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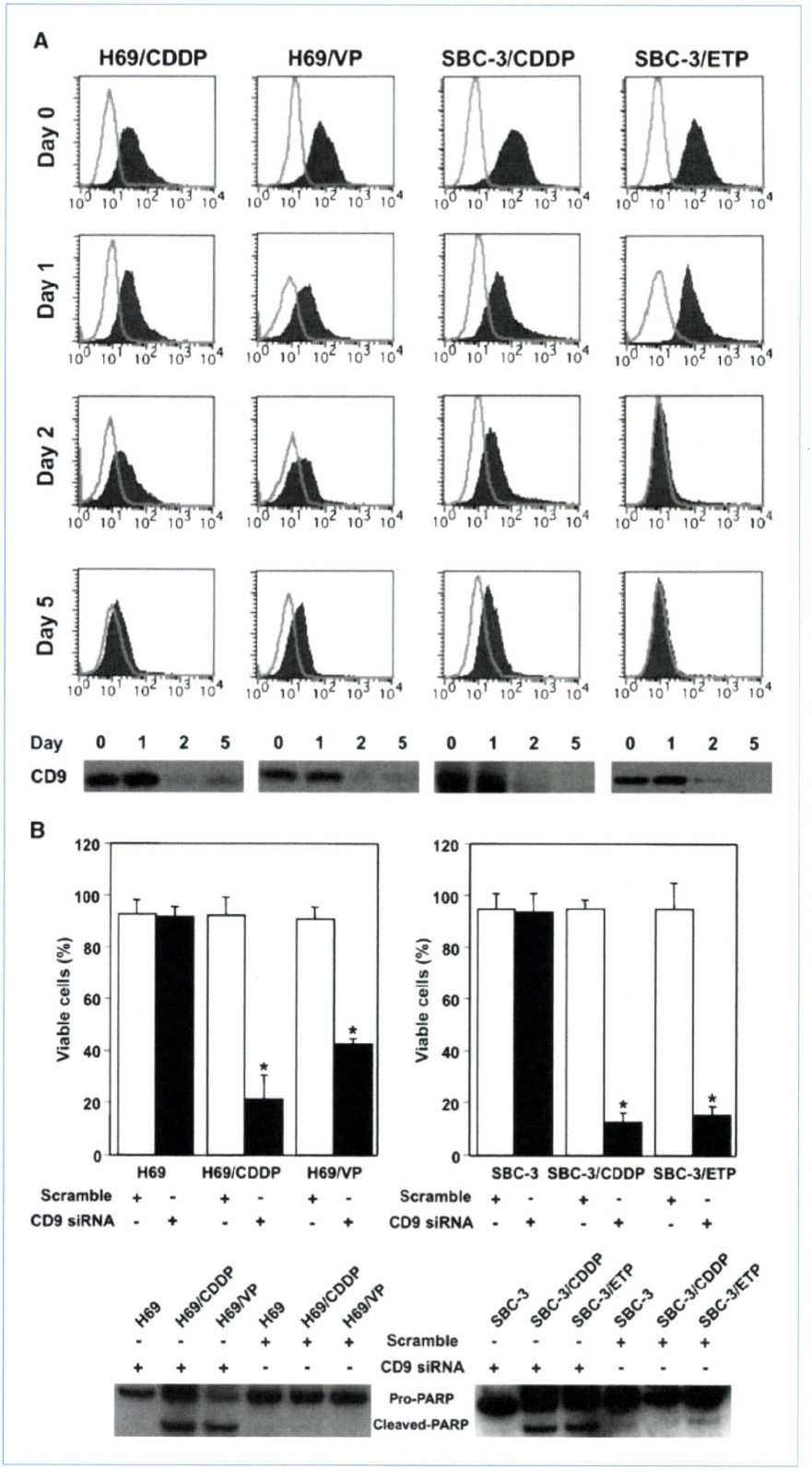

Figure 6. *CD9* siRNA leads chemoresistant SCLC cells to apoptotic cell death. A, successful knockdown of CD9 from day 2 to day 5 was confirmed by immunoblot and FACS analyses. B, relative values of viable cell numbers were quantified by MTT assay on day 5 and shown as mean (columns) ± SD (bars) from three independent experiments. *CD9* siRNA (black) but not scramble RNA (white) induced apoptosis accompanied with PARP cleavage exclusively in chemoresistant clones. *, $P < 0.0001$ versus scramble-transfected cells.

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clones adhered to fibronectin via β1 integrin significantly more tightly than the respective parental clones in which CD9 was strongly concerned (Fig. 3). Therefore, chemoresistant SCLC cells probably strengthen survival signals from ECM in cooperation with upregulated CD9 in their local environment.

Based on the CAM-DR theory, stubborn adhesion to ECM would make cancer cells less mobile but offer them integrin-mediated survival advantage in the face of chemotherapy (38, 39), and in this setting, upregulated CD9 might play a role in strengthening this mechanism. Chemoresistant clones and CD9 transfectants actually became less mobile than their respective parental cells (Fig. 4A and B). Conversely, loose adhesion to ECM would give cancer cells adequate mobility at the sacrifice of increased susceptibility to chemotherapy-induced apoptosis. It is necessary for chemoresistant SCLC cells to restore motility to metastasize to new distant sites during the "treatment holiday." We have previously reported that CXCL12 secreted from stroma in such organs as bone marrow and lymph nodes enhanced the motility of SCLC cells and contributed to their site-specific metastasis to these organs (36). The motility of chemoresistant clones transiently increased to the level of parental cells in response to CXCL12 coupled with downregulation of CD9 (Fig. 4A and B). To form *de novo* metastases without affecting their chemosensitivity, it is reasonable and advantageous for chemoresistant cells that biological activity of CXCL12 on their motility and CD9 expression does not last so long (Fig. 4C). Thus, CD9 and CXCL12 might dynamically regulate cellular motility to provide cancer cells with suitable conditions spatially as well as temporally in the CAM-DR mechanism (Fig. 4D).

Selective targeting of CD9 by ALB6 and siRNA both led CD9-expressing SCLC cells to apoptotic cell death, suggesting that CD9 is a regulator of cell survival (Figs. 5 and 6). Murayama and colleagues reported that CD9 was commonly expressed in cancer cells originating from the digestive system, including stomach, colon and pancreas, and that ALB6 induced the apoptosis of these CD9-expressing cells coupled with transient activation of JNK/SAPK and p38 (40), which was consistent with our results in SCLC (Fig. 5B). ALB6 treatment of mice bearing CD9-expressing human gastric cancer cells has been recently shown to successfully inhibit tumor progression via not only antiproliferative and proapoptotic effects on malignant cells but also antiangiogenic effects on tumor vessels reducing microvessel density (42). Inhibitory effects of anti-CD9 mAb on *in vivo* tumorigenicity as well as *in vitro* proliferation of human colon carcinoma cells has also been reported (43). Regulation of tumor angiogenesis by ALB6 *in vivo* is likely and acceptable because another anti-CD9 mAb (ALMA.1) inhibited human vascular endothelial cell migration toward ECM proteins at wound lesion and impaired its repair (44). Because targeting tumor vessels by bevacizumab provides advantages over traditional antitumor approaches (7), the anti-CD9 antibody is an attractive tool targeting both tumor cells and vessels in one molecule.

Furthermore, antibody-dependent cellular cytotoxicity might have participated in antitumor activity by anti-CD9 antibodies *in vivo* (42, 43), although the authors did not refer to the matter. IgG_1 subclass antibodies such as trastuzumab, rituximab, and cetuximab are well known to preferentially induce this immune reaction because they have longer half-lives and could bind to all subtypes of FcγR on effector cells (45–48). In this context, IgG_1 antibody directed against CD9 such as ALB6 is expected not only as an apoptosis inducer for cancer cells and inhibitor of angiogenesis but also as a potent antibody-dependent cellular cytotoxicity mediator. Thus, targeting CD9 is still the more attractive option in antitumor strategy.

Collectively, our present study clarified the molecular significance of CD9 in CAM-DR mechanism in SCLC. Moreover, we showed the possibility of using this molecule as a novel therapeutic target to overcome drug resistance especially in the refractory stage. Finally, the implication of targeting CD9 for therapeutic strategies in SCLC is promising to bring about better prognosis of this devastating malignancy and is valuable for future clinical applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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BJUI
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Infiltration of tumour-associated macrophages in prostate biopsy specimens is predictive of disease progression after hormonal therapy for prostate cancer

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Study Type – Prognostic (case series)
Level of Evidence 4

OBJECTIVE

To evaluate tumour-associated macrophage (TAM) infiltration in prostate biopsy specimens as a possible prognostic factor for prostate cancer (PCa) after hormonal therapy.

PATIENTS AND METHODS

Immunostaining of TAMs in prostate biopsy specimens was performed using a monoclonal antibody CD68 for 71 patients having PCa treated with hormonal therapy. Six microscopic (×400) fields around the cancer foci were selected for TAM counting.

RESULTS

The median value of serum prostate-specific antigen (PSA) was 50.1 ng/mL, and the median TAM count was 22. Recurrence-free survival was significantly better in patients with fewer TAMs (<22) than in those with higher numbers of TAMs (≥22) ($P < 0.001$). TAM count was higher in those with higher serum PSA (PSA), higher Gleason score, clinical T stage or those with PSA failure. Cox multivariate analysis showed that TAM count is one of the prognostic factors for PCa treated by hormonal therapy ($P < 0.0001$).

CONCLUSIONS

TAM infiltration in prostate needle biopsy specimens is a useful predictive factor for PSA failure or progression of PCa after hormonal therapy.

KEYWORDS

androgen deprivation therapy, prognostic factor, prostate cancer, tumour-associated macrophage

INTRODUCTION

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of cancer-related death in men in the United States [1,2]. Recently, the incidence of PCa has also gradually increased in Japan. Even if patients are diagnosed with clinically localised disease, patients are not always treated by definitive therapy (radical prostatectomy or radiotherapy) because of their limited life expectancy or complications. Primary treatment for metastatic PCa is androgen deprivation therapy (ADT) [3–5], but resistance to ADT is a serious problem in PCa treatment [5]. To date, several clinicopathological factors have been reported as prognostic factors for hormonal therapy [6–13]. However, few studies have reported on prognostic factors that reflect an anti-cancer immune response by the host [14–16].

Leucocytes infiltrate neoplastic tissues, and cells belonging to the monocyte–macrophages lineage are a major component of the leucocyte infiltration of neoplasms. Tumour-associated macrophages (TAMs) originate from circulating blood monocytes. Their recruitment and survival *in situ* is directed by chemokines and by cytokines that interact with tyrosine kinase receptors. TAMs have complex functions in their interaction with neoplastic cells (the 'macrophage balance' hypothesis [17–19]), but strong evidence suggests that they are part of inflammatory circuits that promote tumour progression [17–19].

In the present study, we performed an immunohistochemical analysis of TAMs in biopsy tissues of the prostate to evaluate the prognostic significance of TAM infiltration for PCa.

PATIENTS AND METHODS

One hundred and thirty-one patients who were diagnosed with PCa at our hospital from 1999 to 2002 were selected. Their age at admission ranged from 50 to 88 years (median, 71 years). A diagnosis of PCa was made by histological examination of specimens obtained via transrectal needle biopsy of the prostate. Clinical stage was defined based on the American staging system [20] through DRE, transrectal ultrasonography, X-ray, CT, MRI and bone scintigraphy. Serum PSA levels measured by the immunoenzymatic assay (5.2–5820.0 ng/mL; median, 50.1 ng/mL), and distribution of clinical T stage and Gleason score are shown in Table 1. After diagnosis of PCa and staging, these patients were treated by total androgen blockade with Gn-RH analogue and bicalutamide as initial hormonal therapy.

	Median (range)
Age (years) (n = 71)	74 (50–88)
Prostate-specific antigen (ng/mL) (n = 71)	50.1 (5.2–5820)
	No. of patients (%)
Gleason score	
≤6	21 (29.6)
=7	29 (40.8)
≥8	21 (29.6)
Clinical stage	
T1	9 (12.7)
T2	21 (29.6)
T3	29 (40.8)
T4	11 (15.5)

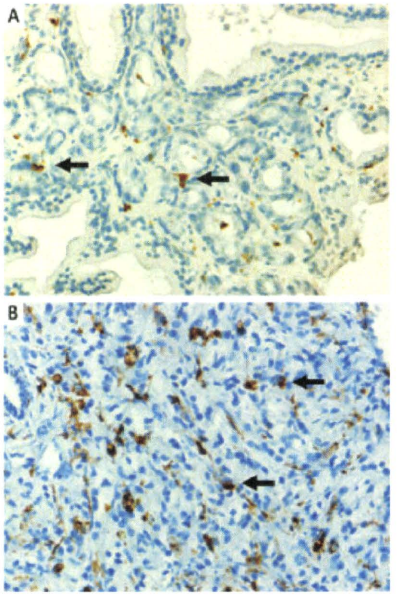
TABLE 1
Patient characteristics

TABLE 2 Association between the TAM count and clinicopathologic factors*

Clinicopathologic factors	Total number of patients (%)	TAM count Mean (SE)	*P* value
Age			
≤74	34 (47.9)	36.58 (23.24)	0.4130
>74	37 (52.1)	31.79 (24.61)	
PSA (ng/mL)			
<50.1	34 (47.9)	30.01 (18.33)	0.0891
≥50.1	37 (52.1)	37.83 (28.66)	
Gleason score			
≤6	21 (29.6)	24.03 (12.92)	0.0437†
=7	29 (40.8)	33.51 (24.96)	0.0025‡
≥8	21 (29.6)	44.94 (28.60)	0.0697§
Clinical T stage			
T≤2	30 (42.3)	25.26 (14.94)	0.0041
T≥3	41 (57.7)	40.54 (27.93)	
PSA failure			
+	29 (32.1)	49.71 (26.29)	<0.0001
–	42 (67.9)	23.29 (15.94)	

**TAM, tumor associated macrophage. †≤6 vs 7, ‡≤6 vs ≥8, §7 vs ≥8. PSA, prostate-specific antigen.*

*FIG. 1. Representative immunostaining for TAMs. Representative cases with low (**A**) and high (**B**) TAM counts are shown. Arrows indicate TAMs in the prostate biopsy specimens.*



Histological specimens from the prostate biopsy were fixed in 10% neutral buffered formalin and routinely processed for paraffin embedding. Serial 5-μm-thick sections were cut and stained with haematoxylin and reviewed by one pathologist (K.A.) to determine Gleason score based on the Gleason grading system [21].

After initial therapy, patients were followed with periodical evaluations of DRE, serum PSA and imaging findings. Progression of PCa was defined by an elevation of serum PSA levels at three consecutive measurements (PSA failure), the existence of local or metastatic recurrent tumours or evidence of symptomatic worsening.

TAMs were immunohistochemically labelled using CD68 monoclonal antibody (1 : 100 dilution). Immunohistochemical analysis of the paraffin sections was carried out using the labelled streptavidin–biotin method (Dako, Glostrup, Denmark). For systematic counting, six ocular measuring fields within a cancer, and each real area of 0.06175 mm^2, were randomly chosen under a microscope at ×400 magnification. The mean number of CD68-positive cells in these six areas was determined as the TAM count for each case.

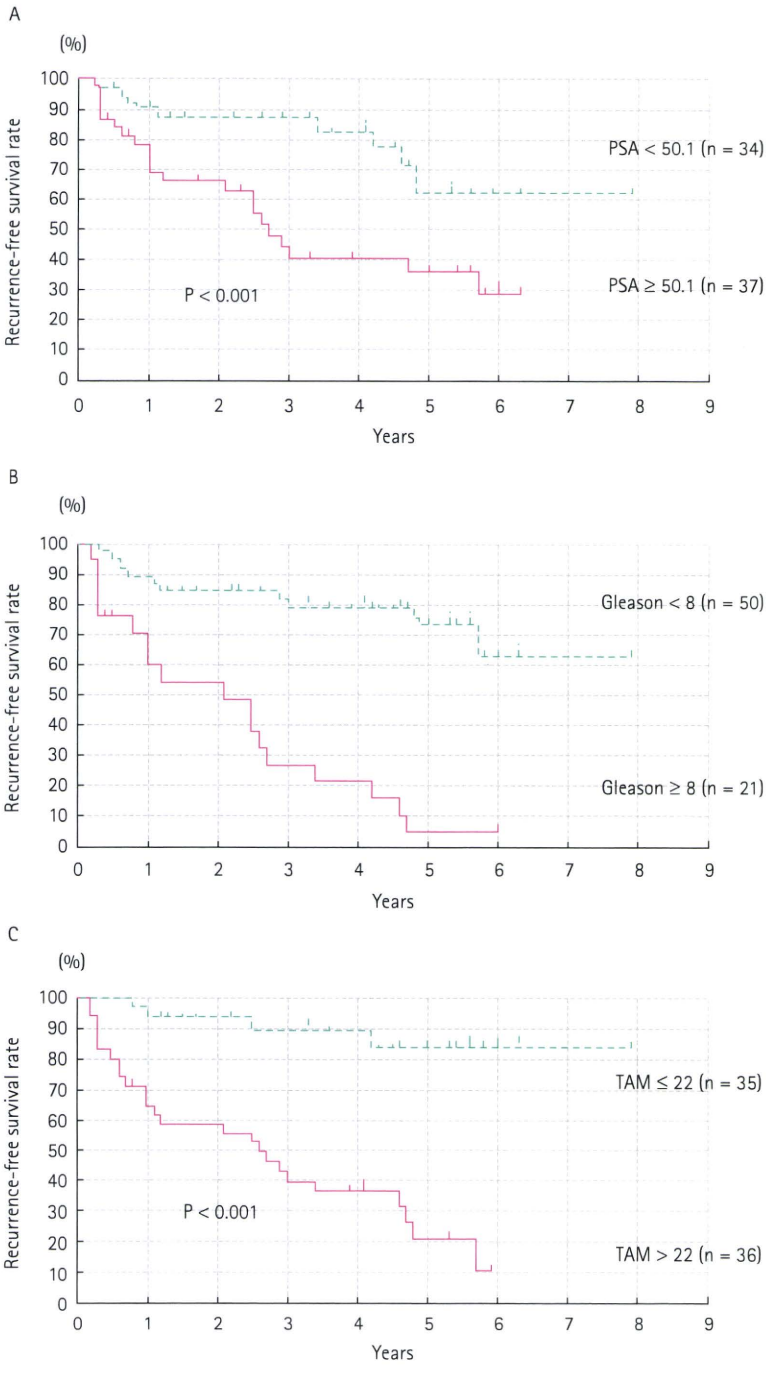
Statistical analysis was performed using StatView software (SAS Institute, Inc., Cary, NC). Correlation between TAM infiltration at immunohistochemistry and clinicopathological parameters was evaluated using the χ^2 test and Fisher's exact probability test. The follow-up period, measured from the date of the start of therapy for survivors, ranged from 2.7 to 181.5 months (mean, 54.5 months). Progression-free and overall survival rates were calculated using the Kaplan–Meier method, and differences in survival curves were estimated with the log-rank test [3]. $P < 0.05$ denoted a statistically significant difference.

RESULTS

CD68-positive cells were observed in all of the specimens tested, and the majority had morphological features of macrophages (Fig. 1). The number of TAMs ranged from 9.00 to 106.33 (median, 22.00). Of the 71 patients, 35 had ≤22 TAMs (lower TAM group), while the TAM count was >22 (higher TAM group) in the other 36 cases.

Table 2 shows the correlation between TAM count and various clinicopathological factors. Patients with a high level of PSA (≥50.1 ng/mL) showed a tendency to have a higher TAM count than those with a low level of PSA, but the difference was not significant (<50.1 ng/mL) (P = 0.0891). Patients with PCa of higher

FIG. 2. ***A****, Progression-free survival of patients with PCa stratified by initial serum PSA levels. The solid line represents the progression-free survival curve of patients with serum PSA not less than 50.1 ng/mL. The dotted line represents the progression-free survival curve of patients with serum PSA <50.1 ng/mL.* ***B****, Progression-free survival of patients with PCa stratified by Gleason scores. The solid line represents the progression-free survival curve of patients with Gleason score not lower than 8. The dotted line represents the progression-free survival curve of patients with Gleason score <8.* ***C****, Progression-free survival of patients with PCa stratified by TAM counts. The solid line represents the progression-free survival curve of patients with a TAM count >22. The dotted line represents the progression-free survival curve of patients with a TAM count not higher than 22.*



stage or higher Gleason score had a higher TAM count. Patients with PSA failure had a significantly higher TAM count than those without ($P < 0.0001$).

The follow-up time ranged from 2.4 to 96.0 months (mean, 34.8 months). Relapse-free survival (RFS) ranged from 2.4 to 68.4 months (mean, 25.2 months). The significance of TAM infiltration as well as serum PSA level and Gleason score for RFS was analyzed. RFS rate in patients with high PSA was significantly lower than in those with lower PSA ($P < 0.001$) (Fig. 2A). RFS rate in patients with a high Gleason score (≥8) was significantly lower than in those with a low Gleason score (<8) ($P < 0.001$) (Fig. 2B). RFS rate was significantly lower in patients with high TAM count than in those with a lower TAM count ($P < 0.001$) (Fig. 2C).

The results of multivariate analysis are shown in Table 3. Cox multivariate analysis revealed that TAM count was a significant prognostic factor ($P < 0.0001$), in addition to PSA ($P = 0.0136$), Gleason score ($P < 0.0001$), extraprostatic extension ($P = 0.0006$), lymph node metastasis ($P = 0.0228$) and distant metastasis ($P = 0.0008$).

DISCUSSION

ADT is now a worldwide accepted therapy for advanced PCa [3–5]. Even localized PCa is often treated with ADT rather than radical prostatectomy or radiotherapy because of life expectancy or complications [22,23]. ADT is one of the effective treatment options for PCa, especially in elderly patients. In Japan, ADT is often chosen by for PCa partly because of its greater efficacy due to racial differences [24]. In using ADT for PCa, it is important to predict its duration of efficacy. To date, clinical stage, PSA level and Gleason score have been reported to be prognostic factors for ADT [8–10]. Time to nadir PSA level (<2 ng/mL) is also reported to be a good predictive factor after ADT [25].

Cancer-associated inflammatory cells, such as macrophages and mast cells, communicate via a complex network of intercellular signalling pathways that are mediated by cell surface adhesion molecules, cytokines and their receptors. These cancer-associated inflammatory cells include the TAMs [17–19]. Activated TAMs have been reported to exert tumouricidal activity directly through the

TABLE 3 Multivariate analysis for prognostic factors

Prognostic factor	Hazard ratio	95% confidence interval	*P* value
TAM* count (>22)	2.697	1.445–5.036	<0.0001
Prostate-specific antigen level (continuous variable)	1.996	1.473–3.999	0.0136
Gleason score (>7)	2.327	1.611–3.362	<0.0001
Extracapsular extention (+)	6.356	2.202–18.353	0.0006
Lymph node metastasis (+)	2.411	1.130–5.142	0.0228
Distant metastasis (+)	3.580	1.700–7.540	0.0008
Digital rectal examination (+)	1.620	0.773–3.398	0.2013

**TAM, tumor associated macrophage.*

production of tumour necrosis factor-α, nitric oxide and reactive nitrogen intermediates, and indirectly through the production of cytokines such as interleukin (IL)-12 and IL-18 [17–19]. TAMs, however, also have been reported to promote tumour progression (the 'macrophage balance' hypothesis) [17–19]. Accumulating evidence suggests that TAMs are part of the inflammatory circuit that promotes tumour progression [17–19]. The presence of TAMs is positively correlated with increased vascularity and disease progression in breast and kidney cancers [26,27]. In our study, TAM infiltration was significantly correlated with serum PSA level, Gleason score or stage among the clinicopathological factors. Shimura *et al.* demonstrated the association between TAM infiltration and disease-free survival after radical prostatectomy using whole mount sections [14]. They demonstrated that disease-free survival is significantly shorter for patients with a high level of TAMs than for those with a low level. The outcome of PCa is variable and difficult to predict before the initiation of therapy. To be able to predict the prognosis of patients with PCa is of great benefit. Our current study was the first to predict the prognosis of patients with PCa before initiation of hormonal therapy, by evaluating infiltration of TAMs in prostate biopsy specimens. This finding is consistent with studies that showed a correlation of high infiltration of TAMs with increased recurrence rate and poor prognosis in other cancers [26,27].

Despite many clinicopathological studies, few have reported on prognostic factors that reflect an immune response by the host against PCa [14–16]. Progression of cancer possibly depends on the aggressiveness of the cancer itself, as well as host immunity such as infiltration of immune cells. That the efficacy of ADT against PCa can also be influenced by the host immune response, such as infiltration of macrophages around cancer cells, is very meaningful.

CONFLICT OF INTEREST

None declared.

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Postoperative whole pelvic radiotherapy plus concurrent chemotherapy versus extended-field irradiation for early-stage cervical cancer patients with multiple pelvic lymph node metastases

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ABSTRACT

Objectives. The aim of this study was to compare the efficacy of postoperative pelvic radiotherapy plus concurrent chemotherapy with that of extended-field irradiation (EFRT) in patients with FIGO Stage IA2–IIb cervical cancer with multiple pelvic lymph node metastases.

Methods. We retrospectively reviewed the medical records of patients with FIGO Stage IA2–IIb cervical cancer who had undergone radical surgery between April 1997 and March 2008. Of these, 55 patients who demonstrated multiple pelvic lymph node metastases were treated postoperatively with pelvic radiotherapy plus concurrent chemotherapy (n = 29) or EFRT (n = 26). Thirty-six patients with single pelvic node metastasis were also treated postoperatively with pelvic radiotherapy plus concurrent chemotherapy. The recurrence rate, progression free survival (PFS), and overall survival (OS) were compared between the treatment groups.

Results. Pelvic radiotherapy plus concurrent chemotherapy was significantly superior to EFRT with regard to recurrence rate (37.9% vs 69.2%, p = 0.0306), PFS (log-rank, p = 0.0236), and OS (log-rank, p = 0.0279). When the patients were treated with pelvic radiotherapy plus concurrent chemotherapy, there was no significant difference in PFS or OS between the patients with multiple lymph node metastases and those with single node metastases. With regards to grade 3–4 acute or late toxicities, no statistically significant difference was observed between the two treatment groups.

Conclusions. Postoperative pelvic radiotherapy plus concurrent chemotherapy is superior to EFRT for treating patients with FIGO Stage IA2–IIb cervical cancer displaying multiple pelvic lymph node metastases.

Introduction

Early stage cervical cancer has traditionally been treated with either radical hysterectomy or primary radiotherapy with similar survival outcomes. According to previous reports, the 5-year survival rate of patients with FIGO Stage Ib–IIa cervical cancer treated with radical surgery ranges from 83 to 91%, which is comparable to the 74–91% reported for those treated with radiotherapy alone [1–3]. In patients with FIGO stage IIb disease, a recent retrospective analysis conducted by a Japanese group suggested similar treatment outcomes for patients treated with radical hysterectomy and those treated by definitive radiotherapy, both of which showed estimated 5-year survival rates of 69% [4].

Several risk factors have been identified that adversely impact on the outcome of patients with early stage cervical cancer who undergo radical surgery as their primary treatment [5–7]. Generally, patients with risk factors such as positive pelvic nodes, parametrial invasion, or a positive surgical margin are regarded as at "high-risk" of recurrence. Moreover, patients with a tumor that is confined to the cervix who display risk factors such as a large tumor, lymph vascular space invasion, or deep stromal invasion are considered to be at "intermediate-risk" of recurrence [5–7]. Postoperative radiotherapy with or without concurrent chemotherapy is usually recommended for patients that display these risk factors.

Among these reported prognostic factors, nodal metastasis remains the single most important prognostic factor in cervical

Abbreviations: MRI, magnetic resonance imaging; OS, overall survival; PFS, progression free interval; ICRT, intracavitary radiotherapy; EBRT, external beam radiotherapy; RT, radiotherapy; CCRT, concurrent chemoradiotherapy; PALN, para-aortic lymph node.

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cancer. It has been reported that pelvic lymph node metastasis is associated with a 30–50% reduction in 5-year survival rates [8].

The number of pelvic node metastases is reported to be a predictor of para-aortic node (PALN) metastasis. According to a previous report, PALN metastasis was observed in 0.5% of patients with one or no metastatic pelvic nodes compared with 27.6% of patients with two or more positive nodes [9]. Moreover, Kim et al. reported that the risk of PALN failure after postoperative pelvic radiotherapy in patients with two or more metastatic pelvic lymph nodes was 10 times higher than that in those with one or no positive nodes [10]. These findings indicate that a significant number of patients with early stage cervical cancer, especially those suffering from multiple pelvic node metastases, harbor occult para-aortic nodal metastases.

With the aim of controlling occult para-aortic nodal metastases and prolonging survival, RTOG initiated a phase III study examining the role of postoperative EFRT for patients with bulky Ib–IIb cervical cancer. The study demonstrated that postoperative EFRT significantly reduced extrapelvic recurrence and improved 10-year overall survival [11]. However, a subsequent RTOG study in the setting of definitive radiotherapy that compared EFRT with pelvic radiotherapy combined with concurrent chemotherapy using cisplatin and fluorouracil demonstrated that pelvic radiotherapy with concurrent chemotherapy is significantly superior to EFRT in terms of survival in patients with FIGO stage Ib–IIb cervical cancer [12]. In addition, a prospective randomized clinical trial (GOG 109/SWOG 87–97) addressing the role of postoperative adjuvant CCRT in early-stage cervical cancer patients with high-risk prognostic factors demonstrated that the addition of concurrent cisplatin-based chemotherapy to pelvic radiotherapy improved survival [13].

On the basis of the results of the above-mentioned trials [12,13], concurrent chemotherapy combined with pelvic radiotherapy has become a standard treatment for cervical cancer [14,15]. However, there has been no direct comparison between postoperative concurrent chemotherapy and pelvic radiotherapy with EFRT in patients with high-risk early stage cervical cancer.

In the current study, we retrospectively evaluated whether postoperative pelvic radiotherapy plus concurrent chemotherapy is superior to EFRT in patients with FIGO Stage IA2–IIB cervical cancer with multiple pelvic node metastases.

Materials and methods

Patients

Permission to proceed with the data acquisition and analysis was obtained from the institutional review board of Osaka University Hospital and Osaka Medical Center for Cancer and Cardiovascular Diseases. A list of patients who had undergone radical hysterectomy (type III) and pelvic lymphadenectomy for FIGO Stage IA2–IIB cervical cancer from April 1998 to March 2008 was generated from our institutional tumor registries. Then, through a chart review, 55 patients who had multiple pelvic nodes metastases and were treated postoperatively with either pelvic radiotherapy plus concurrent chemotherapy (CCRT-group) or extended-field radiotherapy (EFRT-group) were identified, and their clinical data were retrospectively reviewed. A group of patients with early-stage cervical cancer with single pelvic node metastasis that had received postoperative pelvic radiotherapy plus concurrent chemotherapy were also identified through the chart review and used as the control (N1-CCRT-group). Of a total of 36 patients in the N1-CCRT-group, 21 were treated inside the context of the previous clinical study [16].

Treatments

All patients included in this study were treated with radical hysterectomy (type III) and pelvic lymphadenectomy. The lymphadenectomy procedure included complete bilateral pelvic lymphadenectomy with the aim of removing all of the external iliac, internal iliac, common iliac, obturator, suprainguinal, and presacral lymph nodes. Preoperative PALN evaluation was performed in all patients via a CT scan of the pelvis and abdomen as part of the initial evaluation. The intra-operative assessment of PALN was routinely performed by palpation. When PALN metastasis was suspected by CT scan or palpation, a biopsy was taken for confirmation whenever possible. Patients who had biopsy-confirmed PALN metastasis were excluded from the study.

Postoperatively, patients were treated in accordance with the institutional treatment guidelines. In Osaka University Hospital, the patients were treated with pelvic radiotherapy plus concurrent chemotherapy when their pathological report displayed any of the following "high-risk" prognostic factors: a single pelvic lymph node metastasis, positive parametrial involvement, or a positive surgical margin or one of the following "intermediate-risk" prognostic factors: deep stromal invasion, lymphovascular space invasion, or a large tumor (over 4 cm), as reported previously [16]. When a patient's pathological report revealed multiple pelvic node metastases, they were treated with either EFRT, as reported previously [17], or with pelvic radiotherapy plus concurrent chemotherapy according to the physician's preference. In Osaka Medical Center for Cancer and Cardiovascular Diseases, the patients with high-risk or intermediate-risk prognostic factors were all treated with pelvic radiotherapy plus concurrent chemotherapy regardless of the number of pelvic node metastases.

Postoperative radiotherapy was performed within 4 weeks of radical surgery. Pelvic radiotherapy was delivered using a 10 megavolt (MV) X-ray from a linear accelerator using the anteroposterior parallel opposing technique. The superior margin of the external radiation field was located at the top of the fifth lumber vertebra, and the inferior border of the obturator foramen was used as the inferior margin. Laterally, the field extended 2 cm beyond the lateral margin of the bony pelvic wall. We used multi-leaf collimators to block the upper and lower corners of the radiation field. The external irradiation was delivered to the whole pelvis at 2 Gy per fraction in 5 fractions per week, for a total of 25 fractions (50 Gy).

Postoperative EFRT was also delivered to the patients via a 10 megavolt (MV) X-ray from a linear accelerator using the anteroposterior parallel opposing technique. The radiation field encompassed the pelvic and PALN drainage area. The superior margin of the PALN area was located at the bottom of the T12 vertebral body, and the inferior margin was located at the inferior border of the obturator foramen. The lateral margin was 1.5 cm to 2 cm lateral to the widest margin of the bony pelvis. The external irradiation was delivered to the EFRT fields for a total of 45 Gy in 25 fractions, and to the whole pelvis at 1.8 Gy per fraction, for a total of 28 fractions (50.4 Gy).

The patients that displayed vaginal invasion close to the surgical margin also received intracavitary radiotherapy (ICRT) involving 30 Gy in 5 fractions delivered to a depth of 5 mm below the vaginal mucosa after external beam radiotherapy (EBRT).

In our institutions, nedaplatin has been employed as a radiosensitizing agent for patients with cervical cancer [16,18]. Nedaplatin was given intravenously weekly or biweekly during the course of pelvic radiotherapy. Weekly nedaplatin was administered in Osaka University Hospital to 10 patients at a median dose of 40 mg/m^2 for 5 weeks, as described previously [16]. Biweekly nedaplatin was administered in Osaka Medical Center for Cancer and Cardiovascular Disease to 19 patients at a median dose of 70 mg/m^2 for 2 cycles per patient, as recommended by a previous report [19]. The first cycle of nedaplatin was initiated on the first day of radiotherapy treatment.

Toxicity

Clinical data regarding treatment-related complications were also collected. Complications that occurred within 90 days of the start of

primary treatment were considered to be acute complications, and those that occurred more than 90 days after the start of treatment were considered to be late complications. The severity of acute complications was classified according to the NCI Common Terminology Criteria for Adverse Events, Version 2.0. Late complications were graded according to the Radiation Therapy Oncology Group (RTOG) Late Radiation Morbidity Scoring Scheme [20].

Follow-up

The patients were followed-up regularly and observed for acute and late toxicities by both gynecological oncologists and radiation oncologists as reported previously [21]. During the treatment, the patients were evaluated weekly by pelvic examination and complete blood counts. For the patients who were treated with chemoradiation, renal and liver function tests were also performed weekly. After treatment completion, the patients were followed in an outpatient clinic every month in the first year, every 2 months in the second year, every 3 months in the third year, every 6 months in the fourth to fifth year, and annually thereafter until 10 years after treatment. When recurrence was suspected, a biopsy was taken for confirmation whenever possible. Loco-regional recurrence was defined as disease recurring in the pelvis in the CCRT-group or in the pelvis or para-aortic area in the EFRT-group. Recurrences were defined as distant when disease occurred outside the pelvis in the CCRT-group or outside the pelvis excluding the PALN area in the EFRT-group. The median duration of follow-up was 48 months (range: 13–120 months).

Statistical analysis

The differences between groups with respect to clinical stage, histology, parametrial involvement, deep stromal invasion, and surgical margin status were assessed using Fisher's exact test. Age, tumor diameter, and the duration of radiotherapy were analyzed using Wilcoxon's exact test. Pretreatment hemoglobin levels were compared using the Student's t test. Treatment related toxicities and the recurrence rate were compared using Fisher's exact test. The survival analysis was based on the Kaplan–Meier method, and the results were compared using the log-rank test. PFS was defined as the time from the primary diagnosis to the detection of recurrence. OS was defined as the time from the primary diagnosis to death or the latest observation. P-values of <0.05 were considered statistically significant.

Results

Patient characteristics

The characteristics of the 91 patients included in the study are shown in Table 1. Of these, 55 patients displayed multiple pelvic lymph node metastases and were treated with either pelvic radiotherapy plus concurrent chemotherapy (CCRT-group) or EFRT (EFRT-group) postoperatively. Thirty-six patients displayed single pelvic lymph node metastases and received pelvic radiotherapy plus concurrent chemotherapy after surgery (N1-CCRT-group).

The characteristics of the patients in the CCRT- and EFRT-groups are shown in Table 2. Among a total of 55 patients, 26 were treated with EFRT, and 29 were treated with pelvic radiotherapy plus concurrent chemotherapy.

In the CCRT-group, the mean age of the patients was 44 years. Twenty-five patients had SCC, and 4 had non-SCC histology (3 adenocarcinomas and 1 small cell carcinoma). Eight patients displayed parametrial involvement (Stage IIb), and 3 patients demonstrated vaginal invasion close to the surgical margin and were treated with ICRT after pelvic radiotherapy.

Table 1
Patients included in this study.

		CCRT-group	EFRT-group	N1-CCRT-group
Number of patients		29	26	36
Number of positive pelvic nodes	(mean)	3.3	5.0	1
Age	(mean)	46.1	49	49.8
Stage[a]	Ia2–IIa	21	15	21
	IIb	8	11	15
Histology	SCC	25	18	30
	Non-SCC	4	8	6

CCRT, concurrent chemoradiotherapy; EFRT, extended-field radiotherapy.
[a] Pathological stage.

In the EFRT-group, the mean age of the patients was 49 years. Eighteen patients had SCC histology, and 8 had non-SCC histology (8 adenocarcinomas). Eleven patients displayed parametrial involvement (Stage IIb). Two patients demonstrated vaginal invasion close to the surgical margin and received ICRT after EFRT.

There were no statistically significant differences in terms of age, clinical stage, histological distribution, tumor diameter, margin status, pretreatment hemoglobin level, or number of positive lymph nodes between the groups. However, as shown in Table 2, the duration of radiotherapy was significantly longer in the EFRT-group (42 days) than in the CCRT-group (34 days).

Survival outcome

At the time of this report, 8 patients in the CCRT-group (27.6%) and 16 patients in the EFRT-group (61.5%) had died of their disease. When the CCRT-group was compared with the EFRT-group, as shown in Fig. 1 and Table 3, CCRT was significantly superior in terms of recurrence rate ($p=0.0306$), PFS (log-rank; $p=0.0236$), and OS (log-rank; $p=0.0279$). When the patients in the CCRT-group were compared with those in the N1-CCRT-group (Fig. 2 and Table 4), there was no significant difference in recurrence rate ($p=1.0000$), PFS (log-rank; $p=0.9967$), or OS (log-rank; $p=0.7990$). In contrast, when the patients in the EFRT-group were compared with those in the N1-CCRT-group, there were significant differences in recurrence rate ($p=0.0197$), PFS (log-rank; $p=0.0194$), and OS (log-rank; $p=0.0351$).

Table 2
Patient characteristics.

		CCRT	EFRT	P-value
Number of patients		29	26	
Age	(mean)	46.1	49	0.3402
Stage[a]	Ia2–IIa	21	15	0.2730
	IIb	8	11	
Histology	SCC	25	18	0.1924
	Non-SCC	4	8	
Maximal tumor diameter[b]	Median (mm)	35	40	0.3334
Number of positive pelvic nodes	(mean)	3.3	5.0	0.0809
Common iliac nodes involvement	Yes	2	6	0.1306
	No	27	20	
Positive margins	Yes	3	2	1.0000
	No	26	24	
Stromal invasion	Less than one-half	4	4	1.0000
	More than one-half	25	22	
Pretreatment hemoglobin level[c]	Mean (mg/dl)	12.25	12.29	0.8988

CCRT, concurrent chemoradiotherapy; EFRT, extended-field radiotherapy; SCC, squamous cell carcinoma.
[a] Pathological stage.
[b] The maximal tumor diameter was measured three-dimensionally based on T2-weighted images. The longest diameter was considered valid as the maximal tumor diameter.
[c] Hemoglobin level just before the start of adjuvant radiotherapy.

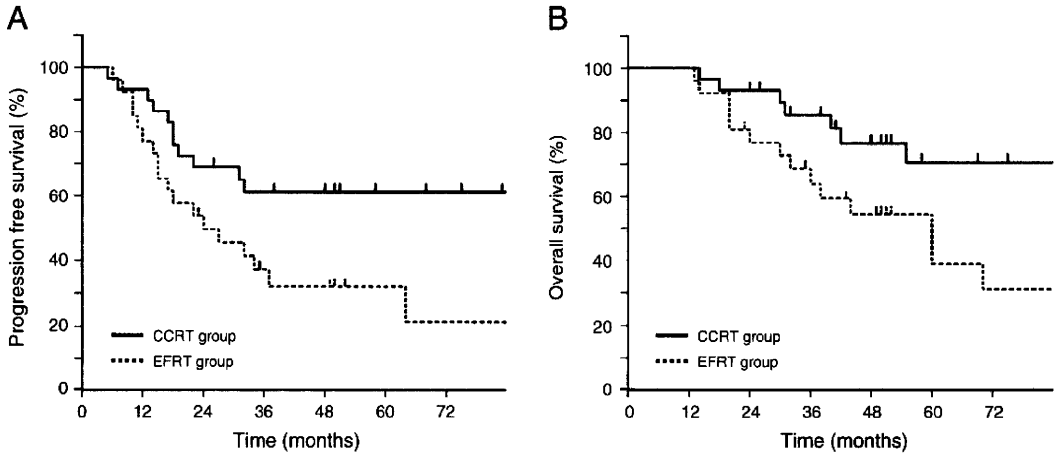

Fig. 1. A: Progression free survival among patients in the CCRT- and EFRT-groups. The progression free survival rate was significantly higher among the patients in the CCRT-group ($p = 0.0236$). B: Overall survival in the CCRT- and EFRT-groups. The overall survival rate was significantly higher among the patients in the CCRT-group ($p = 0.0279$).

When analyzed according to histology, CCRT was significantly superior to EFRT in terms of PFS (log-rank; $p = 0.0462$) and OS (log-rank; $p = 0.0349$) in the SCC-group. However, in the patients with non-SCC histology, no significant difference in recurrence rate, PFS, or OS was observed between the two treatment groups.

Pattern of recurrence

Treatment failure was observed in 11 patients (37.9%) in the CCRT-group and 18 patients (69.2%) in the EFRT-group. In the CCRT-group, one patient developed recurrence inside the irradiated field, 6 developed recurrence outside the irradiated field, and 4 developed recurrence both inside and outside of the irradiated field. In the EFRT-group, 6 patients developed recurrence in the irradiated field, 10 developed recurrence outside the irradiated field, and 2 developed recurrence both inside and outside of the irradiated field. The median interval from surgery to recurrence was 17.5 months (range: 5–32 months) in the CCRT-group and 16 months (range: 6–64 months) in the EFRT-group. The use of pelvic radiotherapy plus concurrent chemotherapy resulted in fewer relapses inside the irradiated field than EFRT; however, the pattern of recurrence did not differ statistically between the two treatment groups ($p = 0.1680$).

Of a total of 11 patients who developed recurrences in the CCRT-group, 6 (54.5%) developed recurrences in PALN area. In contrast, of a total of 18 patients with recurrences in the EFRT-group, recurrences in PALN area were observed in 5 patients (27.7%).

When examined according to histology, a distinctive pattern of recurrence was observed in the non-SCC group. Two out of 8 recurrences in the EFRT-group and 2 out of 4 recurrences in the CCRT-group involved peritoneal dissemination in the non-SCC-group. No peritoneal dissemination was observed in the SCC-group.

Table 3
Treatment outcome.

		CCRT	EFRT	P-value
Number of patients		29	26	
Duration of radiotherapy	Median (days)	34	42	<0.0001
Number of patients with recurrence	(%)	11 (37.9)	18 (69.2)	0.0306
PFS	Median (months)	38.0	23.5	0.0236
OS	Median (months)	51.0	40.5	0.0279
Number of patients with grade 3–4 acute toxicity	(%)	2 (6.9)	3 (11.5)	0.6586
Number of patients with grade 3–4 late toxicity	(%)	2 (6.9)	3 (11.5)	0.6586

CCRT, concurrent chemoradiotherapy; EFRT, extended-field radiotherapy; PFS, progression free survival; OS, overall survival.

Adverse effects

Generally, both pelvic radiotherapy plus concurrent nedaplatin-based chemotherapy and EFRT were well tolerated. All patients completed the planned external beam radiotherapy, and there were no treatment-related deaths. Among a total of 29 patients in the CCRT-group, although grade 1–2 acute toxicities were commonly observed, only two (6.9%) patients had grade 3 or 4 acute toxicities (Table 3): one patient had neutropenia, and the other had thrombocytopenia. Among a total of 26 patients who were treated with EFRT, three patients (11.5%) had grade 3 or 4 acute toxicities, all of which were neutropenia. No patient developed grade 3–4 non-hematologic toxicities. All cases were manageable by conservative treatment.

Grade 3–4 severe late toxicities were observed in three patients in the EFRT-group (bowel obstruction in two patients and radiation dermatitis in one patient). In the CCRT-group, two patients developed grade 3–4 severe late toxicities, both of which were bowel obstruction. All cases were manageable by conservative treatment. Although the absolute number of acute or late toxicities was lower in the CCRT-group, the difference did not reach statistical significance.

Discussion

Although nodal status is not included in the International Federation of Gynecology and Obstetrics (FIGO) staging criteria, nodal metastasis remains the single most important prognostic factor in early-stage cervical cancer [8]. It was reported that failure to control disease in early-stage cervical cancer is primarily due to an inability to sterilize tumor-containing lymph nodes, and central failure was only responsible for 6% or less of the cases [22].

In general, lymphatic metastasis in cervical cancer proceeds in a stepwise and predictable fashion from the lower pelvis to the upper pelvis including the common iliac nodes, followed by the para-aortic nodes (PALN). Although cervical cancer can reach the common iliac and para-aortic nodes directly via the posterior cervical trunk, this pattern of spread is uncommon [9,23,24].

It is generally accepted that patients with multiple pelvic lymph node involvement carry a risk of para-aortic node metastasis. In addition, a previous surgical staging study conducted by the Gynecologic Oncology Group (GOG) demonstrated a clear correlation between the incidence of para-aortic nodal metastasis and advancing

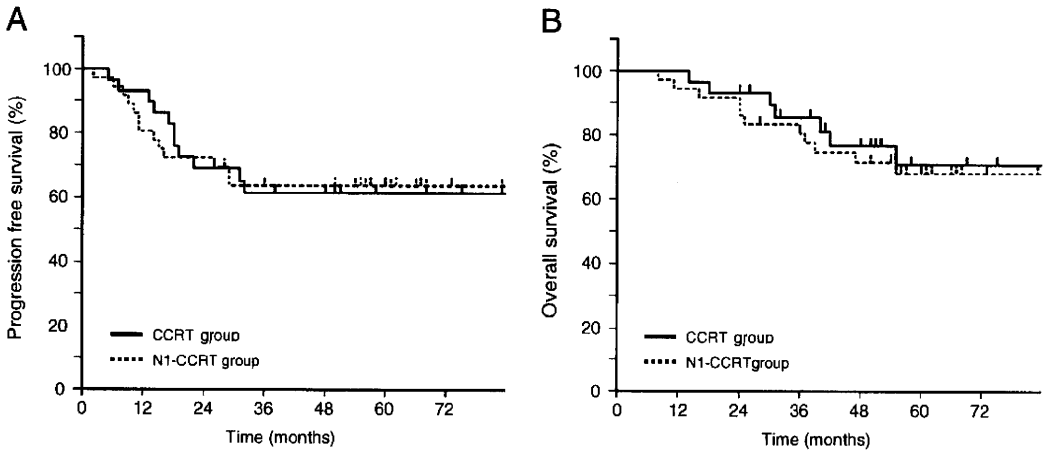

Fig. 2. A: Progression free survival among patients in the N1-CCRT- and CCRT-groups. The progression free survival rate in the CCRT-group was similar to that in the N1-CCRT-group ($p=0.9967$). B: Overall survival of high-risk patients. The overall survival rate in the CCRT-group was similar to that in the N1-CCRT-group ($p=0.7990$).

tumor stage. In this report, 5% of stage I, 17% of stage II, and 25% stage III patients demonstrated biopsy-proven para-aortic metastasis [25]. On the basis of a randomized controlled study [11], the patients at high risk of para-aortic node metastasis have traditionally been treated with prophylactic EFRT after radical surgery in our institution [17]. Although a previous randomized controlled study demonstrated the superiority of pelvic radiotherapy plus concurrent chemotherapy over EFRT in patients with early-stage cervical cancer in the setting of definitive radiotherapy for cervical cancer [12] and pelvic radiotherapy plus concurrent chemotherapy has become a standard treatment for this patient population, no direct comparison of postoperative pelvic radiotherapy plus concurrent chemotherapy and EFRT has been made.

Although our study is retrospective, our results suggested that postoperative pelvic radiotherapy plus concurrent chemotherapy is superior to EFRT with regard to recurrence rate, PFS, and OS for treating early-stage cervical cancer patients with multiple lymph node metastases. Moreover, pelvic radiotherapy plus concurrent chemotherapy resulted in improved outcomes without increasing the incidence of serious side effects. The estimated 5-year overall survival rate of 71% in the CCRT-group in the current study is comparable to the 5-year overall survival rate of 75% observed in the previous randomized clinical trial of cisplatin-based concurrent chemoradiotherapy [13,26].

We employed nedaplatin as a radio-sensitizing agent. Nedaplatin (*cis*-diammine-glycoplatinum), a derivative of cisplatin, was developed in 1983 by Shionogi Pharmaceutical Company with the aim of producing a treatment with a similar effectiveness to cisplatin but decreased renal and gastrointestinal toxicities [27]. In preclinical evaluations of cervical cancer, nedaplatin demonstrated similar antitumor activity to cisplatin [28,29]. Its lower incidence of nephrotoxicity in comparison to cisplatin has been demonstrated to be associated with differences in the kidney distributions of these drugs. When the two agents were administered at the same dose, the amount of nedaplatin that accumulated in the rat kidney was approximately 40% of that of cisplatin, which explains why nedaplatin is associated with less nephrotoxicity than cisplatin [30,31].

The radio-sensitizing properties of nedaplatin in the setting of postoperative adjuvant radiotherapy have been evaluated in one Phase I [32] and two retrospective studies [16,19], in which the authors recommended weekly 35–40 mg/m^2 nedaplatin or biweekly 70 mg/m^2 nedaplatin. In the current study, of a total of 29 patients that were treated with pelvic radiotherapy plus concurrent chemotherapy, 19 received biweekly nedaplatin and 10 received weekly nedaplatin during the course of pelvic radiotherapy. Recurrences were observed in 7 patients (37%) who had received biweekly nedaplatin and in 4 patients (40%) who had received weekly nedaplatin, indicating that treatment failure was not influenced by the concurrent chemotherapy treatment schedule employed in the current study.

As shown in Fig. 2 and Table 4, when the patients were treated with pelvic radiotherapy plus concurrent chemotherapy, there was no significant difference in PFS or OS between the patients with multiple lymph node metastases and those with single node metastases, indicating that the addition of concurrent nedaplatin to pelvic radiotherapy abolished the adverse prognostic impact of multiple lymph node metastases. Our finding is consistent with those of a previous report that demonstrated the effect of the addition of concurrent chemotherapy to pelvic radiotherapy is more profound in patients with multiple lymph node metastases than in patients with single node metastases [26].

Although postoperative EFRT resulted in fewer PALN recurrences than pelvic radiotherapy plus concurrent chemotherapy, it failed to improve the overall recurrence rate and the survival outcome in this patient population. These results may indicate the potential activity of concurrent chemotherapy to control occult systemic metastasis.

The rate of severe late complications in the EFRT-group in our study (11.5%) was similar to those found in previous series [11]. Although not statistically significant, the rate of severe late complications in the EFRT-group (11.5%) was higher than that observed in the CCRT-group (6.9%). As it is well tolerated by patients and displays

Table 4
The activity of postoperative CCRT according to the number of pelvic node metastases.

			CCRT	N1-CCRT	P-value
Patient characteristics	Number of patients		29	36	
	Age	(mean)	46.1	49.8	0.2144
	Stage[a]	Ia2–IIa	21	21	0.3007
		IIb	8	15	
	Histology	SCC	25	30	1.0000
		Non-SCC	4	6	
Treatment outcome	Patients with recurrence	(%)	11 (37.9)	13 (36.1)	1.0000
	PFS	Median (months)	38.0	54.5	0.9967
	OS	Median (months)	51.0	56.5	0.7990

CCRT, concurrent chemoradiotherapy.
[a] Pathological stage.

significant activity, we believe that pelvic radiotherapy plus concurrent nedaplatin-based chemotherapy is a reasonable treatment for this patient population.

We have to recognize the limitations of our study. One is the relatively small sample size. Moreover, due to the retrospective nature of this study, potential biases may have influenced the results, such as the heterogeneity of the patient population and the considerable selection bias exercised by physicians in determining which patients should be considered for pelvic radiotherapy plus concurrent chemotherapy. In addition, the educational level and/or the socio-economical status of the patients might also have affected treatment selection. These factors can only be eliminated in a prospective randomized controlled study.

Although nedaplatin-based CCRT was well tolerated, 7% of patients experienced severe acute or late complications. Thus, further efforts need to be made to reduce severe complications. One strategy is to use intensity-modulated radiation therapy (IMRT) to achieve more conformal dose distributions. According to several retrospective studies, IMRT shows a reduced incidence of acute toxicities compared with conventional techniques in patients with gynecological malignancies including uterine cervical cancer [33]. Thus, to confirm the benefit of IMRT, a clinical trial of pelvic IMRT combined with concurrent weekly nedaplatin in the setting of adjuvant therapy for cervical cancer needs to be conducted in the future.

Although the pelvic radiotherapy plus concurrent nedaplatin-based-chemotherapy resulted in improved survival, a significant number of patients still suffered recurrences and died of their disease. Of the 5 patients in the CCRT-group that developed recurrences inside the irradiated field, only 1 patient developed recurrence at the vaginal apex, indicating that the routine addition of ICBT to the upper vagina only benefits a small proportion of patients. Therefore, to further improve the prognosis of this patient group, novel treatment strategies need to be investigated.

One strategy that might improve the patient outcome is the use of EFRT combined with concurrent chemotherapy. The activity and feasibility of EFRT combined with concurrent chemotherapy has been investigated in several phase I/II clinical studies in cervical cancer patients that were positive or at high risk for PALN metastasis. According to previous clinical trials in which concurrent weekly cisplatin was employed as a radiosensitizer, the reported incidence of acute grade 3–4 gastrointestinal or hematologic toxicities was 10–80% [34–36]. Similarly, in a phase II clinical trial of EFRT plus concurrent nedaplatin, roughly 40% of patients experienced grade 3–4 gastrointestinal or hematologic toxicities [37]. These results indicate the limitation of performing EFRT plus concurrent chemotherapy using conventional radiation techniques. However, recently, preliminary data from small clinical studies suggested a preferable toxicity profile of extended-field IMRT with concurrent cisplatin in patients with cervical cancer [38]. Thus, the activity and feasibility of extended-field IMRT with concurrent cisplatin or nedaplatin should be further investigated in prospective settings. In addition, novel treatments such as the use of new cytotoxic agents in concurrent chemotherapy, the co-administration of nedaplatin with molecularly targeted agents, or the addition of consolidation chemotherapy after postoperative pelvic radiotherapy plus concurrent chemotherapy should also be investigated in future clinical trials.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Expression of aldehyde dehydrogenase 1 (ALDH1) in endometrioid adenocarcinoma and its clinical implications

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Aldehyde dehydrogenase 1 (ALDH1) is expressed in stem/progenitor cells, including cancer-initiating cells (CIC) of various organs. In the present study, ALDH1 expression was immunohistochemically examined in uterine endometrioid adenocarcinoma. The ALDH1 was expressed in a small portion of tumor cells, and these ALDH1-expressing cells were less mature than ALDH1-non-expressing cells. The ALDH1-expressing (ALDH1-hi) cells were more tumorigenic, resistant to anti-cancer agents and more invasive than ALDH1-lo cells. Culture of the sorted ALDH1-hi cells yielded both ALDH1-hi and ALDH1-lo cells, whereas ALDH1-lo cells yielded ALDH-lo cells alone. Clinically, a high-level of ALDH1 expression in tumor cells was correlated with T category, lymphatic invasion, recurrence and prognosis of patients. Patients with high ALDH1 expression showed poorer prognoses than those with low expression ($P = 0.015$ for disease-free survival [DFS] and $P = 0.010$ for overall survival [OS]), and high ALDH1 expression was an independent factor for poor prognosis. Aldehyde dehydrogenase 1 is a candidate for CIC marker for uterine endometrioid adenocarcinoma. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01864.x, 2011)

Tumors consist of heterogeneous cell populations derived from a single clone. Recently, it has been demonstrated that cells with tumorigenic potential are limited to a small population among tumor cells, called cancer-initiating cells (CIC), in cancers of blood (leukemia), breast, brain and colon.[1–11] Cancer-initiating cells efficiently efflux anti-tumor agents and degrade reactive oxygen species that are related to radiation-induced apoptosis. Furthermore, CIC are in a quiescent state for cell division, and thus escape the attack of various anti-cancer drugs targeting the rapidly dividing tumor cells. These characteristics enable CIC to be resistant to anti-tumor drugs and radiation therapy.[12–15]

Endometrioid adenocarcinoma is one of the most common malignancies of the female genital system.[16,17] Despite the advances in methods for detection and treatment, prognosis of patients with endometrioid adenocarcinoma still remains unfavorable. Therapeutic strategies targeting CIC would be necessary to improve cure rates, but studies on CIC of endometrioid adenocarcinoma are limited. Gotte *et al.*[18] demonstrated that Musashi-1, highly expressed in neural stem cells, was co-expressed with Notch-1 in a subpopulation of endometrial cells and endometrioid adenocarcinoma cells. Kato *et al.*[19] demonstrated that the side-population of endometrioid adenocarcinoma, which is considered to contain CIC, possessed higher tumorigenic activities than non side-population cells. To our knowledge, the relationship of stem cell marker expression to prognosis has not been reported in endometrioid adenocarcinoma.

Aldehyde dehydrogenase 1 (ALDH1), a predominant isoform of the ALDH family in mammals, oxidizes retinol to retinoic acid in early stages of stem cell differentiation, and hematopoietic and neural stem cells show high ALDH1 activity.[20–22] Cancer-inducing cells of human multiple myeloma, acute myeloid leukemia and cancers of brain, lung and breast also show high ALDH1 activity.[23–27] The activity of ALDH1 might be a common marker for both normal and malignant stem cell populations. In the present study, ALDH1 expression was immunohistochemically examined in endometrioid adenocarcinoma and its clinical implications were evaluated.

Materials and Methods

Patients. Ninety-eight patients who underwent surgery for uterine endometrioid adenocarcinoma at Osaka University Hospital from January 1998 to January 2007 were examined. Clinicopathological findings in these 98 patients are summarized in Table 1. The age of patients ranged from 22 to 75 years (median, 55.9 years). Resected specimens were macroscopically examined to determine the location and size of the tumors. Histological specimens were fixed in 10% formalin and routinely processed for paraffin embedding. Paraffin-embedded specimens were stored in the dark room in the Department of Pathology of Osaka University Hospital at room temperature, sectioned at 4-μm thickness at the time of staining, and stained with H&E and immunoperoxidase procedures. The histological stage was determined according to the International Federation of Obstetricians and Gynecologists (FIGO) staging system. All patients were followed up with laboratory examinations including routine peripheral blood cell counts at 1- to 6-month intervals, X-ray, computed tomographic scan and pelvic examination at 6- to 12-month intervals. The follow-up period for survivors ranged from 8 to 122 months (median, 89 months). The study was approved by the ethical review board of the Graduate School of Medicine, Osaka University.

Immunohistochemistry for ALDH1, ER, PgR, CD9 and MIB-1. Expression of ALDH1, estrogen receptor (ER), progesterone receptor (PgR) and Cluster Differentiation (CD) was examined with anti-ALDH1 (BD Biosciences, Franklin Lakes, NJ, USA), anti-ER (Dako, Glostrup, Denmark), anti-PgR (Dako) and anti-CD9 (Abcam Ltd, Cambridge, UK) antibodies, respectively. The proliferative activity of cancer cells was examined with monoclonal antibody MIB-1 (Immunotech, Marseilles, France), recognizing the proliferation-associated antigen Ki67. The antigen retrieval with Pascal pressurized heating chamber (Dako) was done for the staining of ER, PgR, CD9 and MIB-1. The

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Table 1. Summary of characteristics in 98 endometrioid adenocarcinoma patients

	Number of patients
Tumor	
T1	70
T2	8
T3	20
Lymph node	
N0	73
N1	25
Tumor histological grade	
Grade 1	38
Grade 2	39
Grade 3	21
Estrogen receptor status	
Positive	40
Negative	58
Progesterone receptor status	
Positive	75
Negative	23
Ki67 labeling index	
≥ 20%	82
<20%	16
Response to chemotherapy	
Non-respond	17
Respond	32
Recurrence	
Positive	20
Negative	78
Prognosis	
Dead	15
Alive	83

sections were incubated with anti-ALDH1 (×100), -ER (×2), -PgR (×6), -CD9 antibody (×100) or MIB-1 (×50), then treated with a ChemMate EnVision kit (Dako). Diaminobenzidine (DAB) (Dako) was used as a chromogen. As the negative control, staining was carried out in the absence of primary antibody. Stained sections were evaluated independently by two pathologists (JI, EM). As described previously,[24] cases with more and <10% of cells positive for ALDH1 were regarded as ALDH1-hi and ALDH1-lo, respectively. The proportion and intensity of ER and PgR expression were evaluated as described previously.[28] The MIB-1 labeling index was defined as the percentage of stained nuclei per 1000 cells. The patients were divided into MIB-1-high and MIB-1-low groups using the median as cut-off value.

Double staining of ALDH1 and CD9, ER and PgR. Double staining of ALDH1 and CD9, ER and PgR was done with the EnVision G/2 doublestain system (Dako) according to the manufacturer's protocol. First, the ALDH1 was stained with DAB, and subsequently the staining of CD9, ER and PgR was done with Permanent Red. Since the red fluorescence is released from Permanent Red, the signal of CD9, ER and PgR was detected with a fluorescence microscope (Biozero, Keyence, Osaka, Japan).

Cell lines and isolation of ALDH1-hi population. Endometrioid adenocarcinoma cell lines HEC-1, -1A, -108, -116, -6, -88nu and -251; and SNG-M and -II were obtained from the Health Science Research Resources Bank of Osaka, Japan. Cells were cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% FCS (Nippon Bio-Supply Center, Tokyo, Japan). To isolate the population with high ALDH1 enzymatic activity, the Aldefluor kit (Stem Cell Technologies, Vancouver, BC, Canada) was used according to the manufacturer's instructions. Briefly, cells were suspended in Aldefluor assay buffer containing ALDH1 substrate and BODIPY–aminoacetaldehyde (BAAA). The BAAA was taken up by living cells and converted by intracellular ALDH1 into BODIPY–aminoacetate, which causes the cells to fluoresce brightly. The brightly fluorescent ALDH1-expressing cells were detected with FACS Calibur or FACS Aria (BD Biosciences). As a negative control, cells were stained under identical conditions with the specific ALDH1 inhibitor, diethylaminobenzaldehyde (DEAB; Sigma, St Louis, MO, USA). Data were analyzed by using Cell Quest software (BD Biosciences). In endometrioid adenocarcinoma cell lines, cells with bright fluorescence were judged as ALDH-hi, and those with no or faint fluorescence as ALDH-lo. The criteria for ALDH1-hi and ALDH1-lo in cell lines were different from those for ALDH1 immunohistochemistry in clinical samples.

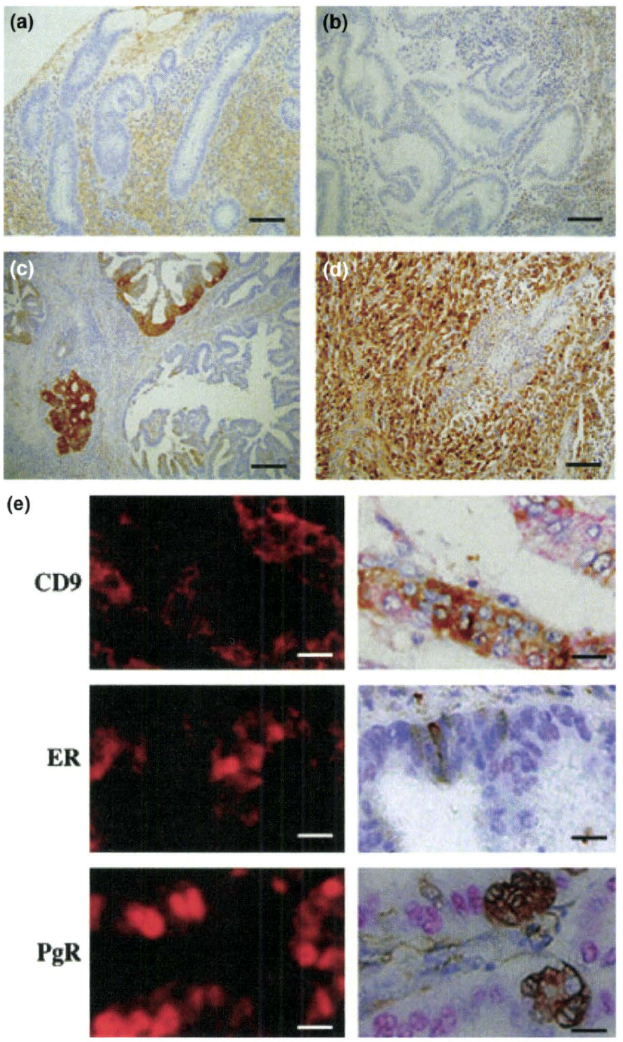

Fig. 1. Expression of ALDH1 in normal endometrium and endometrioid adenocarcinoma. Normal proliferative (a) and secretory (b) phases of endometrial epithels did not express ALDH1. (c) Small portions of endometrioid adenocarcinoma were positive for ALDH1 in some cases. (d) Most tumor cells were positive for ALDH1. (e) Double staining of ALDH1 for CD9, ER or PgR was done. Red fluorescence from CD9, ER or PgR staining signals is shown at left. Scale lines, (a–d) 200 μm, (e) 20 μm.

Effects of the anticancer drug cisplatin. Cisplatin is commonly used for the treatment of endometrioid adenocarcinoma. The effect of cisplatin on ALDH-hi cells was compared to that

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on ALDH-lo cells. Cells (1×10^4) were seeded onto cell culture plates with DMEM-10% FBS, cultured for 20 h, and various concentrations of cisplatin (0, 1, 4, 8 μg/mL) were added. After 24 h, the viability of cells was assessed with the Premix WST-1 cell assay system (Takara Bio Inc., Kyoto, Japan). The absorbance of cisplatin-treated cells at 450 nm was subtracted from the background absorbance (600 nm). The resultant value was divided by that of cells not treated with cisplatin, and the results are shown as the viability index.

Matrigel invasion assay. Invasion of tumor cells into Matrigel was examined with a BD BioCoat Matrigel Invasion Chamber (BD Biosciences). Briefly, cells were seeded in DMEM without FCS in the Matrigel invasion upper chamber and cultured for 72 h. The lower chamber contained DMEM and 10% FBS. Invading cells were stained with a Diff-quick staining kit (Siemens, Munich, Germany). The number of invading cells was counted in four microscopic fields per well at a magnification of ×20 and the extent of invasion was expressed as the average number of cells per square millimeter.

***In vitro* colony formation assay.** Cells were suspended in 0.1 mL of DMEM and 10% FBS, and 1000 cells were plated in culture dishes with 1 mL of methylcellulose-containing DMEM supplemented with 15% FBS. The number of colonies was counted on day 14.

Statistical analysis. Statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC, USA). The Chi-square and Fisher's exact probability tests were used to analyze the correlation between ALDH1 expression and clinicopathological factors in endometrioid adenocarcinoma. Kaplan–Meier methods were used to calculate overall survival (OS) and disease-free survival (DFS) rates, and differences in survival curves were evaluated with the log-rank test. Cox's proportional hazards regression model with a stepwise manner was used to analyze the independent prognostic factors. The P values of <0.05 were considered to be statistically significant.

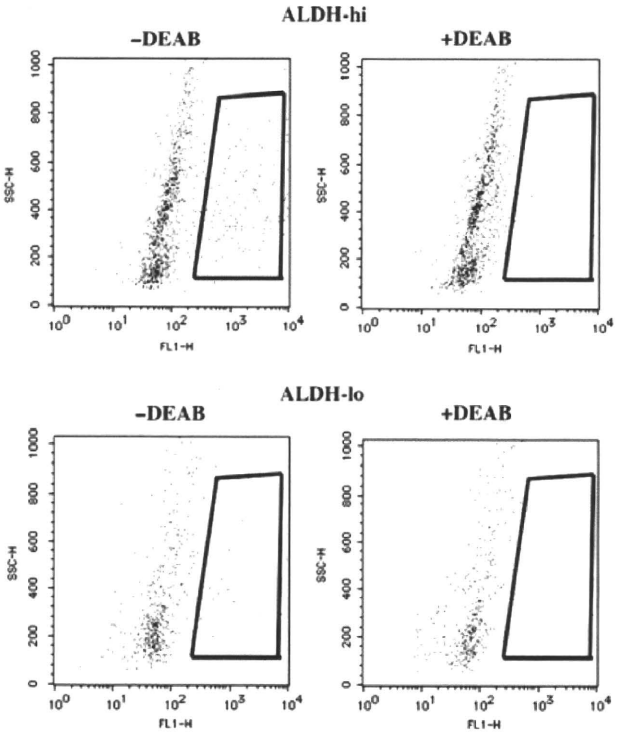

Fig. 3. ALDH1 activity of ALDH-hi and ALDH-lo HEC-1 cells after culture for 5 days. Dot–blot of Aldefluor assay without inhibitor is shown in the left side, and that with inhibitor on the right side.

Results

Immunohistochemical findings. The expression of ALDH1 was examined in normal endometrium and 98 endometrioid adenocarcinoma tissues. No signals were detected in normal proliferating and secretory phase of endometrium (Fig. 1a,b, respectively). Strong signals were found in the cytoplasm of a small portion of tumor cells in several cases (Fig. 1c), whereas signals were found in most tumor cells in other cases (Fig. 1d). The expression of ALDH1 was not detected in some cases. Based on the criteria of Jiang *et al.*,[24] cases with more than

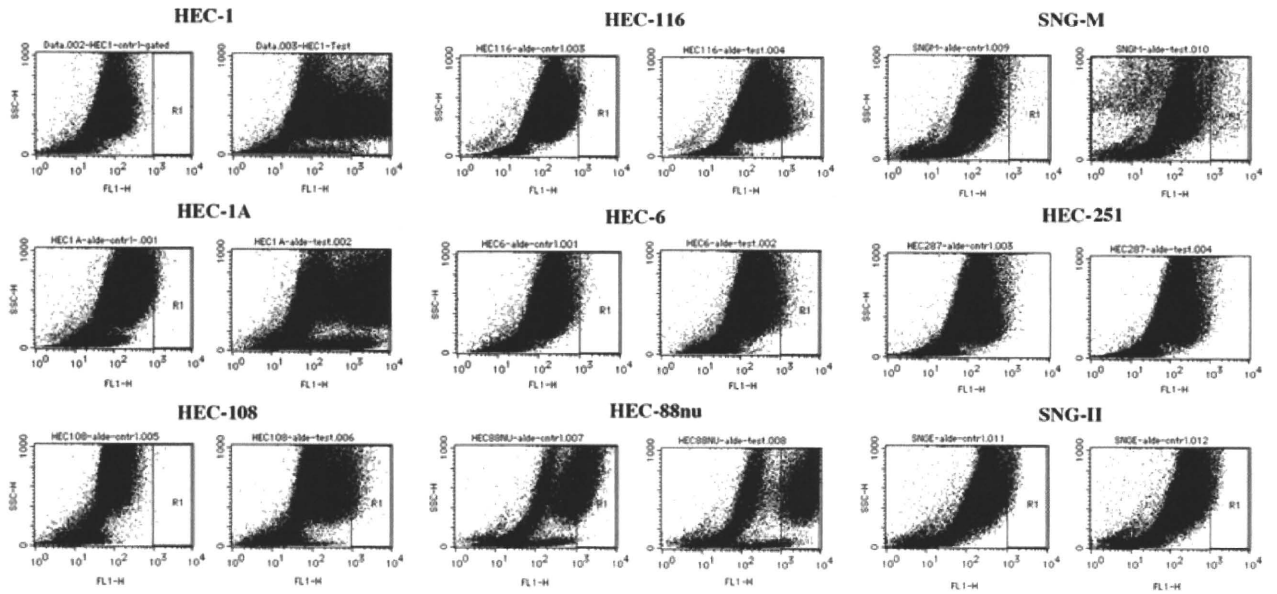

Fig. 2. Activity of ALDH1 in various endometrioid adenocarcinoma cell lines. The left side of each cell line shows dot–blot of Aldefluor assay with inhibitor (DEAB), and the right side shows dot–blot without inhibitor. The ALDH1-hi population is boxed.

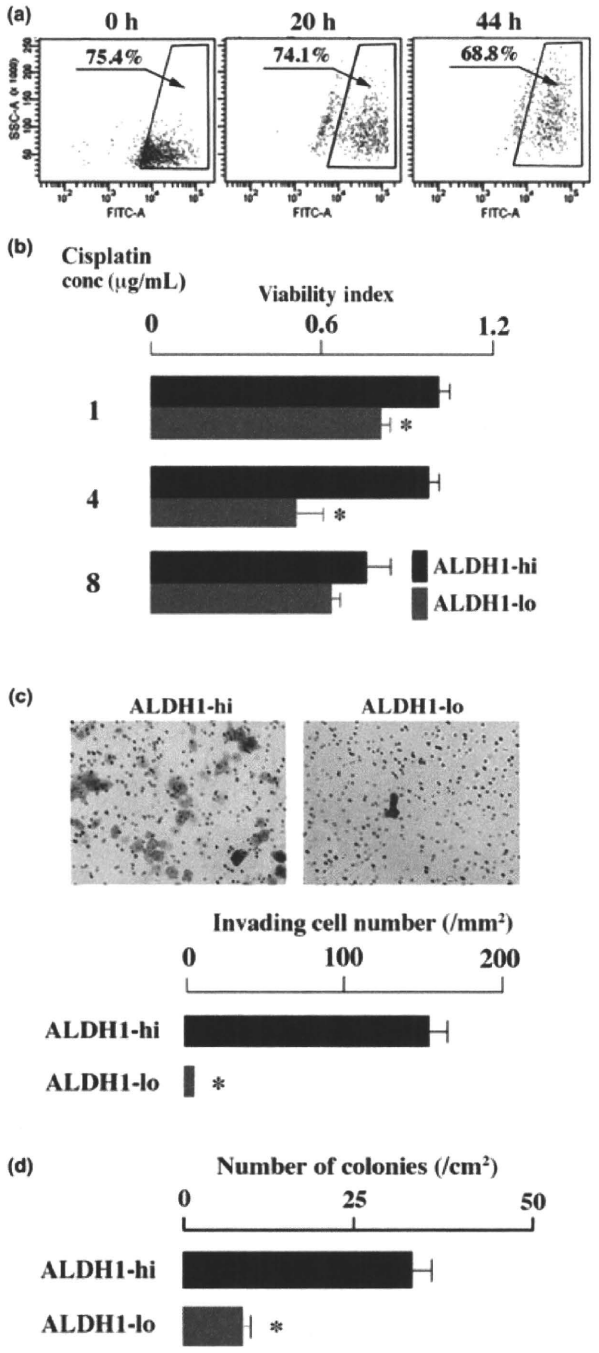

Fig. 4. Effects of ALDH1 on resistance to the anticancer drugs, cell invasion activity and *in vitro* colony formation activity. (a) Percentage of ALDH1-hi population in the sorted cells at 0, 20 and 44 h after sorting. (b) Viabilities of ALDH1-hi and ALDH1-lo HEC-1 cells compared in the presence of various amounts of cisplatin. (c) Matrigel invasion assay. HEC-1 cells invade through Matrigel (×40), and the number of invading cells per square millimeter are shown. (d) Comparison of colony number derived from the ALDH1-hi and ALDH1-lo HEC-1 cells per square millimeter. The values are the means ± SE of three experiments. *$P < 0.05$ (Student's *t*-test).

10% of cells positive for ALDH1 were regarded as ALDH1-hi: 40 (40.8%) of 98 cases were categorized as ALDH1-hi, and the remaining as ALDH1-lo.

Table 2. Correlation between ALDH1 expression in adenocarcinoma and clinicopathological parameters

	ALDH1 expression in cancer		*P*
	Low	High	
Tumor			
T1	43	27	
T2	5	3	
T3	10	10	0.047
Lymph node			
N0	50	23	
N1	8	17	0.002
Tumor histological grade			
Grade 1	28	10	
Grade 2	19	20	
Grade 3	11	10	0.065
Estrogen receptor status			
Positive	10	30	
Negative	11	47	0.474
Progesterone receptor status			
Positive	48	27	
Negative	10	13	0.080
Ki67 labeling index			
≥20%	47	35	
<20%	11	5	0.499
Response to chemotherapy			
Non-respond	4	13	
Respond	20	12	0.009
Recurrence			
Positive	7	13	
Negative	51	27	0.014
Prognosis			
Dead	5	10	
Alive	53	30	0.027

Expression of CD9, ER and PgR is one of the differentiation markers of endometrium. The expression level of CD9, ER and PgR was significantly lower in ALDH1-expressing cells than in non-expressing cells (Fig. 1c).

ALDH1 activity in endometrioid adenocarcinoma cell lines. ALDH1 activity was examined with Aldefluor assay in nine endometrioid adenocarcinoma cell lines: HEC-1, -1A, -108, -116, -6, -88nu and SNG-M contained ALDH-hi population, whereas HEC-251 and SNG-II did not (Fig. 2). In the subsequent experiments, HEC-1, which proliferates rapidly and is easy to handle, was used as a representative endometrioid adenocarcinoma cell line containing an ALDH-hi population.

Cancer-inducing cells are known to yield both CIC and non-CIC, whereas non-CIC does not yield any CIC. To examine whether ALDH1 could be used as a CIC marker for endometrioid adenocarcinoma, ALDH1-hi and ALDH1-lo HEC-1 were sorted separately. After culture for 5 days, cells derived from sorted ALDH1-hi HEC-1 yielded both ALDH-hi and ALDH-lo cells, whereas few ALDH1-hi cells were detected in cells derived from ALDH1-lo cells (Fig. 3).

Comparison of ALDH1-hi cells to ALDH1-lo cells in resistance against anti-tumor drug, abilities of invasion and *in vitro* colony formation. As cisplatin is commonly used for the treatment of endometrioid adenocarcinoma, the effect of ALDH1 on resistance of HEC-1 to cisplatin was examined. The ALDH-hi and ALDH-lo cells were cultured for 20 h, and cisplatin was added. Then, viability was examined at 44 h after sorting. Since 5-day culture of ALDH-hi cells yielded both ALDH-hi and ALDH-lo as described above, there is a possibility that the ALDH-hi cells

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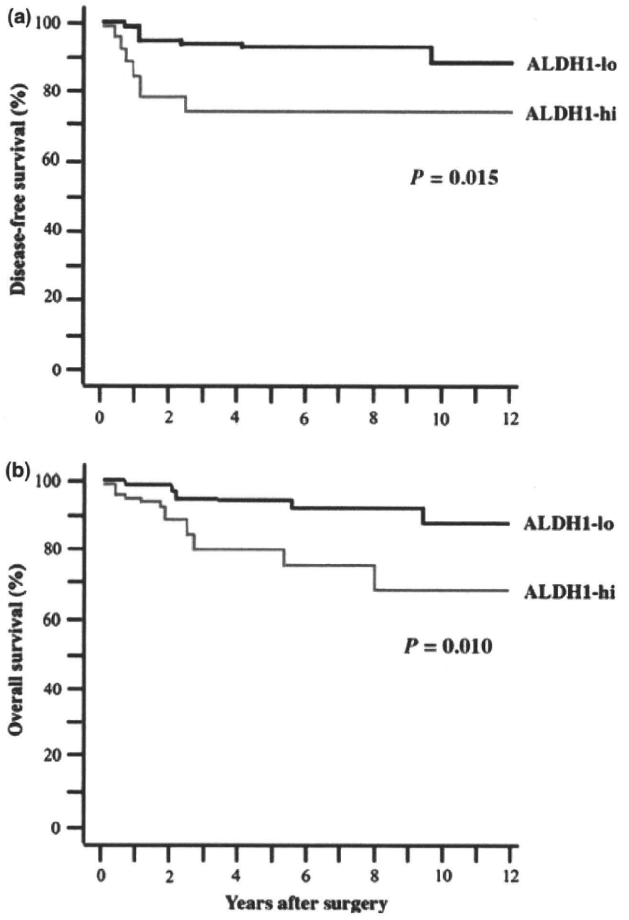

Fig. 5. Kaplan–Meier plots. Disease-free (a) and overall (b) survival curves are shown. The ALDH1-hi cases showed less favorable disease-free survival and overall survival.

yielded significant number of ALDH-lo cells at the time of cisplatin addition and viability check. However, this possibility was unlikely, because the percentage of sorted ALDH-hi cells remained at similar levels at 20 and 44 h to that just after sorting (Fig. 4a). The ALDH1-lo cells were more vulnerable to cisplatin than ALDH-hi cells (Fig. 4b). The invasion ability of ALDH-hi cells was compared to that of ALDH1-lo cells with Matrigel invasion assay: the number of invading cells was lower in ALDH1-lo cells than in ALDH1-hi cells, indicating that ALDH1-hi cells possessed stronger invasive capability than ALDH1-lo cells (Fig. 4c). Next, the ability for *in vitro* colony formation was evaluated. In comparison to ALDH1-hi cells, ALDH1-lo cells formed fewer colonies *in vitro* (Fig. 4d).

Correlation of ALDH1 expression with clinical variables. Correlation of ALDH1 expression with clinicopathological features was evaluated. Positive correlation was observed between ALDH1 expression and T factor ($P = 0.047$), lymph node metastasis ($P = 0.002$), resistance to chemotherapy $P = 0.009$), relapse rate ($P = 0.014$) and poor prognosis ($P = 0.027$). Other parameters including tumor histological grade, ER, PgR and Ki67 labeling index did not correlate with ALDH1 expression (Table 2). The 5-year DFS and OS were 86.7% and 90.6%, respectively. Tumors recurred in 22 patients. Of these, 14 patients died due to the tumors. There was a statistically significant difference in DFS ($P = 0.015$) and OS rates ($P = 0.010$) between patients with ALDH1-hi and ALDH1-lo tumors (Fig. 5).

Univariate analysis showed that T factor, lymph node metastasis, tumor histological grade and ALDH1 expression were significant factors for OS. For DFS, T factor, lymph node metastasis, tumor histological grade, PgR expression and ALDH1 expression were significant factors (Table 3). Multivariate analysis revealed that ALDH1 expression, lymph node metastasis and tumor histological grade were independent prognostic factors for OS, and ALDH1 expression was an independent prognostic factor for DFS (Table 3).

Discussion

Normal stem/progenitor cells of various lineages, such as hematopoietic, neural and mesenchymal stem cells, show high ALDH1 activity.[20–22] In addition, CIC have been reported to show high ALDH1 activity: the ALDH1-hi population is tumorigenic and resistant to chemotherapy in cancers of colon, breast, lung, pancreas, bladder, prostate and ovary.[23–27] To our knowledge, the role of ALDH1 in uterine cancer has never been studied. In the present study, we showed that ALDH1-hi endometrioid adenocarcinoma cells to be more tumorigenic, resistant to anti-cancer agents and invasive than ALDH-lo cells. Culture of the sorted ALDH-hi cells yielded both ALDH-hi and ALDH-lo cells, whereas culture of the ALDH-lo cells yielded ALDH-lo cells alone. These findings suggest that the ALDH-hi population possessed the character of CIC in endometrioid adenocarcinoma of uterus, like cancers of other organs.

In clinical specimens, ALDH1-expressing tumor cells were mostly negative for CD9, ER and PgR, indicating that ALDH1 expression was detected in tumor cells of a less mature state. Since CIC are in the immature state, this was consistent with the notion that ALDH1 was expressed in cells with CIC character.

The expression of ALDH1 was limited to a small portion of endometrioid adenocarcinoma cells, which were randomly

Table 3. Univariate and multivariate analyses of prognostic factors for overall and disease-free survivals

	Overall survival				Disease-free survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Tumor	3.32 (1.58–7.00)	0.002	1.59 (0.66–3.80)	0.302	3.29 (1.58–6.83)	0.002	1.49 (0.61–3.61)	0.383
Lymph node	2.64 (1.66–4.21)	<0.001	2.19 (1.21–3.97)	0.010	1.59 (1.26–2.00)	<0.001	1.29 (0.97–1.70)	0.077
Tumor histological grade	3.32 (1.58–7.00)	0.002	2.93 (1.11–7.74)	0.029	3.29 (1.58–6.83)	0.002	2.43 (0.97–6.12)	0.060
Estrogen receptor status	0.68 (0.23–2.00)	0.482			0.64 (0.22–1.89)	0.421		
Progesterone receptor status	0.40 (0.14–1.10)	0.076			0.36 (0.13–0.99)	0.048	0.44 (0.15–1.28)	0.132
Ki67 labeling index	2.56 (0.34–19.5)	0.364			2.85 (0.38–21.7)	0.311		
ALDH1 expression	3.78 (1.28–11.2)	0.016	4.89 (1.37–17.5)	0.014	3.51 (1.19–10.4)	0.023	3.65 (1.03–13.0)	0.045

CI, confidence interval; HR, hazard ratio.