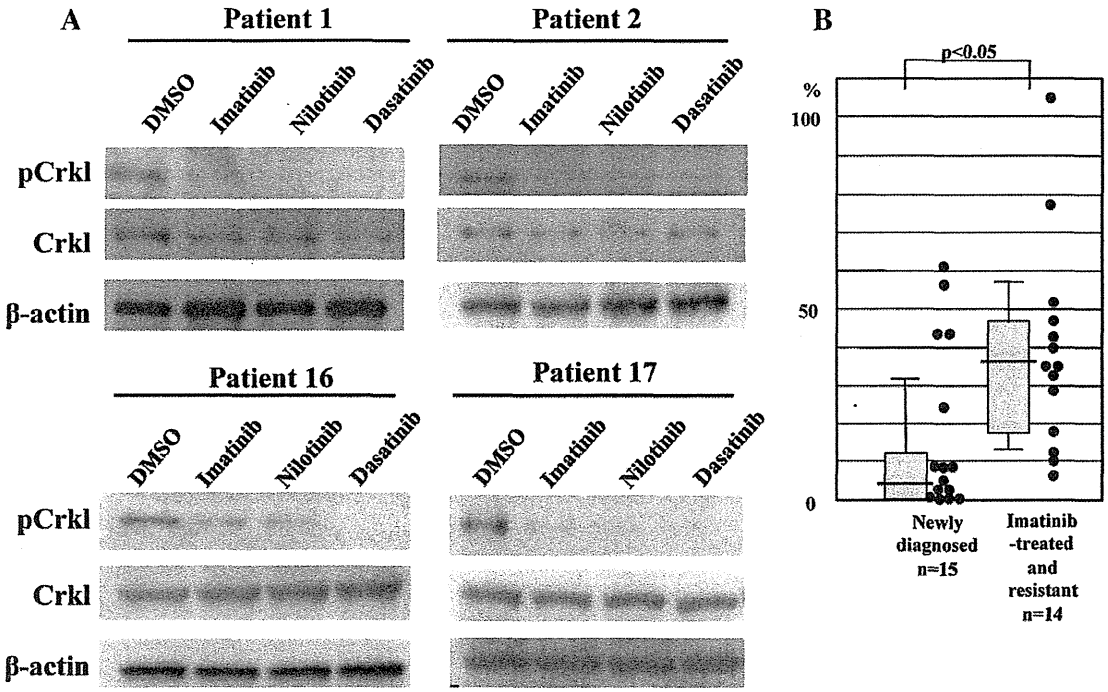

Fig. 3. Different RI values against imatinib between patients at diagnosis and patients showing imatinib-resistance. (A) Four typical data of immunoblots were represented. PB cells from newly diagnosed patients (Patient 1 and 2) or patients (Patient 16 and 17) who had been receiving imatinib-therapy 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

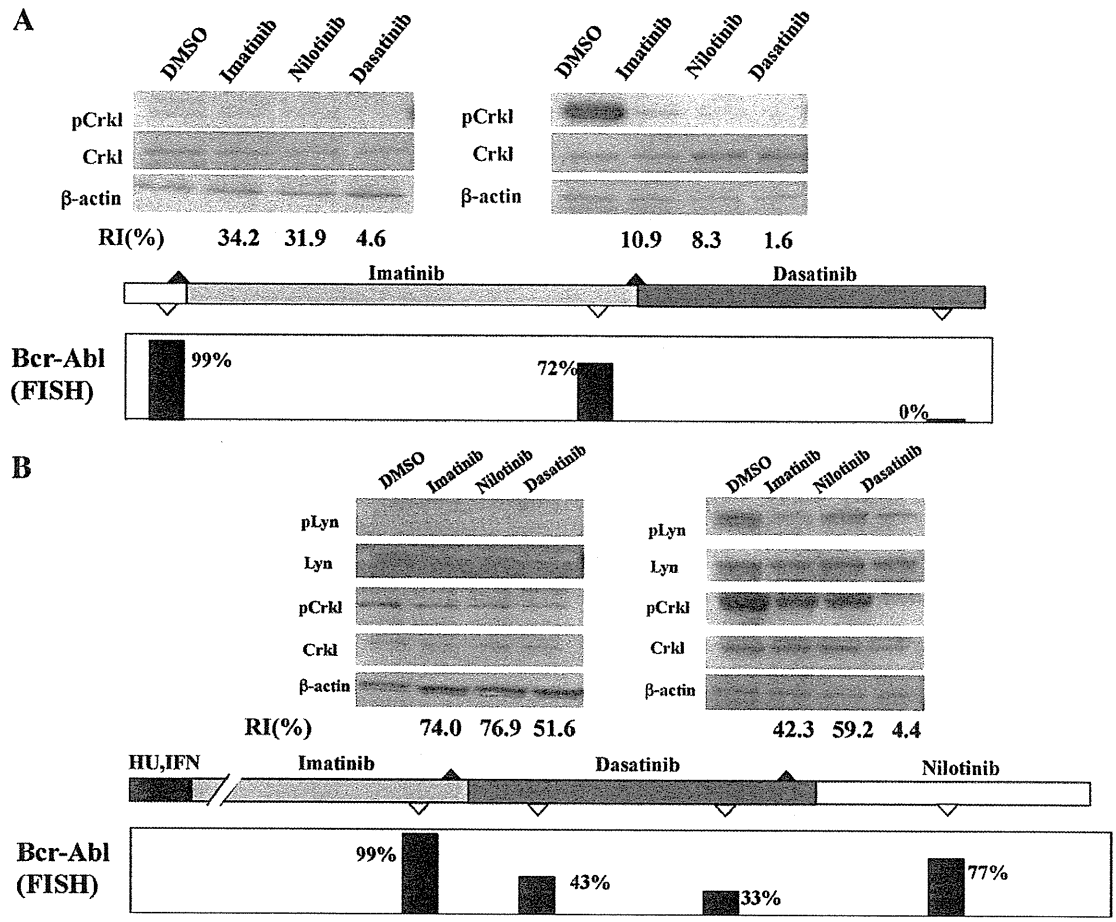

Fig. 4. Sequential examinations of RI values during clinical treatments in two patients. Immunoblots were sequentially analyzed during CML-treatment in two patients who showed resistance to TKIs. Data of immunoblots using the indicated antibodies are shown with their clinical course. FISH analyses are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

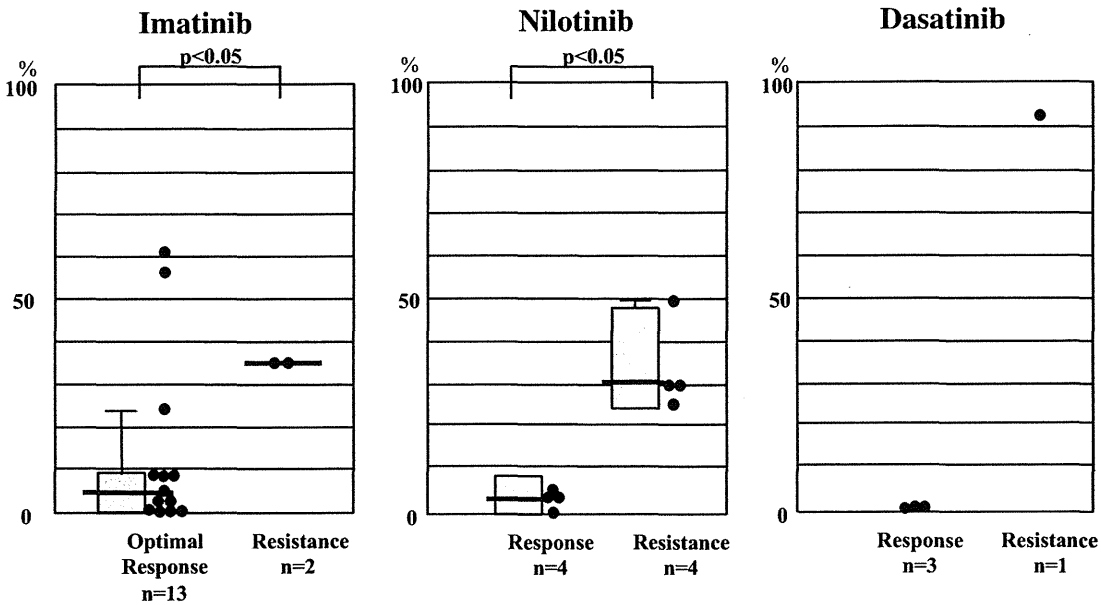

Fig. 5. RI values in patients grouped by clinical response to each TKI-therapy. Fifteen patients were newly diagnosed as CML, and their PB cells were obtained just before the beginning of imatinib-therapy. The patients were divided into two groups: "optimal response" in imatinib-treated patients means *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.

Acknowledgements

We would like to thank Shibano M. (Sakai Munincipal Hospital, Sakai, Japan), Sugahara H. (Sumitomo Hospital, Osaka, Japan), Moriyama Y. (Ikeda City Hospital, Ikeda, Japan), Azenishi Y. (Minoh City Hospital, Minoh, Japan), Ishida N. (Itami City Hospital, Itami, Japan), and Yamada M. (Suita Munincipal Hospital, Suita, Japan), who kindly provided blood samples.

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Clinical Study

Oncology

Oncology 2012;82:242–248
DOI: 10.1159/000337225

Received: December 22, 2011
Accepted after revision: February 2, 2012
Published online: April 12, 2012

A Phase I Study of Infusional 5-Fluorouracil, Leucovorin, Oxaliplatin and Irinotecan in Japanese Patients with Advanced Colorectal Cancer Who Harbor *UGT1A1*1/*1, *1/*6* or **1/*28*

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Key Words
FOLFOXIRI · UDP-glucuronosyltransferase 1A1 · Phase 1 study · Irinotecan · Oxaliplatin · Colorectal cancer · Combination chemotherapy

Abstract
Objective: To evaluate the safety and efficacy of combination chemotherapy with 5-fluorouracil (5-FU), leucovorin, irinotecan and oxaliplatin (FOLFOXIRI) in Japanese patients with advanced colorectal cancer. ***Methods:*** This phase I dose-finding study was designed to determine the maximum tolerated dose (MTD), recommended dose (RD) or both of FOLFOXIRI. Patients with *UDP-glucuronosyltransferase (UGT) 1A1*6/*6, *28/*28* and **6/*28* genotypes were excluded, because these *UGT1A1* genotypes are linked to severe neutropenia in Japanese. ***Results:*** A total of 10 Japanese patients with advanced colorectal cancer were studied. The MTD of FOLFOXIRI in these Japanese patients was 165 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU. Accordingly, the RD of FOLFOXIRI was determined to be 150 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU. Toxic effects, evaluated until the completion of 4 cycles, were manageable. Grade 3–4 neutropenia occurred in 27% of cycles, but there was no febrile neutropenia. Among the 9 assessable patients, the objective response rate was 89%. ***Conclusions:*** We thus determined the RD of FOLFOXIRI in Japanese patients with advanced colorectal cancer who do not have *UGT1A1*28/*28, *6/*6* or **6/*28* genotypes. Our results indicate that FOLFOXIRI is a well-tolerated regimen for these Japanese patients.

Introduction

The key cytotoxic antitumor drugs for colorectal cancer are 5-fluorouracil (5-FU), irinotecan and oxaliplatin. Exposure to all three of these active cytotoxic drugs during the course of treatment has been associated with longer overall survival (OS) [1, 2]. However, several studies have demonstrated that only 60–80% of patients can receive second-line treatments in sequential strategies and therefore are not exposed to all three agents [1]. These fac-

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tors have fostered attempts to develop potentially more active first-line regimens combining 5-FU with both irinotecan and oxaliplatin.

A combination of 5-FU with leucovorin (LV), irinotecan and oxaliplatin (FOLFOXIRI) has been demonstrated to be more effective than 5-FU-LV and irinotecan (FOLFIRI) in randomized clinical trials performed in Western countries [3, 4], suggesting that first-line treatment with FOLFOXIRI might improve survival in metastatic colorectal cancer. FOLFOXIRI has produced a higher response rate (RR) of 66% and longer OS of 23.4 months than any other regimen evaluated in randomized studies of metastatic colorectal cancer reported to date [5–10]. FOLFOXIRI might also thus facilitate the radical surgical resection of metastases initially considered unresectable [11, 12]. The FOLFOXIRI regimen is considered an initial therapy for advanced colorectal cancer and is included in the National Comprehensive Cancer Network guidelines version 2.2012. However, no study has reported on the safety and efficacy of FOLFOXIRI in Japanese patients with advanced colorectal cancer.

Polymorphisms of the *UDP-glucuronosyltransferase (UGT) 1A1* gene have been established to underlie irinotecan-related toxicity [13, 14]. Asians have a lower allele frequency of *UGT1A1*28* than Whites, although this polymorphism is seen in both ethnic groups (16% in Asians and 39% in Whites) [15]. In contrast, a single-nucleotide polymorphism in exon 1 of the *UGT1A1* gene, *UGT1A1*6*, which is related to decreased catalytic activity for SN-38 glucuronidation [16], occurs at a relatively high allele frequency in Asians (approximately 20%), but not in Whites [17–19]. Since *UGT1A1*28* and **6* are separately located on two different alleles, *UGT1A1*6/*6*, **28/*28* and **6/*28* genotypes are found in Japanese patients with cancer at a frequency of approximately 10% [17]. These *UGT1A1* genotypes have been linked to severe neutropenia [18, 19]. Because FOLFOXIRI is a potent regimen with a relatively high frequency of grade 3–4 neutropenia, occurring in 35–50% of patients [3, 4], it might be prudent to exclude patients with these *UGT1A1* genotypes to avoid irinotecan-related severe neutropenia. To gain further insight into these issues, we performed a dose-finding phase I study to assess the safety and efficacy of FOLFOXIRI in Japanese patients with advanced colorectal cancer who harbor *UGT1A1*1/*1*, **1/*6* or **1/*28*. The primary endpoint of our phase I study was to determine the maximum tolerated dose (MTD), the recommended dose (RD) or both. The secondary endpoint was to clarify the objective RR.

Table 1. Dose adaptation schedule

Level	Irinotecan mg/m^2	Oxaliplatin mg/m^2	5-FU mg/m^2
2	180	85	3,200
1	165	85	3,200
0	165	85	2,400
–1	150	85	2,400
–2	120	85	2,400

Patients and Methods

Patient Selection

Enrolled patients were required to meet the following eligibility criteria: histologically confirmed adenocarcinoma of the colon or rectum, unresectable recurrent or metastatic disease, age 20–70 years, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, measurable disease according to the Response Evaluation Criteria in Solid Tumors, version 1.0 [20], leukocyte count ≥3,500/mm^3, neutrophil count ≥1,500/mm^3, platelet count ≥100,000/mm^3, serum creatinine ≤1.5 mg/dl, serum bilirubin ≤1.2 mg/dl and serum aspartate aminotransferase and alanine aminotransferase ≤5 times the respective upper limits of normal. Previous adjuvant or palliative chemotherapy with 5-FU with or without LV was allowed. Exclusion criteria were previous chemotherapy with irinotecan or oxaliplatin, symptomatic cardiac disease, myocardial infarction, uncontrolled arrhythmias, active infections and inflammatory bowel disease. Patients with *UGT1A1*6/*6*, **28/*28* or **6/*28* genotypes were excluded. The study was approved by the Institutional Review Board of the Saitama Medical University, and patients were informed of the investigational nature of the study and provided their written informed consent before registration (trial registration ID: UMIN000000883).

Dose Adaptation Schedule

The dose adaptation schedule is shown in table 1. If the MTD was not reached at the initial dose level (level 0: 165 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2400 mg/m^2 5-FU), the next group of patients received an escalated dose level (level 1: 165 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 3,200 mg/m^2 5-FU). If the MTD was also not reached at level 1, subsequent patients received level 2 (180 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 3,200 mg/m^2 5-FU). If the MTD was not reached at level 2, no further dose escalations of any drugs were planned because the respective doses of irinotecan and oxaliplatin at level 2 were approximately their RDs as single agents or in combination with 5-FU. This level was defined to be the RD. If the MTD was reached at level 0, the dose could be reduced down to level –2 (level –1: 150 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU; level –2: 120 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU). If level –2 was the MTD, the study was concluded.

Dose-Limiting Toxicity and MTD

Dose-limiting toxicity (DLT) was defined as any grade 3 or 4 nonhematologic toxicity, except for nausea, vomiting, anorexia,

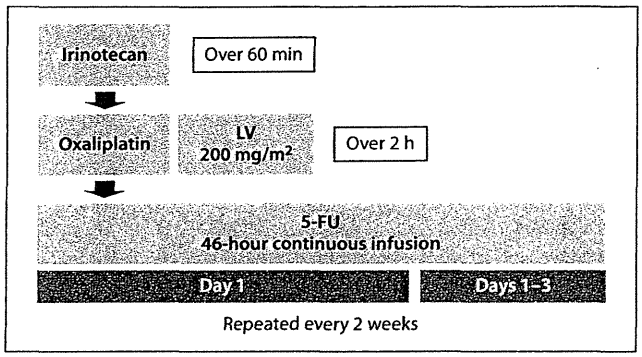

Fig. 1. Treatment schedule for FOLFOXIRI.

fatigue and constipation, any grade 4 neutropenia lasting more than 4 days or associated with fever (≥37.5°C) and any grade 4 thrombocytopenia or grade 3 bleeding tendency in the first cycle of treatment, as defined by the National Cancer Institute Common Toxicity Criteria, version 3.0. DLT was also defined as toxicity precluding administration of the fourth cycle of treatment within 9 weeks from day 1 of cycle 1, or toxicity precluding administration of the next cycle of treatment within 4 weeks from the completion of the previous cycle. If DLT occurred in 1 of the first 3 patients assigned to a given dose level, 3 additional patients were assigned to the same dose level. The MTD was defined as the dose level associated with DLT in at least 2 of 3 or 2 of 6 patients. The RD was defined as the dose level one rank below the MTD.

Treatment Plan

Irinotecan in 5% dextrose (250 ml) was administered over the course of 60 min and was followed immediately by a concomitant infusion of oxaliplatin in 5% dextrose (250 ml) and 200 mg/m^2 LV in 5% dextrose (250 ml), given over the course of 2 h through a Y connector, which was then followed immediately by a continuous infusion of 5-FU over the course of 46 h (fig. 1). Treatment was repeated every 2 weeks until disease progression. The maximum number of cycles was not specified.

Treatment was delayed if patients had any of the following findings on the planned day of treatment: neutrophil count <1,500/mm^3, hemoglobin <8.0 g/dl, platelets <100,000/mm^3, peripheral neuropathy >grade 2 or nonhematologic toxicity >grade 1, except for alopecia, nausea, vomiting, anorexia, fatigue and constipation. To prevent nausea and vomiting, a 5-hydroxytryptamine-3 antagonist plus dexamethasone was administered intravenously before chemotherapy. Prophylactic use of granulocyte colony-stimulating factor was prohibited.

Toxicity and Efficacy Assessments

Pretreatment evaluations included disease history, ECOG PS, white blood cell counts with differential and platelet counts, complete blood profile, carcinoembryonic antigen, CA19-9, urinalysis, electrocardiogram, chest radiograph, computed tomographic scan and any other appropriate diagnostic procedures to evaluate metastatic sites. During treatment, the following examinations were performed every week until the completion of cycle 4: physical examination, complete blood cell count, blood profile and urinalysis.

Toxicities were monitored weekly until cycle 4 and were scored according to the National Cancer Institute Common Toxicity Criteria, version 3.0.

Responses were evaluated every 2 cycles according to the Response Evaluation Criteria in Solid Tumors, version 1.0 [20], as complete response (CR), partial response (PR), stable disease or progressive disease (PD). The duration of response was calculated from the date of starting treatment to the date of the first confirmation of PD or the last examination. Progression-fee survival (PFS) was calculated from the date of starting therapy to the date of its discontinuation because of PD or death.

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). The *UGT1A1*6* polymorphism was analyzed by the polymerase chain reaction-restriction fragment length polymorphism method as described elsewhere [21]. The *UGT1A1*28* polymorphism was determined by direct sequencing as described by Fujita et al. [21].

Results

Patients

A total of 10 patients with advanced colorectal cancer were enrolled in this study from October 2007 through April 2009. Median age was 54 years (range 33–69), and ECOG PS was 0 in all patients. Six patients (60%) had liver metastases, 6 (60%) had multiple metastatic sites and 3 (30%) had received previous adjuvant chemotherapy with 5-FU-LV (table 2). In this study, a total of 111 cycles of chemotherapy were administered, with a median of 8.5 cycles per patient (range 1–36).

Dose Adaptation Results

The first patient was assigned to dose level 0 and had DLT (grade 3 infection with normal neutrophils). On day 8 after the first cycle of chemotherapy, the patient had severe diarrhea with a body temperature of 40°C and a C-reactive protein level of 27 mg/dl and therefore received antibiotics by intravenous infusion. Therefore, 3 additional patients were assigned to receive dose level 0. The first additional patient had DLT (a treatment delay of 14 days due to neutropenia). Because 2 of 4 patients had DLT at dose level 0, the next 3 patients were given dose level −1. One of these patients had DLT (a treatment delay of 6 days due to neutropenia). Therefore, 3 additional patients received dose level −1. Only 1 of the 6 patients who received dose level −1 had DLT. On the basis of these results, dose level 0 and dose level −1 were determined to be the MTD and RD, respectively.

Table 2. Patient characteristics

Characteristic	
Total patients	10
Median age (range), years	54 (33–69)
Male/female	9/1
ECOG PS	
0	10
1	0
Primary tumor site	
Colon/rectum	6/4
Previous surgery on primary tumor	7
Number of metastatic sites	
Single	4
Multiple	6
Metastatic sites	
Lung	6
Liver	6
Lymph nodes	4
Others	1
Previous chemotherapy	
Adjuvant	3
Palliative	0

Table 3. Maximum toxicity per cycle observed in a total of 37 cycles

Event	NCI-CTC grade					
	1[a]	2[a]	3[a]	4[a]	1–4[b]	3–4[b]
Leukopenia	7	11	1	0	51	3
Neutropenia	8	7	7	3	68	27
Anemia	12	0	1	0	35	3
Thrombocytopenia	3	0	0	0	8	0
Fatigue	13	1	0	0	38	0
Anorexia	17	3	1	0	57	3
Nausea	17	3	1	0	57	3
Vomiting	10	0	1	0	30	3
Diarrhea	3	3	0	0	16	0
Constipation	5	0	0	0	14	0
Mucositis	4	0	0	0	11	0
Alopecia	19	1	–	–	54	–
Neuropathy (sensory)	13	0	0	0	35	0
AST	8	0	0	0	22	0
ALT	12	1	0	0	35	0
Infection with normal ANC	0	0	1	0	3	3
Febrile neutropenia	–	–	0	0	–	0

Maximum toxicity in each patient was evaluated until the completion of 4 cycles. A total of 37 cycles were completed. NCI-CTC = National Cancer Institute Common Toxicity Criteria, version 3.0; – = not defined in the National Cancer Institute Common Toxicity Criteria, version 3.0; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ANC = absolute neutrophil count.

[a] Values represent numbers of cycles.

[b] Values represent percentages.

Toxicity

Toxicity was assessable in all patients until the completion of 4 cycles (table 3). The most common toxicities were neutropenia, anorexia, nausea, vomiting and alopecia. However, grade 3 and 4 toxicities were uncommon, except for neutropenia. In particular, 27% of cycles were associated with grade 3–4 neutropenia, although no patient had febrile neutropenia. When we evaluated the maximum toxicity per patient over 4 cycles, 5 patients (50%) had at least one episode of grade 3–4 neutropenia. No patient required hospitalization or died because of toxicity.

The causes of treatment discontinuation were PD in 4 patients, delayed recovery from toxicity such as neuropathy and nausea in 2 patients, allergic reaction to oxaliplatin in 2 patients, conversion therapy in 1 patient and DLT in 1 patient.

Efficacy

Response was assessable in 9 patients. Response was not assessed in 1 patient because of DLT (infection) occurring during the first cycle of treatment. One of the 9 patients (11%) had a CR, and 7 (78%) had PRs, resulting in an objective RR of 89% (95% confidence interval 56–98%; table 4). The location of responses were metastases of abdominal lymph nodes in a patient who had a CR and metastases of lung (n = 2), liver (n = 2) or both (n = 3) in patients who showed PRs. The median number of cycles required to reach PR was 3.5 (range 2–6). Residual liver metastases were surgically removed after chemotherapy in 1 patient whose metastases were initially considered unresectable. The median PFS was 11.6 months, after a median follow-up of 34.7 months, as calculated by the Kaplan-Meier method with the use of JMP software, version 6 (SAS Institute Inc., Cary, N.C., USA). The median dose intensities of irinotecan, oxaliplatin and 5-FU during the first 4 courses of treatment were 73 mg/m^2/week (88% of planned), 37 mg/m^2/week (88% of planned) and 1,066 mg/m^2/week (88% of planned), respectively, among the 3 patients given level 0, and 59 mg/m^2/week (79% of planned), 33 mg/m^2/week (79% of planned) and 950 mg/m^2/week (79% of planned), respectively, among the 6 patients given level –1.

Table 4. Objective responses

Response	Level 0 (n = 4)	Level –1 (n = 6)	Total
Complete response, n	0	1	1
Partial response, n	3	4	7
Stable disease, n	0	1	1
Progressive disease, n	0	0	0
Not evaluable, n	1	0	1
RR, %			89

Responses were assessed by computed tomography or magnetic resonance imaging according to the Response Evaluation Criteria in Solid Tumors.

Discussion

This is the first study to demonstrate the feasibility and activity of FOLFOXIRI in Japanese patients with advanced colorectal cancer who harbor *UGT1A1*1/*1, *1/*6* or **1/*28*. The RD of FOLFOXIRI in these Japanese patients was determined to be 150 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin and 2,400 mg/m^2 5-FU. The FOLFOXIRI regimen showed a manageable safety profile.

In the present study, patients with *UGT1A1*1/*1* and *UGT1A1*1/*28* or **1/*6* were enrolled. We reported previously that the efficacy and toxicity of irinotecan did not differ significantly between patients with *UGT1A1*1/*1* and **1/*28* or **1/*6* [22]. Exclusion of patients with *UGT1A1*28/*28, *6/*6* or **28/*6* might be one of the reasons for the manageable toxicities in our study, since these *UGT1A1* genotypes have been linked to severe irinotecan-induced toxicity [18, 19].

In this study, we used the treatment schedule described by the Gruppo Oncologico Nord Ovest (GONO). Two randomized phase III trials of FOLFOXIRI have been performed in Western countries. In the GONO study, median PFS and OS obtained with FOLFOXIRI as first-line treatment in patients with metastatic colorectal cancer were significantly longer than those obtained with FOLFIRI [3]. Furthermore, an updated analysis of the GONO study showed that FOLFOXIRI was associated with a clinically significant improvement in long-term outcomes as compared with FOLFIRI [23]. In contrast, the Hellenic Oncology Research Group (HORG) failed to demonstrate statistically significant benefits of FOLFOXIRI as compared with FOLFIRI, although some improvements in RR, time to disease progression and OS were obtained with FOLFOXIRI [4]. There was a major difference between the GONO and HORG studies with regard to the regimen of FOLFOXIRI used. Intravenous bolus 5-FU was included in the treatment regimen used by HORG but not that used by GONO. Consequently, the HORG study used lower doses of oxaliplatin and irinotecan than those used in the GONO study. We therefore decided to use the treatment schedule adopted by GONO. In our study, the doses of irinotecan and oxaliplatin at the initial dose level were the same as those used in the GONO study, and that of 5-FU was the approved dose (2,400 mg/m^2) in Japan. This may have contributed to the high RR and long PFS obtained in our study.

In this dose-finding study, 2 criteria for severe DLT were met during the search for the optimal RD of FOLFOXIRI: (1) the fourth cycle of treatment was not administered within 9 weeks from day 1 of cycle 1 and (2) the next cycle was not started within 4 weeks after completion of the prior cycle. When FOLFOXIRI was administered at the RD determined in the initial phase I–II study performed by GONO, 35% of cycles required dose reductions of at least one drug and 16% of cycles were delayed by at least 1 week because of toxicities [24]. GONO therefore performed another phase II study using slightly lower doses of irinotecan, oxaliplatin and 5-FU to improve the dose intensity. Use of the modified RD resulted in lower incidences of both hematologic and nonhematologic toxic effects, and the median dose intensity increased from 78 to 88% [25]. We considered the initial RD found in the phase I–II study by GONO to be too high to maintain adequate dose intensity. We therefore used the severe DLT criteria described above to determine the optimal RD that allowed a high dose intensity to be delivered safely, potentially enhancing treatment effectiveness.

First-line FOLFOXIRI might reduce the efficacy of second-line treatments, since all three key cytotoxic drugs used for the management of colorectal cancer (irinotecan, oxaliplatin and 5-FU) are used simultaneously. However, the GONO study showed that the FOLFOXIRI regimen did not negatively affect the outcomes of patients who received second-line chemotherapy [26]. Among patients given second-line treatment, median PFS and OS have been shown to be better in those who initially receive FOLFOXIRI than in those who initially receive FOLFIRI [23]. In our study, all of the 9 patients in whom response was assessable (excluding the 1 patient with a CR, who received FOLFOXIRI for the duration of treatment) received second-line treatments (5-FU-LV and oxaliplatin plus bevacizumab in 2, FOLFIRI plus bevacizumab in 2, infusional 5-FU plus bevacizumab in 2,

FOLFOXIRI in 1, 5-FU-LV and oxaliplatin in 1 and FOLFIRI in 1). We obtained a RR of 44% and median PFS of 9.1 months, similar to the results of the GONO study. We did not observe any toxicities which were life-threatening or grade 3 or worse neurotoxicity with second-line treatment. Our study also showed no evidence suggesting a negative impact of first-line FOLFOXIRI on treatment benefits in patients who subsequently received second-line chemotherapy.

Although our results suggested that FOLFOXIRI is a promising regimen for Japanese patients with advanced colorectal cancer, it remains unclear whether first-line treatment with three cytotoxic agents is better or worse than that with two cytotoxic agents plus one monoclonal antibody. Because the FOLFOXIRI regimen has a high RR, it might facilitate the radical surgical resection of metastases in patients with initially unresectable metastatic colorectal cancer [11, 12]. Although excessive chemotherapy can cause liver injury and further increase perioperative morbidity and mortality, one study assessing hepatic toxicity reported no treatment-related liver damage in patients who underwent surgery for metastases after they received FOLFOXIRI [12]. These results have suggested that FOLFOXIRI is a 'conversion therapy' for patients in good general condition who have potentially resectable disease [23]. The addition of cetuximab to FOLFOXIRI [27] may be a promising treatment strategy to increase the RR in patients without *Kras* mutations. Phase II studies of FOLFOXIRI combined with cetuximab should be performed to improve the surgical resection rate of metastases in Japan.

In conclusion, we determined the RD of FOLFOXIRI in Japanese patients with advanced colorectal cancer who do not have *UGT1A1*28/*28*, **6/*6* or **6/*28* genotypes. Our results showed that FOLFOXIRI is a well-tolerated regimen even in Japanese. Exclusion of patients with *UGT1A1*28/*28*, **6/*6* or **6/*28* might have contributed to the good tolerance in our study. FOLFOXIRI was also shown to be a very active regimen for the initial treatment of colorectal cancer.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan (21S-8-1) and in part by a Grant-in-Aid for the 'Support Project of Strategic Research Center in Private Universities' from the Ministry of Education, Culture, Sports, Science and Technology to the Saitama Medical University Research Center for Genomic Medicine.

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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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DOI: 10.1002/cncr.26378, **Received:** March 30, 2011; **Revised:** May 25, 2011; **Accepted:** June 2, 2011, **Published online** July 19, 2011 in Wiley Online Library (wileyonlinelibrary.com)

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

possibility of malignancy). These evaluations were made by the 3 same instructors certified by the Japanese Urological Association in all examinations, and high-level reproducibility was demonstrated in the previous report.[29]

After hemostasis of the biopsied region, the tumor was resected using a resectoscope. In the test design, first the tumor was resected under the conventional white light source. The light source was then changed to the fluorescence, and the excited region was in addition resected. Operations were performed by 3 physicians certified by the Japanese Urological Association under instruction by the same 3 instructors certified by the Japanese Urological Association in all operations.[30]

Diagnostic accuracy based on semiquantitative evaluation was analyzed by comparing the level on images of ALA-induced fluorescence with the pathological diagnosis according to the General Rule for Clinical and Pathological Studies on Bladder Cancer, third edition.[31] Diagnostic capability was assessed by the area under the receiver operative characteristic curve (AUC) in PDD compared with that in conventional white light endoscopic examination. These comparisons were analyzed using Fisher exact test (2×2), chi-square test, 2-sample test for equality of proportions, and the Wilcoxon rank sum test.

Moreover, in these cases, multivariate analysis using the Cox proportional-hazards model was performed to detect the clinicopathological factors including the factors based on the European Organization for Research and Treatment of Cancer risk tables[32] that contribute independently to improving prognosis.

Routine Follow-up

Periodic tests were performed as postoperative follow-up using the conventional white light examination. Basically, cystoscopy was performed every 3 months for 1 year after the operation, every 6 months thereafter until 3 years, and then every year in all patients.

RESULTS

Pathological Evaluation

The diagnostic accuracy and also ability in blue light (fluorescence) mode was higher than in white light (conventional) mode in all 1372 specimens in 210 cases of PDD, including 534 specimens from 75 cases with intravesically applied ALA and also 838 specimens from 135 cases of PDD with orally applied ALA. Among the 1372 specimens from 210 cases obtained by transurethral biopsy, 485 specimens (35.3%) were pathologically diagnosed as malignant epithelium, including 106 specimens (7.7%) of CIS and 77 specimens (5.6%) of severe dysplasia detected pathologically (Tables 4 and 5). In semiquantitative analysis in conventional mode, macroscopic impression of malignancy was shown to be statistically significantly correlated with tumor grade, regardless of the method of ALA administration ($P < .001$) (Table 4). In semiquantitative analysis in fluorescence mode, fluorescence intensity was shown to be statistically significantly correlated with the tumor grade, regardless of the route of ALA administration ($P < .001$). Moreover, 132 samples (72.1%), including 44 dysplasia lesions and 88 CIS lesions, could be detected only in fluorescence mode in 183 flat lesions, 77 dysplasia lesions, and 106 CIS lesions. The percentage of flat lesions that could be detected only in fluorescence mode of PDD by orally applied ALA (74.3%) was higher than with PDD by intravesically applied ALA (68.6%; Table 5).

Diagnostic Accuracy and Capability

The diagnostic accuracy of ALA-PDD, including the positive rate, predictive accuracy, sensitivity, and specificity, was examined in all 1372 biopsy samples of all 210 cases. The sensitivity of PDD (93.4%) was significantly higher than the 44.7% sensitivity in white light mode, whereas the specificity of PDD (58.9%) was significantly lower than the 94.1% specificity in white light mode ($P < .05$). Regardless of the method of ALA administration, the sensitivity of ALA-PDD is significantly higher than that of conventional white light examination, whereas the specificity of ALA-PDD is low, which means that there are many false-positive findings in ALA-PDD. Both endoscopic examinations are equivalent in predictive accuracy (Table 6). The AUC in blue light (fluorescence) mode was greater than that in white light (conventional) mode in not only all PDD cases ($P < .01$) but also in PDD with intravesically applied ALA ($P < .01$) and PDD with orally applied ALA ($P < .01$) (Fig. 1).

Recurrence-Free Survival

The median follow-up period was 22.0 (range, 0.2-68.7) months in 99 patients who underwent PDD-TURBT. Thirty-three of 99 patients recurred, and the recurrence-free survival rate was 86.9% (at 12 months), 74.7% (24 months), 69.7% (36 months), 67.7% (48 months), and 66.7% (60 months). The median follow-up period was 21.5 (range, 0.2-204.1) months in 99 patients who underwent conventional TURBT. Sixty of 99 patients recurred, and the recurrence-free survival rate was 58.6% (at 12

Table 4. Pathological Evaluation and Macroscopic Impression of Malignancy in the Examination in White Light Mode in All 1372 Biopsy Samples

White Light Mode (Conventional)	Macroscopic Impression of Malignancy			Samples, Total No.	*P*
	None	Weak	Strong		
All cases					<.001[a]
Normal epithelium	835	29	23	887	
Dysplasia	60	7	10	77	
UC G1	5	2	13	20	
UC G2	42	4	112	158	
UC G3	60	3	61	124	
UC G3-pTis	99	3	4	106	
Samples, total No.	1101	48	223	1372	
Intravesically applied ALA					<.001[a]
Normal epithelium	312	24	14	350	
Dysplasia	30	7	3	40	
UC G1	5	2	11	18	
UC G2	3	3	44	50	
UC G3	19	2	25	46	
UC G3-pTis	26	3	1	30	
Samples, total No.	395	41	98	534	
Orally applied ALA					<.001[a]
Normal epithelium	523	5	9	537	
Dysplasia	30	0	7	37	
UC G1	0	0	2	2	
UC G2	39	1	68	108	
UC G3	41	1	36	78	
UC G3-pTis	73	0	3	76	
Samples, total No.	706	7	125	838	

Abbreviation: ALA, 5-aminolevulinic acid.
Correlation between pathological evaluation and macroscopic impression of malignancy in the examination in white light mode is shown. In semiquantitative analysis in conventional mode, macroscopic impression of malignancy was shown to be statistically significantly correlated with tumor grade, regardless of the method of ALA administration ($P < .001$).
[a] Fisher exact test (2 × 2).

months), 49.5% (24 months), 41.4% (36 months), 41.4% (48 months), and 40.4% (60 months). There was a statistically significant difference in the recurrence-free survival rate between these 2 therapeutic groups ($P < .001$) (Fig. 2).

The median follow-up period was 32.4 (range, 0.2-68.7) months in 32 patients who underwent PDD-TURBT with intravesically applied ALA. Sixteen of 32 patients recurred, and the recurrence-free survival rate was 84.4% (at 12 months), 68.8% (24 months), 59.4% (36 months), 53.1% (48 months), and 50.0% (60 months). The median follow-up period was 17.1 (range, 1.9-40.8) months in 67 patients who underwent PDD-TURBT with orally applied ALA. Nineteen of 67 patients recurred, and the recurrence-free survival rate was 86.6% (12 months), 76.1% (24 months), and 71.6% (36 months). There was no statistically significant difference in the recurrence-free survival rate between these 2 therapeutic groups ($P = .980$) (Fig. 3).

The median follow-up period was 18.1 (range, 3.7-38.8) months in 18 T1G3 patients who underwent PDD-TURBT. Nine of 18 patients recurred, and the recurrence-free survival rate was 72.2% (at 12 months), 55.6% (24 months), and 50.0% (36 months). The median follow-up period was 27.6 (range, 0.2-185.1) months in 18 T1G3 patients who underwent conventional TURBT. Thirteen of 18 patients recurred, and the recurrence-free survival rate was 47.3% (at 12 months), 33.3% (24 months), and 27.8% (36 months). There was no statistically significant difference in the recurrence-free survival rate between PDD-TURBT for T1G3 and conventional TURBT for T1G3 ($P = .062$) (Fig. 4).

A deferred cystectomy because of recurrence and progression was performed for 1 patient in the PDD-TURBT group and 2 patients in the conventional TURBT group. Median time to cystectomy was 7.9 months in the PDD-TURBT group, and 3.9 months and 10.4 months in the conventional TURBT group (Fig. 4).

Table 5. Pathological Evaluation and Fluorescence Intensity in the Examination in Blue Light Mode in All 1372 Biopsy Samples

Blue Light Mode (Fluorescence)	Fluorescence Intensity			Samples, Total No.	*P*
	None	Weak	Strong		
All cases					<.001[a]
Normal epithelium	550	279	58	887	
Dysplasia	18	45	14	77	
UC G1	0	6	14	20	
UC G2	11	75	72	158	
UC G3	4	45	75	124	
UC G3-pTis	12	52	42	106	
Samples, total No.	595	502	275	1372	
Intravesically applied ALA					<.001[a]
Normal epithelium	195	136	19	350	
Dysplasia	8	25	7	40	
UC G1	0	5	13	18	
UC G2	4	23	23	50	
UC G3	2	17	27	46	
UC G3-pTis	0	18	12	30	
Samples, total No.	209	224	101	534	
Orally applied ALA					<.001[a]
Normal epithelium	355	143	39	537	
Dysplasia	10	20	7	37	
UC G1	0	1	1	2	
UC G2	7	52	49	108	
UC G3	2	28	48	78	
UC G3-pTis	12	34	30	76	
Samples, total No.	386	278	174	838	

Abbreviation: ALA, 5-aminolevulinic acid.
Correlation between pathological evaluation fluorescence intensity in the examination under blue light mode is shown. In semiquantitative analysis in fluorescence mode, fluorescence intensity was shown to be statistically significantly correlated with tumor grade, regardless of the method of ALA administration ($P < .001$). Moreover, 132 samples (72.1%), including 44 dysplasia lesions and 88 CIS lesions, could be detected only in fluorescence mode in 183 flat lesions, including 77 dysplasia lesions and 106 CIS lesions.
[a] Fisher exact test (2 × 2).

Table 6. Diagnostic Accuracy of ALA-PDD

	Positive Rate	Diagnostic Accuracy, %		
		Predictive Accuracy	Sensitivity	Specificity
All cases (1372 samples/210 cases)				
Blue light mode (fluorescence)	51.1	49.0	93.4	58.9
White light mode (conventional)	19.7	80.8	44.7	94.1
P	<.05[a]	<.05[a]	<.05[a]	<.05[a]
Intravesically applied ALA (534 samples/75 cases)				
Blue light mode (fluorescence)	60.3	53.8	92.0	53.8
White light mode (conventional)	26.0	72.7	54.9	89.1
P	<.05[a]	<.05[a]	<.05[a]	<.05[a]
Orally applied ALA (838 samples/135 cases)				
Blue light mode (fluorescence)	78.5	59.7	89.7	66.1
White light mode (conventional)	15.8	89.8	38.6	97.4
P	<.05[a]	<.05[a]	<.05[a]	<.05[a]

Abbreviations: ALA, 5-aminolevulinic acid; ALA-PDD, photodynamic diagnosis mediated by ALA.
The diagnostic accuracy of ALA-PDD including the positive rate, predictive accuracy, sensitivity, and specificity regarding diagnostic accuracy was examined in all 1372 biopsy samples of all 210 cases. Regardless of the method of ALA administration, the sensitivity of ALA-PDD was significantly greater than that of conventional white light examination, whereas the specificity of ALA-PDD was low, which means that there were many false-positive findings in ALA-PDD. Both endoscopic examinations were equivalent in predictive accuracy.
[a] Two-sample test for equality of proportion.