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.

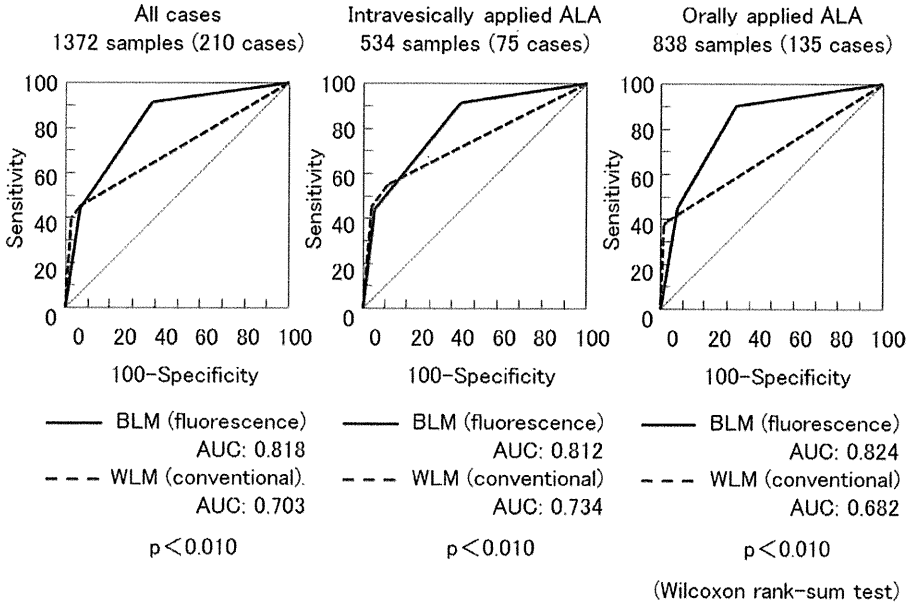

Figure 1. Diagnostic capability of photodynamic diagnosis (PDD) mediated by 5-aminolevulinic acid (ALA) (ALA-PDD) is defined by the area under the receiver operating characteristic curve (AUC). The AUC in blue light (fluorescence) mode (BLM) was more than that in white light (conventional) mode (WLM) in not only all PDD cases ($P < .01$) but also in PDD with intravesically applied ALA ($P < .01$) and PDD with orally applied ALA ($P < .01$).

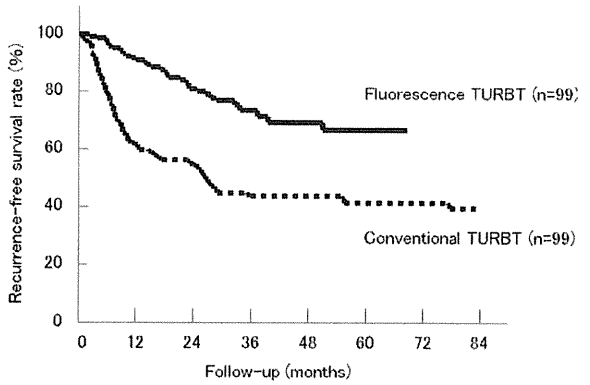

Figure 2. Recurrence-free survival in all cases is shown. Median follow-up period was 22.0 (range, 0.2-68.7) months in 99 patients who underwent transurethral resection of bladder tumor (TURBT) guided by photodynamic diagnosis mediated by 5-aminolevulinic acid. Thirty-three of 99 patients recurred, and 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). 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 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 ($P < .001$).

Moreover, multivariate analysis revealed that PDD-TURBT was the only independent factor that contributed to improving the intravesical recurrence rate (hazard ratio, 0.578; 95% confidence interval, 0.371-0.888; $P = .012$) (Table 7).

Adverse Events

Although no special precaution, such as liver support and light shielding, was implemented throughout PDD, there were mild and transient adverse events in conformity with the Common Terminology Criteria for Adverse Events version 3.0.[27] Urinary frequency and/or urgency occurred in 13 (17.3%) of 75 cases of PDD with intravesically applied ALA, photosensitivity reaction in 6 cases (4.4%), elevated level of AST and/or ALT in 4 cases (3.0%), and nausea and/or vomiting in 4 of 135 cases (3.0%) of PDD with orally applied ALA.

DISCUSSION

ALA-PDD is a cancer diagnostic method by fluorescence navigation, which has been clinically recognized as an effective procedure to detect various cancers, such as brain tumor[33] and bladder cancer.[10-17] In particular, PDD with hexaminolevulinate, the hexyl ester derivative of 5-ALA for bladder cancer, has already been approved and

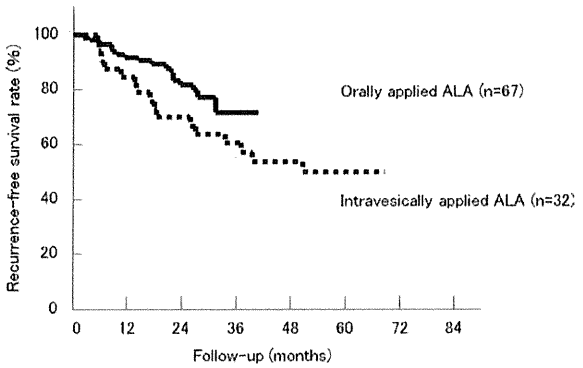

Figure 3. Recurrence-free survival stratified by 5-aminolevulinic acid (ALA) administration route is shown. Median follow-up period was 32.4 (range, 0.2-68.7) months in 32 patients who underwent transurethral resection of bladder tumor guided by photodynamic diagnosis mediated by ALA (PDD-TURBT) with intravesically applied ALA. Sixteen of 32 patients recurred, and 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). Median follow-up period was 16.8 (range, 1.2-41.8) months in 70 patients who underwent PDD-TURBT with orally applied ALA. Nineteen of 67 patients recurred, and the recurrence-free survival rate was 86.6% (at 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 ($P = .980$).

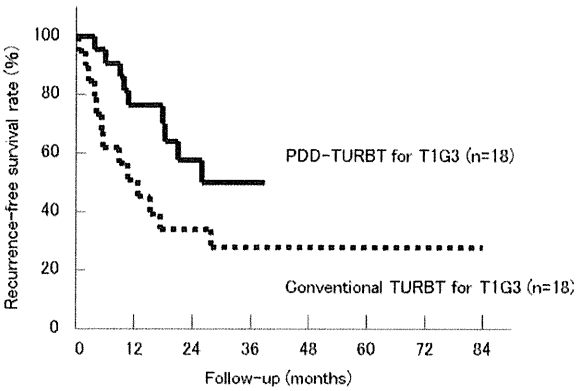

Figure 4. Recurrence-free survival is stratified by fluorescence transurethral resection of bladder tumor (TURBT) and conventional TURBT in T1G3. Median follow-up period was 18.1 (range, 3.7-38.8) months in 18 T1G3 patients who underwent TURBT guided by photodynamic diagnosis mediated by 5-aminolevulinic acid (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). 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 ($P = .062$). A deferred cystectomy because of recurrence and progression was performed in 1 case in the PDD-TURBT group and 2 cases 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.

implemented as a legitimate medical practice in Europe and the United States, whereas in Japan, PDD with intravesically and orally applied ALA was performed for the first time in 2004,[29] and has just been approved and implemented as advanced medical technology in registered institutions by the Ministry of Health, Labor, and Welfare in 2010. In this study, we demonstrated the clinical effectiveness and safety of ALA-PDD and also of TURBT guided by ALA-PDD with a large population in Japan.

At transurethral biopsy, intravesical observation using ALA-PDD was useful in detecting CIS, avoiding an incorrect decision on adjuvant therapy. The diagnostic accuracy of ALA-PDD was first reported with a sensitivity of 100.0% and specificity of 68.5% in 68 cases of bladder cancer by Kriegmair et al[10] in 1994. Subsequently, many clinical trials of ALA-PDD have been performed, mainly in Europe. Hungerhuber et al[17] reported 1713 procedures of ALA-PDD with a sensitivity of 92.0% and specificity of 55.6% in 875 cases of bladder cancer, the largest number of ALA-PDD cases. Including these reports, a summary of previous reports revealed that the sensitivity of PDD (94.6%; range, 77.8%-100%) was significantly higher than the 76.0% (range, 67.5%-84.0%) sensitivity of conventional white light examination, whereas the specificity of PDD (59.0%; range, 33.0%-87.1%) was significantly lower than the 68.5% (range, 66.4%-78.0%) specificity of conventional white light examination.[10-17] In particular, the reports of flat lesions revealed that 34.2% (range, 14.9%-42.1%) of all lesions, 43.4%-57.0% of CIS, and 30.3%-44.0% of dysplasia could be detected by ALA-PDD, but not conventional white light

Table 7. Multivariate Analysis of All Cases

Factors	Hazard Ratio	95% Confidence Interval	*P*
EORTC recurrence score[32] (0, 1-4, 5-9, 10-17)	1.240	0.482-3.234	.657
BCG intravesical instillation	0.832	0.516-1.400	.475
PDD-TURBT	0.578	0.371-0.888	.012

Abbreviations: BCG, Bacillus Calmette-Guerin; EORTC, European Organization for Research and Treatment of Cancer; PDD-TURBT, transurethral resection of bladder tumor guided by photodynamic diagnosis.
Multivariate analysis revealed that the only independent factor contributing to improving prognosis was PDD-TURBT (hazard ratio, 0.578; 95% confidence interval, 0.371-0.888; $P = .012$).

examination.[15-17,34,35] The results of this study support the results of these clinical studies of ALA-PDD. The AUC in PDD with a sensitivity of 93.4% and specificity of 58.9% was significantly greater than that in conventional white light examination. Only PDD could detect 72.1% of flat lesions including dysplasia and CIS. Moreover, it was demonstrated that regardless of the ALA administration route, there was no significant difference in the diagnostic accuracy and ability of PDD in this study.

Causal lesions of intravesical recurrence are endoscopic invisible lesions. In TURBT, additional resection under ALA-PDD could avoid insufficient resection of tumors, reducing the rate of intravesical recurrence. The rate of residual tumor in PDD-TURBT with a median rate of 8.0% (0%-32.7%) was significantly reduced compared with conventional TURBT under white light, with a median rate of 37.0% (19.2%-53.1%) in the summary of previous reports.[14,18,21,36,37] Denzinger et al[22] made a comparative review of intravesical recurrence-free survival between 88 cases of PDD-TURBT and 103 cases of conventional TURBT, with 96 months as the follow-up duration. They reported that the intravesical recurrence-free survival of 90.9% (at 12 months after PDD-TURBT), 90.9% (24 months), 85.0% (48 months), 79.0% (72 months), and 71.0% (96 months) in PDD-TURBT was significantly greater than that in conventional TURBT of 78.6% (at 12 months after conventional TURBT), 69.9% (24 months), 60.7% (48 months), 54.0% (72 months), and 45.0% (96 months) ($P = .0003$). Moreover, it was demonstrated that PDD-TURBT was an independent prognostic factor for improving the intravesical recurrence rate, with a hazard ratio of 0.29 (95% confidence interval, 0.15-0.56; $P = .0002$) by multivariate analysis using the Cox proportional hazards model. The results of this study support the results of previous clinical studies of PDD-TURBT.[18-22] In this study, it was revealed that the intravesical recurrence-free survival was significantly greater than that in conventional TURBT at up to 60 months follow-up ($P < .001$). It was also revealed that PDD-TURBT was the only independent factor that contributed to improving the intravesical recurrence rate (hazard ratio, 0.578; 95% confidence interval, 0.371-0.888; $P = .012$) by multivariate analysis. Moreover, it was demonstrated that regardless of the ALA administration route, there was no significant difference in intravesical recurrence-free survival in this study.

In this study, only 18 cases of T1G3 were known to show highly aggressive behavior. PDD-TURBT for T1G3 had a greater tendency to reduce the intravesical recurrence rate, but with no statistically significant difference ($P = .062$) compared with conventional TURBT for T1G3 in this study population. However, more recently, the effectiveness of tumor control has been demonstrated in 77 cases of PDD-TURBT for T1G3[38]; therefore, more data compilation may show the usefulness of PDD-TURBT for T1G3 in improving recurrence-free survival and also avoiding deferred cystectomy because of tumor recurrence and progression.

Currently ALA and hexaminolevulinate have been approved as photosensitizers of PDD by authorities around the world. In Europe, ALA was approved under the trade name Gliolan as an optical imaging agent to enhance intraoperative detection of malignant glioma (World Health Organization grade III and IV) by the European Medicines Evaluation Agency.[9] Conversely, hexaminolevulinate was approved under the trade names Hexvix and Cysview as an optical imaging agent to enhance intraoperative detection of papillary bladder cancer by the European Medicines Evaluation Agency and the Food and Drug Administration in Europe and the United States, respectively.[9] More accumulation of biosynthesized protoporphyrin IX was revealed by administration of hexaminolevulinate compared with ALA by in vivo spectrophotometric measurement[39]; therefore, it was reported that hexaminolevulinate yielded higher fluorescence intensity and contrast between normal and malignant urothelium, with less photobleaching. In 2002, Dalton et al investigated the pharmacokinetics of ALA administered intravenously, orally, and intravesically, in which <1% of ALA was absorbed through the urinary bladder, and the cumulative ALA amount in urine was about 20,000× higher than that in plasma.[40] This finding indicates the low possibility of the occurrence of systemic adverse events induced by the intravesical administration of ALA, but does not demonstrate the usefulness for diagnosis compared with oral ALA administration, that is, differences in the fluorescence intensity between lesions and nonlesions (ie, contrast) and the fluorescence attenuation level with excitatory light irradiation (photobleaching) are parameters of diagnostic usefulness, and the diagnostic accuracy is based on these. We performed a clinical comparison of ALA administered intravesically and orally, and showed that the diagnostic accuracy of PDD was superior to that of white light examination excluding the predictive accuracy using intravesical ALA administration. In addition, there were no significant differences because of the variation in the route of administration (intravesical and oral ALA administrations) in diagnostic accuracy,

diagnostic performance, or recurrence-free survival. To pharmacologically demonstrate this subjective evaluation of clinical data and fluorescence intensity, we are planning to compare intravesical and oral ALA administrations with regard to tissue ALA and protoporphyrin IX levels in lesions and nonlesions, and the time course changes of these. Moreover, it was demonstrated in the retrospective series that currently, although PDD with ALA and hexaminolevulinate applied intravesically were 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 used ALA orally and intravesically to evaluate the clinical value in PDD and TURBT guided by ALA-PDD for bladder cancer. As described above, in this study, it was demonstrated that regardless of the ALA administration route, there was no significant difference in diagnostic accuracy, effectiveness of PDD, and recurrence-free survival, and all procedures were well tolerated by all patients without any severe adverse events. Although it is subjective, the intensity of ALA-induced fluorescence in PDD with orally applied ALA was higher than that in PDD with intravesically applied ALA. Moreover, PDD with orally applied ALA had less photobleaching. Hence, in the urological field, oral administration of ALA may be useful in PDD for malignancies other than bladder cancer. Further technical development of ALA-PDD is required to avoid photobleaching, improve diagnostic accuracy, and establish a standard intraoperative diagnostic system in the near future in Japan.

Conclusions

PDD with intravesically and also orally applied ALA is equally effective in detecting endoscopic invisible lesions such as dysplasia and carcinoma in situ, avoiding an incorrect decision on adjuvant therapy. In TURBT also, additional resection under ALA-PDD could avoid insufficient resection of tumors, reducing the rate of intravesical recurrence. It was suggested that regardless of the ALA administration route, ALA-PDD might be remarkably helpful in detection and intraoperative navigation programs.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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Neoadjuvant endocrine therapy of breast cancer: which patients would benefit and what are the advantages?

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Abstract Aromatase inhibitors (AIs) were more effective than tamoxifen as a neoadjuvant endocrine therapy (NAE) for postmenopausal women with estrogen receptor (ER)-positive breast cancer. Neoadjuvant AIs were shown to reduce tumor volume and to allow the performance of breast-conserving surgery (BCS) in cases that would normally require mastectomy. Predictive markers of neoadjuvant AIs may be ER-rich, progesterone receptor (PgR)-rich and human epidermal growth factor receptor 2 (HER2)-negative tumors. However, the ability of HER2 expression to predict a response to neoadjuvant AIs is controversial. Pathological tumor size, nodal status, Ki67 level, and ER score are predictive for the survival of postmenopausal women with breast cancer who have been treated with NAE. These factors could be useful in order to select patients who do not require chemotherapy. Indeed, neoadjuvant AIs are a potential treatment option for postmenopausal women with ER-rich breast cancer who prefer BCS despite having large tumors suitable for mastectomy.

Keywords Neoadjuvant endocrine therapy · Aromatase inhibitor · Tamoxifen · Neoadjuvant chemotherapy · Tumor response

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Introduction

The objectives of neoadjuvant systemic therapy for operable breast cancers are to minimize the extent of surgery [i.e., to convert a total mastectomy to breast-conserving surgery (BCS)] and to improve survival. Neoadjuvant systemic therapy is an in vivo sensitivity test, and ineffective drugs can be changed to other drugs based on tumor responses, which may lead to better survival rates in breast cancer patients.

Compared with postoperative chemotherapy, randomized controlled trials revealed that neoadjuvant chemotherapy (NAC) increased BCS rates but did not improve survival rates of women with breast cancer [1, 2]. The survival rates of breast cancer patients whose tumors showed a pathological complete response (pCR) after NAC were significantly better than those of patients whose tumors did not show pCR [1]. Neoadjuvant chemotherapy, however, has not been shown to achieve pCR in most women with estrogen receptor (ER)-positive breast cancer [3].

Recently, the aromatase inhibitors (AIs) have been used as a neoadjuvant endocrine therapy (NAE) for postmenopausal women with ER-positive breast cancer. However, randomized controlled trials have not investigated whether NAE can improve the survival of women with ER-positive breast cancer compared with postoperative endocrine therapy. In addition, the optimal duration of NAE has not been clarified. Although there remain some unresolved issues concerning NAE, neoadjuvant AIs have been shown to be as effective as NAC in women with ER-positive breast cancer [4] (Table 1). Therefore, neoadjuvant AIs will likely be a standard treatment option for postmenopausal women with ER-positive breast cancer.

Table 1 Clinical trials comparing the efficacy of aromatase inhibitors with other agents in the neoadjuvant setting

References	Treatment duration (months)	Patients	End points	Results			*P* value
				LET, *N* = 154	**TAM, *N* = 170**		
Eiermann et al. [20]	4	ER +ve or PgR +ve	Response rates by caliper	55%	36%		<0.001
		T2-4 N0-2	Response rates by ultrasound	35%	25%		0.042
		Ineligible for BCS	Response rates by mammography	34%	16%		<0.001
		N = 324	BCS rate	45%	35%		0.022
				ANA, *N* = 113	**TAM, *N* = 108**	**ANA + TAM, *N* = 109**	
Smith et al. [21]	3	ER +ve	Response rates by caliper	37% (39%)	36% (28%)	39% (36%)	NS (NS)
		N = 330	Response rates by ultrasound	24% (33%)	20% (19%)	28% (29%)	NS (NS)
			(%) = Patients not suitable for BCS				
			PD rates by ultrasound	10%	9%	7%	
			BCS rates	44% (46%*)	31% (22%*)	24% (26%)	NS (0.03*)
			(%) = Surgeon's assessment				
				EXE, *N* = 76	**TAM, *N* = 75**		
Semiglazov et al. [22]	3	ER +ve	Response rates by caliper	76.3%	40.0%		0.05
		Stage IIB–IIIB	Response rates by ultrasound	60.5%	37.3%		0.092
		N = 151	Response rates by mammography	64.0%	37.3%		0.082
			BCS rates	36.8%	20.0%		0.05
			pCR rates	2.6%	2.7%		NS
			3-year disease-free survival rates	78.9%	74.6%		NS
				ANA, *N* = 163	**TAM, *N* = 151**		
Cataliotti et al. [23]	3	ER +ve or PgR +ve	Response rates by caliper	49.7% (48.6%)	39.7% (35.8%)		0.08 (0.04)
		T2-4 N0-2	Response rates by ultrasound	36.2% (36.6%)	26.5% (24.2%)		0.07 (0.03)
		N = 314	BCS rates	43.0%	30.8%		0.04
			(%) = Patients not suitable for BCS or surgery				
				ANA, *N* = 17	**TAM, *N* = 28**		
Akashi-Tanaka et al. [9]	TAM 4	ER +ve or PgR +ve	Clinical response rates	76%	46%		0.05
	ANA 5	T2-4 ≥3 cm	Pathological response rates	94%	64%		0.02
		N = 45					
				EXE/ANA, *N* = 121	**AP, *N* = 118**		
Semiglazov et al. [4]	3	ER +ve or PgR +ve	Response rates by caliper	64.5% (70%)	63.6% (60%)		>0.5 (0.068)
		Stage IIA–IIIB	Response rates by ultrasound	40%	46%		0.8
		N = 239	Response rates by mammography	60% (66%)	63% (60%)		0.5 (0.088)
			BCS rates	33% (43%)	24% (24%)		0.058 (0.054)
			(%) = Patients with ER-rich tumors (Allred score ≥6 or ≥120 fmol/mg)				

ANA anastrozole, *AP* doxorubicine and paclitaxel, *BCS* breast-conserving surgery, *ER* estrogen receptor, *EXE* exemestane, *LET* letrozole, *NS* not significant, *pCR* pathological complete response, *PD* progressive disease, *PgR* progesterone receptor, *TAM* tamoxifen

* Statistical significance was found only between ANA and TAM groups

Neoadjuvant endocrine therapy for premenopausal women with breast cancer

Several studies have investigated the effects of NAE administration to premenopausal women with ER-positive breast cancer. Tamoxifen, luteinizing hormone-releasing hormone analogue (LH-RH analogue), or a combination of these drugs was used in premenopausal women with metastatic breast cancer, and the combination of these drugs was superior to each drug alone in terms of progression-free survival [5]. Torrisi et al. [6] investigated that activity of NAE with letrozole in combination with LHRH analogue in premenopausal women with T2-4 N0-2 breast cancer, whose tumors expressed both ER and progesterone receptor (PgR). They showed that a partial response rate was 50% in 32 patients, with no progressive disease. Recently, a double-blind, randomized controlled trial was conducted in Japan for premenopausal women with operative, ER-positive breast cancer. LH-RH analogue plus anastrozole was compared with LH-RH analogue plus tamoxifen in a neoadjuvant setting, and the tumor response was measured [7]. The results have not been reported yet.

Neoadjuvant tamoxifen for postmenopausal women with breast cancer

Tamoxifen has been used as a first-line endocrine treatment of breast cancer for approximately 3 decades. In previous studies, tamoxifen was used as an NAE. Hoff et al. [8] reported that a clinical tumor response was observed in 47% of patients (women over 75 years old or comorbid women with stage II or III breast cancer) after 3–6 months of neoadjuvant tamoxifen, and the 5-year overall survival rate was 59%. Similarly, Akashi-Tanaka et al. [9] reported that the clinical tumor response rate to 4 months of neoadjuvant tamoxifen therapy was 46% (Table 1). Another study compared neoadjuvant tamoxifen with surgery, or surgery plus postoperative tamoxifen using randomized controlled trials in women over 70 years of age with operable breast cancer [10]. These trials showed that the overall survival rates were the same between the two groups. Compared with an initial surgery, these results suggested that neoadjuvant tamoxifen might not worsen the overall survival rates of elderly women with ER-positive breast cancer.

Neoadjuvant aromatase inhibitors for postmenopausal women with breast cancer

Single-arm trials

The clinical tumor responses of neoadjuvant AIs were analyzed in single-arm trials [11–19], which are chronologically listed in Table 2. Three to 12 months of neoadjuvant use of AIs revealed that partial response or complete response rates evaluated by palpation were 41–80%, and progressive disease rates were 0–7%. Remarkably, 48–88% of patients treated with neoadjuvant AIs were able to undergo BCS rather than mastectomy suitable at baseline.

Previous studies have investigated the optimal duration of neoadjuvant letrozole therapy [12, 13, 15, 19]. In all but one patient, Renshow et al. [12] reported that tumor responses were maintained over 12 months in cases where a tumor response was found within 3 months of beginning neoadjuvant letrozole therapy. Llombart et al. [13] showed that 4.2 months was the median duration of neoadjuvant letrozole treatment needed to achieve a maximal tumor response. Krainick-Strobel et al. [15] reported that a higher clinical response rate was observed after 8 months of neoadjuvant letrozole therapy compared with treatment for 4 months. In addition, Mustacchi et al. [19] reported that neoadjuvant exemestane therapy for 6 months was more effective than that for 3 months. All of these results suggested that the optimal duration of neoadjuvant therapy might be at least 4 months.

Randomized controlled trials

Previous randomized controlled trials compared the efficacy of neoadjuvant AIs with neoadjuvant tamoxifen [20–23] (Table 1). Measures of objective response rates and BCS rates in these trials revealed that AIs had a greater efficacy than tamoxifen. Objective response rates evaluated by palpation were 1–36% higher in patients treated with AIs than in patients treated with tamoxifen. In addition, BCS rates were 10–17% higher in patients treated with AIs than in patients treated with tamoxifen. Furthermore, Kurosumi et al. [24] showed that the pathological tumor response rate to 3 months of neoadjuvant anastrozole treatment was higher than 3 months of neoadjuvant tamoxifen treatment.

Evaluation of the tumor response

Clinical tumor response rates evaluated by palpation were likely to be higher than those evaluated by ultrasound or mammography. Dixon et al. [11] reported that tumor sizes measured by ultrasound were more closely related to pathological tumor sizes compared with tumor sizes measured by palpation or mammography.

Physicians primarily use the Response Evaluation Criteria in Solid Tumors (RECIST) [25] or the World Health Organization (WHO) [26] criteria for the evaluation of clinical tumor responses to NAE. According to these criteria, a complete response is very rare after NAE, and a partial response is considered as a best response.

Table 2 Single-arm trials analyzing the efficacy of neoadjuvant aromatase inhibitors

References	Treatment duration	Patients	Results	
Dixon et al. [11]	ANA	ER +ve (histoscore > 80)	Median % reduction by caliper, ultrasound, mammography	89%, 81%, 74%
	3 months	>3 cm	Conversion rate from mastectomy to BCS	88%
		$N = 12$		
Renshow et al. [12]	LET	ER +ve (Allred ≥6)	Median % reduction from baseline to 3 months	52%
	3–12 months	$N = 142$	Median % reduction from 3 to 6 months	57%
			Median % reduction from 6 to 12 months	66%
			cCR rates at 3/6/12 months	10%/29%/36%
Llombart et al. [13]	LET	ER +ve or PgR +ve	Median time to objective response	3.5m
	3–12 months	Stage II to IIIB	Median time to maximal response	4.2m
		Non-suitable for BCS	Objective response rate	80%
		$N = 30$		
Tubiana-Hulin et al. [14]	EXE	ER +ve or PgR +ve	Response rates by caliper, ultrasound, mammography	73%, 45%, 36%
	4 months	T2-4 N0-2	PD rate	0%
		Ineligible for BCS	BCS rate	57%
		$N = 45$	pCR rate	0%
Krainick-Strobel et al. [15]	EXE	ER +ve or PgR +ve	Clinical response rate at 4 months	70%
	4–8 months	≥T2 ≥N0 ≥M0	BCS rate at 4 months	71%
		Ineligible for BCS		
		$N = 29$		
Takei et al. [16]	EXE	ER +ve or PgR +ve	Objective response rate	66%
	4 months	T2-4 (≥3 cm) N0-2	PD rate	5%
		$N = 44$	Pathological response rate (pCR rate)	43% (0%)
Mlineritsch et al. [17]	EXE	ER +ve or PgR +ve	Objective response rate	41%
	4 months	$N = 80$	BCS rate	76%
			pCR rate	3%
Barnadas et al. [18]	EXE	ER +ve	Clinical response rate (cCR rate)	61% (6%)
	6 months	T2-4 N0-2	Radiological response rate	51%
		Ineligible for BCS	PD rate	2%
		$N = 55$	BCS rate	56%
			Pathological response rate (pCR rate)	56% (2%)
Mustacchi et al. [19]	EXE	ER +ve or PgR +ve	Objective response rate (cCR rate)	70% (2%)
	6 months	Aged >70 years	PD rate	7%
		T1-3, N0-1	Conversion rate from mastectomy to BCS	48%
		$N = 117$		

AI aromatase inhibitor, *ANA* anastrozole, *BCS* breast-conserving surgery, *cCR* clinical complete response, *ER* estrogen receptor, *EXE* exemestane, *LET* letrozole, *pCR* pathological complete response, *PD* progressive disease, *PgR* progesterone receptor, *TAM* tamoxifen

The pathological criteria for tumor responses after neoadjuvant systemic therapy in breast cancer patients were proposed by the Japanese Breast Cancer Society (JBCS) [27]. These criteria define a grade 3 response as pCR irrespective of a remaining ductal carcinoma in situ. Although grade 3 responses have often been observed after NAC, they have rarely been seen after NAE. Therefore, pathological tumor responses other than grade 3 responses may be important for the prediction of prognoses in patients treated with NAE. Takei et al. [16] (Table 2) and Kurosumi et al. [24], using grade 1b as the cutoff level, reported that pathological tumor responses were observed in 43.3% and 37.2% of patients treated with exemestane for 4 months or with anastrozole for 3 months, respectively. In another study, Akashi-Tanaka et al. [9] (Table 1), using grade 1a as the cutoff level, reported that pathological tumor responses were observed in 64.3 and 94.1% of patients treated with tamoxifen for 4 months or anastrozole for 5 months, respectively. Indeed, pathological tumor response rates depended on the cutoff levels of the JBCS criteria. Another pathological scale proposed by Sataloff et al. [28] (Table 3) was originally used for pathological tumor responses to NAC and was predictive for prognoses of patients with locally advance breast cancer who were treated with NAC. Tubiana-Hulin et al. [14] used this scale for pathological tumor responses to neoadjuvant exemestane treatment and

reported that grades A, B, C, and D of the primary site were observed in 0.0, 16.7, 80.9, and 2.4%, respectively.

These pathological criteria need to be assessed for correlation with clinical tumor responses, biomarker changes, and prognoses of patients treated with NAE. At present, tumor responses after neoadjuvant AIs must be evaluated by a combination of palpation, imaging modalities, and pathological findings.

Ki67 and predictive markers of the tumor response

Dowsett et al. [29] compared Ki67 levels at baseline, 2 weeks, and 12 weeks after the initiation of neoadjuvant anastrozole or tamoxifen therapy. Reduction rates of the Ki67 levels from baseline to 2 or 12 weeks were higher in patients treated with anastrozole compared with those treated with tamoxifen. These reduction rates increased according to the increased ER levels elicited by anastrozole or tamoxifen. The reduction rates were higher in progesterone receptor (PgR)-positive tumors compared with PgR-negative tumors following either anastrozole or tamoxifen treatment. The reduction rates tended to be higher in human epidermal growth factor receptor 2 (HER2)-negative tumors compared with HER2-positive tumors in response to either anastrozole or tamoxifen treatment.

Tubiana-Hulin et al. [14] and Takei et al. [16] reported that PgR positively correlated with the clinical tumor response in neoadjuvant exemestane trials. Takei et al. [16] reported that HER2 scores might be inversely correlated with clinical tumor responses; however, Mlineritsch et al. [17] reported that the tumor response was independent of HER2 status.

Based on these results, the reduction rates of Ki67 levels from baseline were considered as a possible marker of the tumor response to NAE. In addition, ER, PgR, and HER2 were predictive markers of the response to NAE. Indeed, higher ER levels and higher PgR levels were more sensitive to neoadjuvant AIs. However, further analyses are needed to determine whether HER2 expression correlates with the tumor response to neoadjuvant AIs.

Prognostic markers

Dowsett et al. [30] analyzed the prognoses of breast cancer patients who received neoadjuvant anastrozole and found that tumor sizes, ER levels, and Ki67 scores at baseline, as well as ER levels and Ki67 scores at 2 weeks after the initiation of neoadjuvant anastrozole therapy, were significantly predictive for recurrence-free survival. In another study, Ellis et al. [31] reported that pathological tumor size, node status, Ki67 level, and ER status 4 months after the initiation of neoadjuvant letrozole or tamoxifen therapy significantly predicted relapse-free survival and breast cancer-specific survival. Furthermore, clinical tumor response and histological grade also predicted relapse-free survival; however, this finding was not statistically significant by multivariate analysis. Akashi-Tanaka et al. [9] reported that Ki67 level at baseline, as well as pathological tumor response and nodal status after NAE, were significantly predictive for relapse-free survival.

Tumor size, nodal status, and ER are standard prognostic and predictive markers of breast cancer. Ki67 levels are also important to predict the prognoses of patients treated with neoadjuvant AIs. In addition, clinical tumor responses, histological grades, and probably pathological tumor responses after administration of neoadjuvant AIs may be important to predict the prognoses.

Ways to avoid unnecessary chemotherapy

Semiglazov et al. [4] did not discover any differences in clinical tumor responses between treatment with NAE (anastrozole or exemestane for 3 months) and NAC (doxorubicin and paclitaxel, 4 cycles) in postmenopausal women with ER-positive, stage II or III breast cancer (Table 1). A median age of patients in this study was high

Table 3 Pathologic examination for therapeutic effect proposed by Sataloff et al. [28]

Grade	
Primary site	
T-A	Total or near total therapeutic effect
T-B	Subjectively greater than 50% therapeutic effect but less than total or near total
T-C	Less than 50% therapeutic effect, but effect evident
T-D	No therapeutic effect
Axillary lymph node	
N-A	Evidence of therapeutic effect, no metastatic disease
N-B	No nodal metastasis or therapeutic effect
N-C	Evidence of therapeutic effect but nodal metastasis still present
N-D	Viable metastatic disease, no therapeutic effect

(68 years in NAE group and 67 years in NAC group). They also reported a higher clinical response rate for NAE compared with NAC only in patients with ER-rich tumors. After a mean follow-up of <36 months, the incidence of local relapse was the same between the two groups (NAE 3.3 vs. NAC 3.4%). These results suggest that chemotherapy should be avoided in elderly women with ER-rich breast cancer because AIs are so effective.

Neoadjuvant AIs may select for postmenopausal women with breast cancer who do not require chemotherapy. Ellis et al. [31] proposed a preoperative endocrine prognostic index (PEPI) that included the sum of the scores determined for tumor size, nodal status, Ki67 level, and ER status in breast cancer patients treated with neoadjuvant letrozole, anastrozole, or tamoxifen. They showed that PEPI scores predicted the prognoses of these patients and suggested that breast cancer patients with lower PEPI scores, especially those with a score of zero, having excellent survivals, might not require postoperative chemotherapy.

The National Surgical Adjuvant Study of Breast Cancer (N-SAS BC) 06 study randomizes postmenopausal women with ER-positive breast cancer who received 6 months of neoadjuvant letrozole therapy into two groups: one group would receive additional chemotherapy and the other letrozole alone. This trial could resolve whether chemotherapy is needed for postmenopausal women with ER-positive breast cancer whose tumors have responded to neoadjuvant letrozole.

Conclusion

Although NAE is not a standard treatment for premenopausal women with ER-positive breast cancer, neoadjuvant AIs are an available treatment option for postmenopausal women with ER-positive breast cancer. Neoadjuvant AIs are suitable for the following characteristics: (1) invasive carcinoma, (2) ER-rich breast cancer, (3) PgR-rich breast cancer (if possible), and (4) HER2-negative breast cancer (if possible). However, the ability of HER2 expression to predict the response to neoadjuvant AIs remains controversial. In addition to the above-mentioned characteristics, neoadjuvant AIs are a potential treatment option for patients who prefer BCS despite having large tumors suitable for mastectomy.

Conflict of interest There is no conflict of interest.

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Missense mutations in *PML-RARA* are critical for the lack of responsiveness to arsenic trioxide treatment

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Arsenic trioxide (As_2O_3) is a highly effective treatment for patients with refractory/relapsed acute promyelocytic leukemia (APL), but resistance to As_2O_3 has recently been seen. In the present study, we report the findings that 2 of 15 patients with refractory/relapsed APL treated with As_2O_3 were clinically As_2O_3 resistant. Leukemia cells from these 2 patients harbored missense mutations in promyelocytic leukemia gene–retinoic acid receptor-α gene (*PML-RARA*) transcripts, resulting in amino acid substitutions of A216V and L218P in the PML B2 domain. When wild-type or mutated PML-RARA (PR-WT and PR-B/L-mut, respectively) were overexpressed in HeLa cells, immunoblotting showed SUMOylated and/or oligomerized protein bands in PR-WT but not in PR-B/L-mut after As_2O_3 treatment. Protein-localization analysis indicated that PR-WT in the soluble fraction was transferred to the insoluble fraction after treatment with As_2O_3, but PR-B/L-mut was stably detected in fractions both with and without As_2O_3. Immunofluorescent microscopy analysis showed PR-WT localization as a microgranular pattern in the cytoplasm without As_2O_3 and as a macrogranular pattern with As_2O_3. PR-B/L-mut was diffusely observed in the cytoplasm with and without As_2O_3. Nearly identical localization patterns were observed in patients' primary cells. Therefore, B2 domain mutations may play an important role in aberrant molecular responses to As_2O_3 and may be critical for As_2O_3 resistance in APL. (*Blood.* 2011;118(6):1600-1609)

Introduction

Acute promyelocytic leukemia (APL) is characterized by the reciprocal chromosomal translocation t(15;17)(q22;q21), leading to fusion of the promyelocytic leukemia gene (*PML*) on chromosome 15 and the retinoic acid receptor-α gene (*RARA*) on chromosome 17.[1] *PML-RARA* fusions are detectable in > 95% of patients with APL. In 1985, *all-trans* retinoic acid (ATRA) was introduced for the treatment of APL as a differentiation therapy, and a dramatic improvement in the overall survival of patients with APL has been obtained.[2-4] However, approximately 10%-30% of patients eventually relapse after treatment with combination chemotherapies with ATRA.[5-7]

Arsenic trioxide (As_2O_3) is a critical drug for the treatment of APL and is clinically effective even in ATRA-resistant patients.[8] As_2O_3 is a natural substance that has been used medically for over 2400 years. In the 1970s, a group in China identified As_2O_3 as a component of an anticancer reagent.[9] Over the last 18 years, clinical trials conducted worldwide have demonstrated the efficacy of As_2O_3 for the treatment of relapsed patients with APL.[10,11] Recently, it was also reported that As_2O_3 improves event-free survival and overall survival of adult APL when As_2O_3 is used as a consolidation treatment after obtaining the first remission.[12] Currently, the role of As_2O_3 in frontline therapy is under investigation.[10,13]

Rapid degradation of PML-RARA via targeting of PML has been reported as a molecular mechanism for the effectiveness of As_2O_3.[14] Furthermore, As_2O_3 induces posttranslational modifications of PML-RARA with small ubiquitin-related modifier (SUMO) and ubiquitin, resulting in the transfer of PML-RARA from the soluble fraction to the insoluble nuclear matrix[14] and the degradation of both PML and PML-RARA.[14-17] In addition to the significant clinical effectiveness of As_2O_3 for patients with APL, acquired resistance to As_2O_3 therapy has been recognized in clinical practice.[18] Several studies have indicated that arsenic-resistant NB4 cells in vitro show higher glutathione levels than in parental cells.[19-21] However, the detailed molecular mechanisms of resistance to As_2O_3 remain unclear.

Very recently, 2 studies reported that As_2O_3 binds directly to cysteine residues in zinc fingers located within the RBCC motif that contains 3 cysteine-rich zinc-binding domains, a RING-finger (R), 2 B-box motifs (B1 and B2), and a coiled-coil (CC) domain,[22,23] in PML-RARA and PML.[24,25] An intriguing hypothesis is that impairment of As_2O_3 binding to PML-RARA due to conformational changes may result from genetic mutations and/or abnormal posttranslational modifications. These events may be related to resistance to As_2O_3 therapy.

We report the clinical significance and frequency of As_2O_3 resistance in patients with APL. Fifteen patients with APL were treated with As_2O_3 after combination chemotherapy with ATRA, and 2 patients showed clinical As_2O_3 resistance. Interestingly, in both of these As_2O_3-resistant patients, missense genetic mutations in the *PML-RARA* fusion transcript were observed in the leukemia cells. We demonstrated that the mutations, which were located in the PML RBCC region, were critical for PML localization and As_2O_3 responsiveness in vitro. Our observations suggest that acquired genetic mutations in the *PML-RARA* transcript may be a critical molecular mechanism of resistance to As_2O_3 therapy.

Methods

Patients

From January 2000 to December 2008 at Nagoya University Hospital, Japan, 15 patients with APL who showed relapse or disease progression

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Table 1. APL patients treated with As_2O_3 at Nagoya University Hospital

No.	Age, y	Sex	Diagnosis	Treatments prior to As_2O_3	Disease status at As_2O_3	Treatments after As_2O_3	Outcome	Survival after As_2O_3	As_2O_3 resistance
1	61	M	M3v	A+CT	Rel1	(−)	D	6 y, 1 mo	+
2	35	M	M3	A+CT	Rel1	sib-PBSCT	A	3 y, 8 mo	−
3	30	M	M3	A+CT	Rel2	auto-PBSCT	A	6 y, 9 mo	−
4	41	M	M3	A+CT	Rel1	auto-PBSCT	A	5 y, 8 mo	−
5	62	M	M3	A+CT, HD-Ara-C	Rel3	CBT	D	10 mo	−
6	42	F	M3	A+CT, aPBSCT	Rel2	CBT	D	4 mo	+
7	46	F	M3	A+CT	2nd CR	auto-PBSCT	A	6 y, 7 mo	−
8	54	F	M3	A+CT	Rel2	auto-PBSCT	A	5 y, 9 mo	−
9	19	M	M3	A+CT	Rel1	UR-BMT	A	6 y, 1 mo	−
10	42	M	M3	A+CT, UR-BMT	Rel3	DLI	A	6 y	−
11	61	M	M3v	A+CT	Rel1	(−)	D	4 mo	−
12	48	M	M3	A+CT, HD-Ara-C	Rel2	auto-PBSCT	A	4 y, 9 mo	−
13	39	M	M3v	A+CT, UR-BMT	Rel3	CBT	D	5 mo	−
14	20	M	M3	A+CT	Rel1	auto-PBSCT	A	4 y, 9 mo	−
15	36	M	M3	A+CT	Rel1	auto-PBSCT	A	4 y, 2 mo	−

Fifteen patients were treated with As_2O_3 at Nagoya University Hospital during the period of January 2000–December 2008. Outcomes were confirmed on December 1, 2009. Patients 5, 6, and 13 received cord blood transplantation after As_2O_3. Patients 5 and 13 died of complications of transplantation without any relapse sign. Patient 6 died of relapse just after transplantation. Patient 11 died of brain bleeding due to APL relapse with disseminated intravascular coagulation.

Rel1 through 3 indicates the first to third relapse; A+CT, ATRA with combination chemotherapy; PBSCT, peripheral blood stem cell transplantation; CBT, cord blood transplantation; BMT, bone marrow transplantation; D, dead; and A, alive.

after treatment with chemotherapy with ATRA were treated with As_2O_3 (Table 1). The diagnosis of APL and its relapse were confirmed by bone marrow morphology according to the FAB classification, chromosomal abnormality t(15;17) in peripheral blood and/or bone marrow cells, positive RT-PCR assay for *PML-RARA* transcripts, and/or FISH analysis of *PML* and *RARA*. As_2O_3 was diluted in 500 mL of 5% dextrose and administered intravenously over 2 hours at a dose of 0.15 mg/kg daily for a cumulative maximum of 60 days.

Patient 1 (Table 1 and Figure 1A) was diagnosed with APL (the microgranular variant M3v) in October 1998 (Figure 1A), and complete remission (CR) was obtained after combination chemotherapy with ATRA (45 mg/m^2/d). However, relapse with insufficient response to ATRA was observed (Figure 1A) after the end of consolidation therapy in August 1999. As_2O_3 (0.15 mg/kg/d) was started as a salvage chemotherapy, and a molecular response was obtained. The effectiveness of As_2O_3 gradually decreased during the patient's 7-year clinical course. Am80 (6 mg/m^2/d) was started in July 2005 in addition to As_2O_3 therapy. However, the effectiveness was poor, and Am80 was discontinued in October 2005. Thereafter, only As_2O_3 was used. At the terminal stage of his clinical course (Figure 1A), As_2O_3 was administered under the condition that > 90% of his peripheral blood cells were considered APL blast cells, but no response to the elevated blast count was observed. We diagnosed this condition as secondary As_2O_3 resistance. The patient died of disease progression in January 2006. During his 7-year clinical course, clinical samples from his bone marrow and/or peripheral blood were obtained repeatedly (Figure 1A).

Patient 6 was diagnosed with APL in January 2000, and CR was obtained after combination chemotherapy with ATRA. Relapse was observed in February 2002. After obtaining a second CR with combination chemotherapy, autologous peripheral blood stem cell transplantation was performed in October 2002. An early relapse was observed after transplantation in February 2003, and then As_2O_3 (0.15 mg/kg/d) was started. Partial cytoreduction was confirmed, but CR was not obtained. We diagnosed this condition as primary refractory disease to As_2O_3. As_2O_3 was used for 42 days and then discontinued. Cord blood transplantation was performed during non-CR, but the patient died of relapsed disease in July 2003. Genomic DNA was obtained from leukemia cells harvested 27 days after starting As_2O_3 therapy. Although the patient's bone marrow showed hypocellularity at this time, 97.1% of her marrow cells were positive for the *PML-RARA* fusion gene with FISH analysis.

Cell preparation from patients

After obtaining informed consent from each patient in accordance with the Declaration of Helsinki, the patients' primary cells were obtained from their peripheral blood and/or bone marrow. All experiments were conducted with institutional review board approval from the Nagoya University School of Medicine. Mononuclear cells were separated with Ficoll Paque (GE Healthcare) and preserved with CP-1 (Kyokuto Pharmaceutical Industrial) in liquid nitrogen until further analysis.

RNA extraction and RT-PCR

Total RNA was isolated from each sample using TRIzol (Invitrogen). cDNA was synthesized from 5 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) as described previously.[26,27] PCR was performed using LA-Taq polymerase (Takara) under the following conditions: one cycle of 95°C for 4 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. PCR primers for amplification of the coding sequences of *PML-RARA* are as follows: forward PR-U619: 5′-TGT TCC AAG CCG CTG T-3′, reverse PR-L2189: 5′-CAT CTT CAG CGT GAT CA-3′. Amplified PCR fragments were purified with a Wizard PCR prep DNA purification kit (Promega) and cloned into the pCR2.1-TOPO cloning vector (Invitrogen). At least 20 clones were sequenced with an ABI 310 automated DNA sequencer (Applied Biosystems). Genetic mutations were confirmed using MacVector Version 10.5.1 software.

Expression vectors

The coding sequence of *PML-RARA* was amplified with PCR, and the Flag sequence was added with the forward primer as described previously.[26,28] The PCR fragment was cloned into the pcDNA4-His-Max-TOPO mammalian expression vector (Invitrogen) to generate the following expression vectors: pcDNA4-XF-PR-WT for Xpress-tagged wild-type PML-RARA, pcDNA4-XF-PR-B/L-mut for Xpress-tagged PML-RARA with mutations resulting in A216V and G391E substitutions, pcDNA4-XF-PR-B2-mut for PML-RARA with the A216V substitution, and pcDNA4-XF-PR-L-B2-mut2 for the long-form PML-RARA with the L218P substitution. To express the long form of the wild-type PML-RARA protein, pcDNA4-XF-PR-L was used as described previously.[29] Expression vectors pcDNA-F-Ubc9, pcDNA-F-SUMO, and pcDNA-HA-PML for Flag-tagged Ubc9 and SUMO1, and HA-tagged PML, respectively, have also been described previously.[30]

Cell culture

Cells of the human cervical cancer cell line HeLa were cultured in DMEM containing 10% FCS. U937 cells, a human monocytic leukemia cell line, were cultured in RPMI containing 10% FCS.

Protein extraction and antibodies for immunoblotting

HeLa cells (1.0×10^5/well) were cultured in a 12-well plate for 12 hours before transfection. Transfection of the expression vectors was carried out using Effectene (Invitrogen) according to the manufacturer's instructions. The cells were washed with DMEM 24 hours after transfection, and then incubated for 8 hours with or without 10μM As_2O_3 (Sigma-Aldrich) until protein extraction. Whole-cell protein samples for immunoblotting were obtained using 250 μL of Laemmli sample buffer (200mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue). After boiling for 5 minutes, samples were subjected to SDS-PAGE.

U937 cells were cultured in a 12-well plate for 12 hours before transfection. Transfection of the expression vectors pcDNA4-XF-PR-WT and pcDNA4-XF-PR-B/L-mut was carried out using Nucleofector Kit C (Lonza) according to the manufacturer's instructions. After 12 hours, immunofluorescent analysis was performed.

To separate PML-RARA protein into soluble and/or insoluble fractions, cells were lysed in 200 μL of RIPA lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2mM PMSF, and a complete mini protease inhibitor tablet [Roche]). After centrifugation at 10 000*g* for 10 minutes, the supernatants were placed into new tubes, and 200 μL of 2× SDS sample buffer was added (soluble fraction). PBS (20 μL) and 200 μL of 2× SDS sample buffer were added to the pellets (insoluble fraction). After boiling for 5 minutes, samples were analyzed by SDS-PAGE followed by immunoblotting. Antibodies used in this assay are as follows: rabbit anti-hemagglutinin (anti-HA; Sigma-Aldrich), mouse anti-Xpress tag (Invitrogen), and mouse anti–FLAG-M2 (Sigma-Aldrich).

Immunofluorescence microscopy

HeLa cells expressing Flag- and Xpress-tagged PML-RARA and its mutated proteins were cultured on Chamber Slides (Lab-Tek) with or without 10μM As_2O_3. U937 cells expressing Flag- and Xpress-tagged PML-RARA and its mutated proteins and the primary leukemia cells were placed on slide glasses using Cytospin (Shandon Southern Products), air dried, and fixed in acetone/methanol for 10 minutes at −20°C. Cells were then blocked with 1% BSA (Sigma-Aldrich) in PBS for 1 hour, incubated with primary antibodies for 3 hours, and incubated with Alexa Fluor 488 (green)- or 568 (red)–conjugated secondary antibodies for 1 hour at room temperature. Antibodies used in this assay are as follows: rabbit anti–human PML (Santa Cruz Biotechnology), mouse anti–FLAG-M2 (Sigma-Aldrich), rabbit Alexa Fluor 488 (Invitrogen), and mouse Alexa Fluor 568 (Invitrogen). The slides were examined with an Axioskop 2 fluorescence microscope (Carl Zeiss), photos were taken and analyzed with AxioVision FRET Release 4.5, and images were processed with Adobe Photoshop CS3 software.

Results

Two of 15 patients showed clinical As_2O_3 resistance

From January 2000 to December 2008 in Nagoya University Hospital, 15 relapsed patients with APL, including 3 with M3v,[31] were treated with As_2O_3 (Table 1). As a first-line treatment, combination chemotherapies with ATRA were administered to all patients. Thirteen patients received autologous or allogeneic stem cell transplantation after treatment with As_2O_3, and long-term remission (range, 44-81 months) was confirmed in 10 patients. One patient (patient 1; see also "Patients") showed resistance to As_2O_3 after repeated therapy. Another patient (patient 6; see also "Patients") showed resistance to the first course of As_2O_3 therapy. Disease progression (elevation of the blast count in the peripheral blood) was observed in patient 1 after long-term treatment with As_2O_3, and primary refractory disease that was resistant to As_2O_3 was confirmed in patient 6. Both patients died after disease progression.

Acquired missense mutations in PML-RARA transcripts observed in the 2 patients with As_2O_3 resistance

To determine the molecular mechanisms of the resistance to As_2O_3, we first focused on patient 1 who had a long clinical course and whose clinical samples had been preserved several times at each disease stage.

Total RNA was extracted from each sample, and RT-PCR for *PML-RARA* transcripts was performed. DNA sequencing analysis using the sample obtained from the terminal stage was performed first (Figure 1A sample 5). The *PML-RARA* transcript was a short-form type ($bcr3^{32}$) that lacked the nuclear localizing signal (NLS) in PML. Missense mutations resulting in the A216V substitution in the PML-B2 domain and the G391E substitution in the RARA ligand-binding domain (LBD; Figure 1B) were detected. The predicted mutated PML-RARA protein resulting from these mutations is shown in Figure 2A.

We then determined the sequence of *PML-RARA* in patient #6, who showed primary refractory disease to As_2O_3. Only a limited clinical sample obtained at 27 days after starting the As_2O_3 treatment was available for genomic analysis. A missense mutation resulting in an L218P substitution in the PML-B2 domain was confirmed in 2 of 20 clones with PCR cloning using genomic DNA PCR (Figure 1C). The mutations in the PML-B2 domain are indicated as B2-mut and B2-mut2 in Figure 1D. The predicted mutated PML-RARA protein designated as PR-L-B2-mut2 is also shown in Figure 2A. The location of these mutations is very close to the As_2O_3-binding cysteine-cysteine (CC) motif reported by Jeanne et al.[25]

Clonal expansion of PR-B/L-mut at the terminal stage of APL disease progression

To confirm the clonal expansion of the genetic mutations in *PML-RARA*, we performed sequencing analysis using the RT-PCR fragments of *PML-RARA* transcripts from the serial clinical samples obtained from patient 1 (samples 1-5 in Figure 1A). PCR fragments were cloned into the vector, and at least 20 clones were picked for sequencing analysis. Genetic mutations resulting in PR-B2-mut, PR-LBD-mut, and PR-B/L-mut (Figure 2A) were confirmed in samples 4 and 5 obtained at this patient's terminal stage when As_2O_3 resistance and the expansion of the blast count were clinically observed (Figure 2B). These clones were not confirmed in samples 1-3, and the partial response to As_2O_3 treatment was confirmed at the periods 2-3. The samples 2 and 3 had blasts showing FISH-positive *PML-RARA* clones (33.9% and 74.0%, respectively). This result strongly suggests that these mutations were closely related to disease progression during As_2O_3 treatment.

Lack of multimerization of PR-B/L-mut with and without As_2O_3

Posttranslational modification of PML, including SUMOylation, is reported to be critical for the responsiveness to As_2O_3.[24,25,33] To confirm the functional difference between PR-WT and its mutant, we performed an in vitro SUMOylation assay in HeLa cells. HA-tagged PML, Xpress-tagged PR-WT, PR-B/L-mut, and SUMO1/Ubc9 were expressed in HeLa cells with or without 10μM As_2O_3 (Figure 3A-B). PML, PR-WT, and PR-B/L-mut were detected with immunoblotting. SUMO1/Ubc9 is coexpressed with PML, and therefore, SUMOylated PML bands were observed (indicated with black triangles in Figure 3A lane 2). The intensity of the mobility-shifted bands was increased with As_2O_3 treatment (Figure 3A lane 3). When using PR-WT, multimerized/SUMOylated

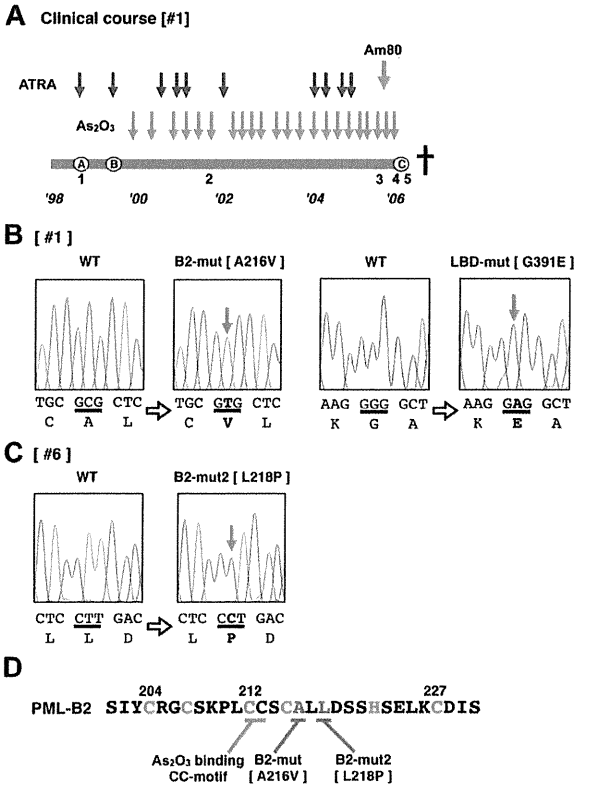

Figure 1. Additional genetic mutations in *PML-RARA* in patients showing an As_2O_3 refractory/resistant phenotype. (A) Clinical course of patient 1, who showed As_2O_3 resistance in the terminal stage of disease progression. The total clinical duration of the patient was almost 7 years. Detailed information is described in "Methods." Black and gray arrows indicate the course of chemotherapies with ATRA, As_2O_3, and Am80. The letters A-C enclosed in circles indicate clinically important time points: A indicates diagnosis with APL, B indicates relapse with inadequate response to ATRA, and C indicates confirmation of clinical As_2O_3 resistance. This patient died of disease progression. Leukemia cells from the bone marrow and peripheral blood were obtained at time periods 1-5. Genetic mutations in *PML-RARA* were confirmed in patients 1 (B) and 6 (C). Missense point mutations in the PML B2 domain and RARA LBD domain were confirmed in the leukemia cells from time point 5. DNA sequences and the genetic code are indicated in capital letters. Bold letters indicate mutations. (D) The zinc finger motif of the PML-B2 domain. Amino acid substitutions in patients 1 and 6 are depicted. The CC motif critical for As_2O_3 binding[25] is also indicated. The "C" and "H" in red are the cysteine and histidine, respectively, which are important for zinc finger formation.

PR-WT bands were confirmed with and without As_2O_3 (Figure 3B lanes 4-6 black triangles). However, when using PR-B/L-mut, oligomerized/SUMOylated PML-RARA protein was not observed with SUMO1/Ubc9 with or without As_2O_3 (Figure 3B lanes 8 and 9). When PR-B2-mut was used in the same assay system, nearly the same result was obtained (Figure 3B lanes 11 and 12). These results indicate that the genetic mutation leading to amino acid alteration of the PML-B2 domain is critical for the appropriate posttranslational modification of the PML-RARA protein, including SUMOylation and oligomerization.

Distinct cellular localization of PR-WT and PR-B/L-mut in soluble/insoluble fractions

Recently, it has been reported that As_2O_3 promotes PML-RARA multimerization via disulfide-mediated covalent binding, leading to formation of PML nuclear bodies; these multimers are subsequently SUMOylated.[25] To confirm the cellular localization (soluble/insoluble fractions, see also "Methods") of PML, PR-WT, and PR-B/L-mut with or without As_2O_3, we performed immunoblotting using HeLa cells that were transiently overexpressing each protein. Cells were lysed using RIPA buffer, as described previously,[14,24] and the whole-cell lysate was separated into 2 fractions, soluble (supernatant) and insoluble (pellet). In the absence of As_2O_3, PML was localized in both the soluble and insoluble fractions (Figure 4A lanes 1 and 3). With As_2O_3, PML was detected mostly in the insoluble fraction (Figure 4A lanes 2 and 4). Nearly the same results were obtained with PR-WT (short form of PML-RARA, Figure 4B lanes 5, 6, 11, and 12) and PR-long (long form of PML-RARA, Figure 4B lanes 9, 10, 15, and 16). Conversely, PR-B/L-mut was localized in both the soluble and insoluble fractions with and without As_2O_3, and protein modification including multimerization was not observed with this assay system (Figure 4B lanes 7, 8, 13, and 14). Protein expression levels were quantitated with BioMax software, and the relative intensity is depicted in Figure 4C. These findings indicate that As_2O_3 did not affect PR-B/L-mut compared with PML and PR-WT in this assay system, and this phenomenon was closely related to insufficient SUMOylation (Figure 3).

Distinct cellular localization of PR-WT and PR-B/L-mut with or without As_2O_3 in HeLa and U937 cells

We then performed immunofluorescent (IF) staining to examine the cellular localization pattern of wild-type PML, PR-WT, and PR-B/L-mut in HeLa cells (Figure 5). Wild-type PML was overexpressed in HeLa cells, which were incubated with or without As_2O_3. PML localization was confirmed by IF staining using an anti-PML antibody. PML was localized in PML nuclear bodies showing a speckled pattern without As_2O_3 (Figure 5Ai,iii). In the presence of As_2O_3, the localization pattern was clearly altered to a macrogranular pattern (Figure 5Aiv,vi).

Similar analyses using PR-WT- and PR-B/L-mut–expressing HeLa cells were also performed (Figure 5B-C). Anti-PML antibody was used to detect endogenous PML, overexpressed PR-WT, and PR-B/L-mut, and anti-Flag antibody was used to detect PR-WT and PR-B/L-mut. In the absence of As_2O_3, PR-WT, a PML-RARA short form without an NLS, was detected around the nucleus as a microgranular pattern (Figure 5Bi,ii,iv), and as a macrogranular pattern in the presence of As_2O_3 (Figure 5Bv,vi, and viii). Surprisingly, PR-B/L-mut was localized differently in the cytoplasm as a diffuse pattern without As_2O_3 (Figure 5C,i,ii,iv), and the localization of PR-B/L-mut was not altered in the presence of As_2O_3 (Figure 5Cv,vi,viii). With As_2O_3 treatment, endogenous PML was confirmed in the nucleus with the macrogranular pattern (Figure 5Cv,viii). Transfected PR-B/L-mut (red fluorescence) could not be confirmed in the nucleus (Figure 5Cvi). The localization of PR-B2-mut showed almost the same pattern as PR-B/L-mut (data not shown). These data strongly suggest that the point mutation in the PML-B2 domain disrupts or inhibits PML body formation and the responsiveness to As_2O_3 treatment.

Nearly identical analyses using U937 cells were performed to confirm the significance in hematopoietic cells. As indicated in Figure 5D, PR-WT was observed in the cytoplasm with the microgranular pattern (Figure 5Di,ii,iv), and PR-B/L-mut showed a diffuse pattern, as observed in HeLa cells (Figure 5Dv,vi,viii).

Distinct cellular localization of PR-L and PR-L-B2-mut2 with or without As_2O_3 in HeLa cells

We also performed a similar analysis using PR-L and PR-L-B2-mut2 to show the molecular significance of the L218P mutation

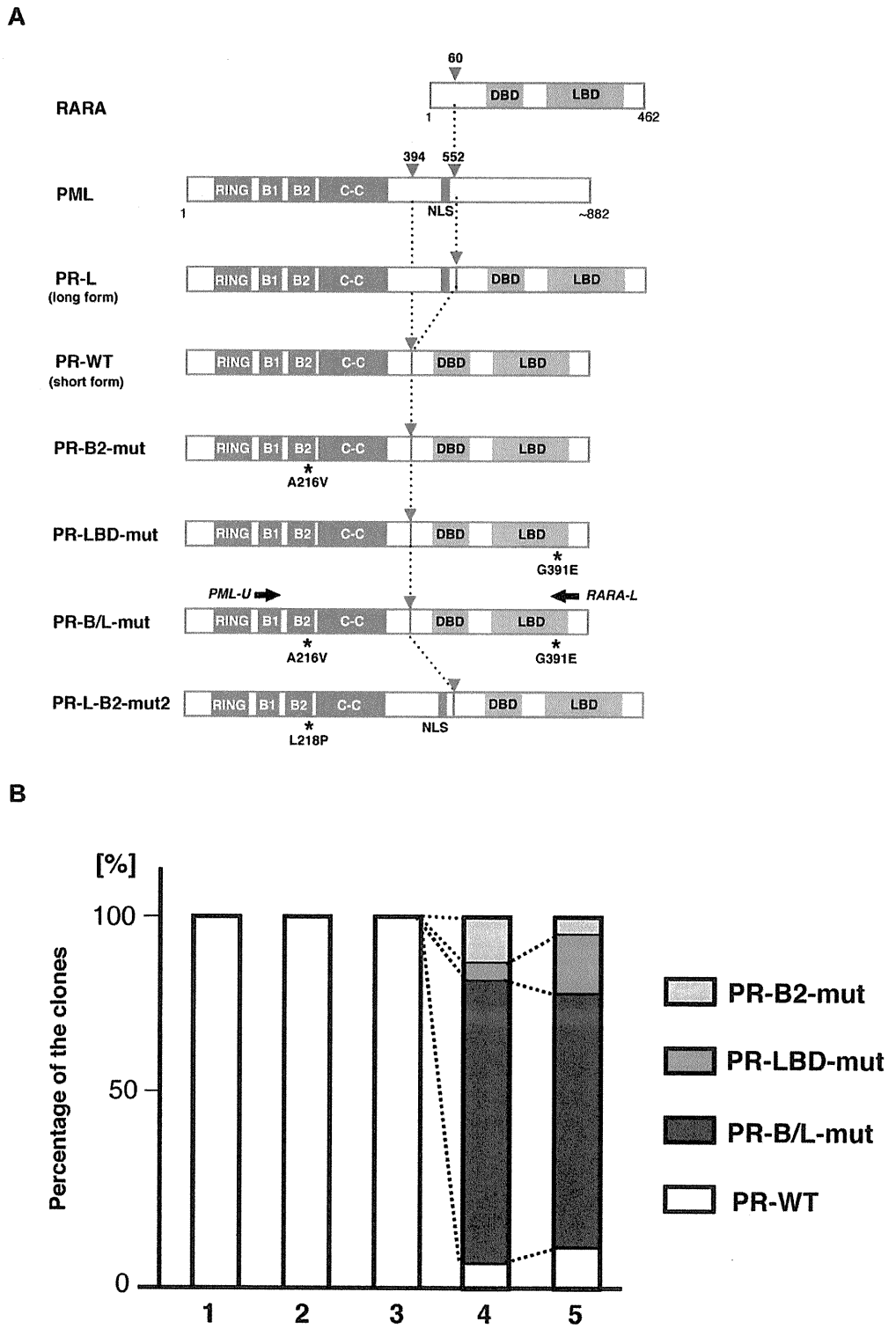

Figure 2. Clonal expansion of PML-B2 and RARA-LBD mutations during disease progression. (A) Schematic representation of PML, RARA, and its fusion proteins with or without mutations. Functional domains are indicated. Gray arrowheads indicate the break points of the fusion proteins. Black asterisks depict amino acid substitutions resulting from genetic mutations. Black arrows indicate the positions of the PCR primers for amplifying *PML-RARA* fusion transcripts. RING indicates the RING finger; B1 and B2, B-box motifs; C-C, coiled-coil; and DBD, DNA-binding domain. (B) Clonal expansion of *PML-RARA* mutants. Using the clinical samples obtained at time points 1-5 in Figure 1A, RT-PCR using PCR primers (PML-U and RARA-L in Figure 1A) followed by cloning was performed. At least 20 clones were sequenced for each sample. The percentages of the clones are depicted in the bar graph.

in the B2 domain confirmed in patient 6 (Figure 6). PR-L, a PML-RARA long form with an NLS, was detected in the nucleus as a microgranular pattern without As_2O_3 (Figure 6Ai,ii,iv) and as a macrogranular pattern with As_2O_3 (Figure 6Av,vi,viii). In contrast, PR-L-B2-mut2 was localized in the nucleus as a diffuse pattern without As_2O_3 (Figure 6Bi,ii,iv). The localization was not altered in