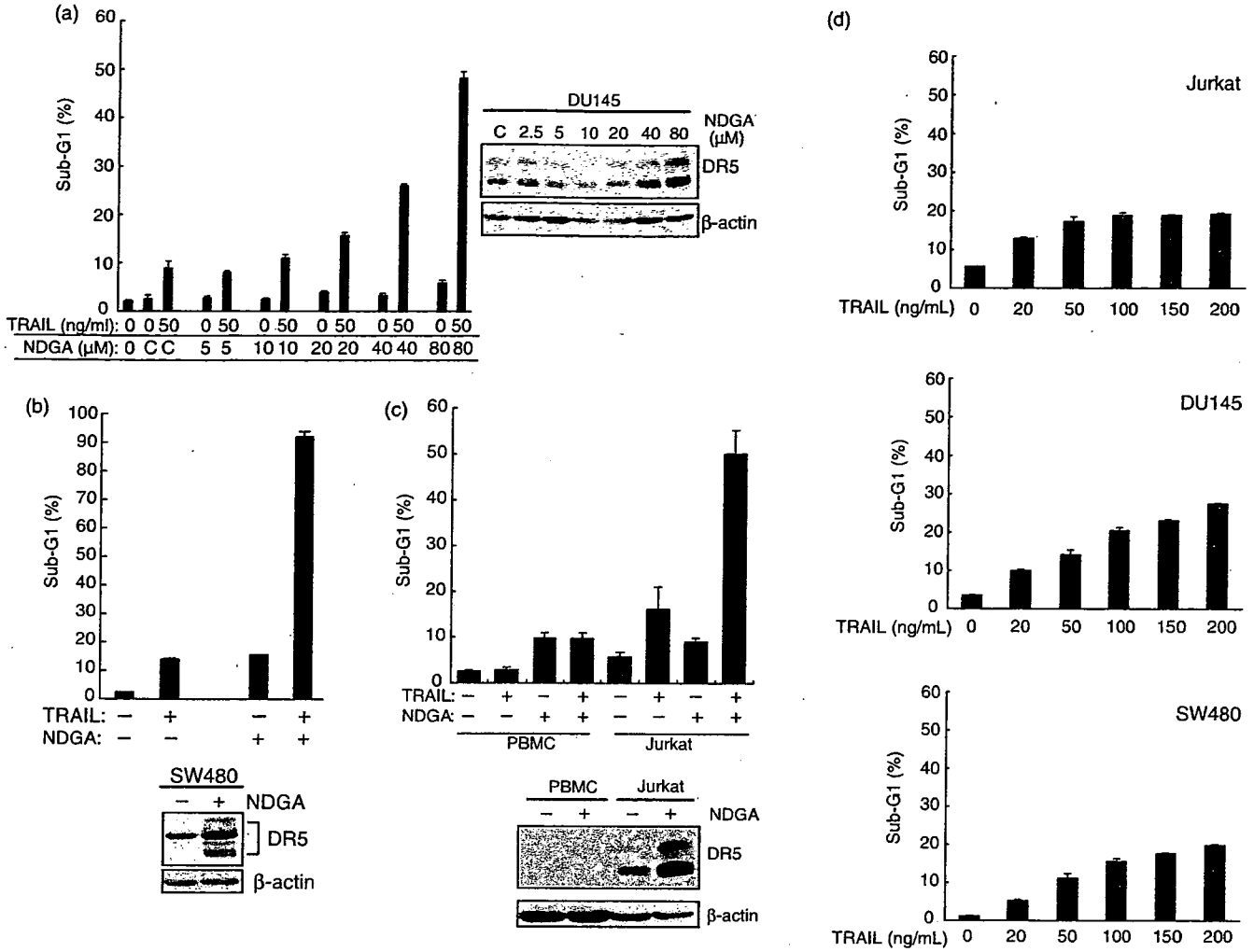

Fig. 4. Nordihydroguaiaretic acid (NDGA) enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in prostate cancer DU145 and colon cancer SW480 cells but not in normal peripheral blood mononuclear cells (PBMC). (a) DU145 cells were treated with various concentrations of NDGA and/or 50 ng/mL TRAIL. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD. In western blotting, DU145 cells were treated with dimethylsulfoxide (DMSO) or NDGA for 24 h and analyzed with anti-death receptor 5 (DR5) antibody. β-Actin is shown as a loading control. C, treated with DMSO. (b) SW480 cells were treated with 80 μM NDGA and/or 50 ng/mL TRAIL. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD. –, treated with DMSO. Western blotting was carried out as shown in (a). (c) Normal human PBMC or Jurkat cells were treated with 80 μM NDGA and/or 50 ng/mL TRAIL. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD. PBMC and Jurkat cells were treated with DMSO or 80 μM NDGA for 24 h and analyzed by western blotting for DR5 up-regulation. β-actin is shown as a loading control. –, treated with DMSO. (d) Dose effects of TRAIL in malignant tumor cells. Jurkat, DU145 and SW480 cells were treated with various concentrations of TRAIL for 12 h. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD.

AA861 also up-regulates DR5 and enhances TRAIL-induced apoptosis in DU145 and Jurkat cells. NDGA has been known to inhibit lipoxygenase activity. To examine whether the inhibition of lipoxygenase up-regulates DR5, another lipoxygenase inhibitor, AA861, which is a 5-lipoxygenase inhibitor, was used. As shown in Fig. 6, AA861 increased DR5 protein in DU145 and Jurkat cells. Furthermore, AA861 sensitized DU145 and Jurkat cells to TRAIL-induced apoptosis. These results suggest that the inhibition of lipoxygenase enhances TRAIL-induced apoptosis through DR5 up-regulation.

Discussion

To date, many agents that possess anti-tumor effects have been reported, whereas the efficacy of a single agent is limited. The combination of two different agents generates new effects rather than just the additive effects of the two agents. Lipoxygenase inhibitors, such as NDGA, are promising anti-tumor agents.[11–18] Moreover, TRAIL is also a promising anti-tumor agent with tumor-selective cell death.[19–21] Both NDGA and TRAIL induce apoptosis in malignant tumor cells, however, each agent very weakly induces apoptosis in a single use. In the present report, the authors showed that the combination of NDGA and TRAIL markedly induces apoptosis in malignant tumor cells at suboptimal concentrations for each agent. Furthermore, it was demonstrated that NDGA up-regulated the expression of DR5 at mRNA and protein levels. This increase of DR5 protein partly contributes to the sensitization of TRAIL-induced apoptosis by NDGA, because DR5 but not DR4 siRNA prevented the apoptosis induced by the combination of NDGA and TRAIL. However, DR5/Fc chimera protein more effectively attenuated the combined effect than DR5 siRNA, suggesting that the silencing of DR5 by siRNA may be insufficient or that DR4 may have a supporting function for DR5.

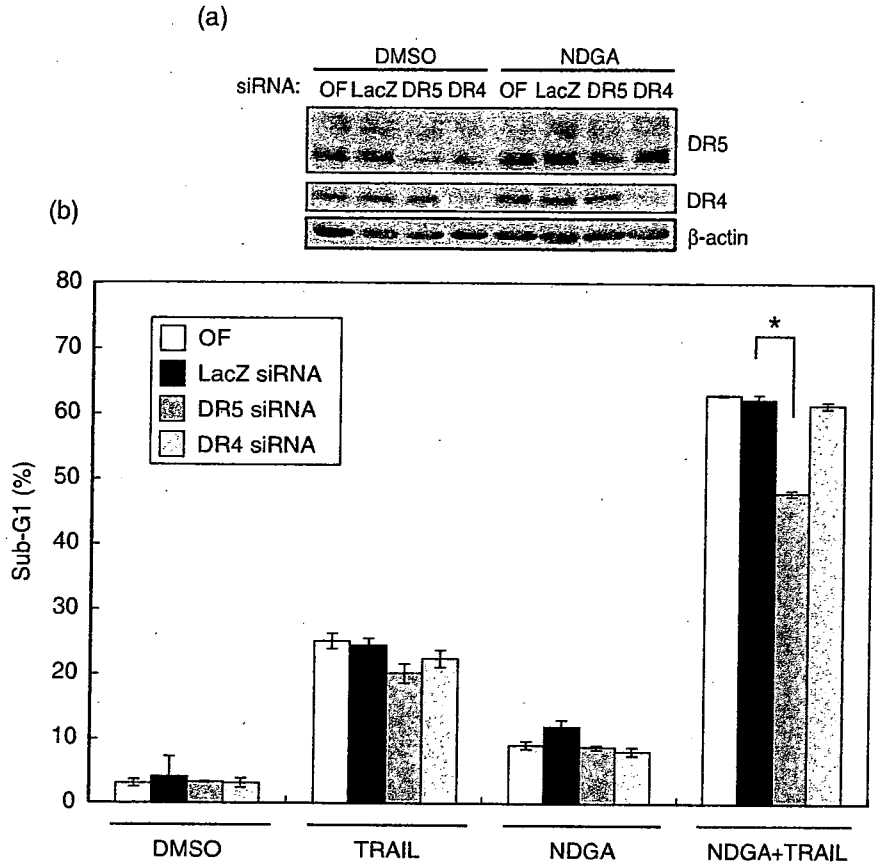

Fig. 5. Down-regulation of death receptor 5 (DR5) by small interfering (si)RNA prevents the apoptosis induced by the combination of nordihydroguaiaretic acid (NDGA) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). (a) Reduction of DR5 protein by DR5 siRNA. DU145 cells were treated with 20 nM DR5, DR4 or LacZ siRNA. 24 h after the transfection, cells were treated with 80 μM NDGA for 24 h. Cell lysates were analyzed by western blotting. β-actin is shown as a loading control. (b) DU145 cells were treated with 20 nM DR5, DR4 or LacZ siRNA. 24 h after transfection, cells were treated with 80 μM NDGA and/or 50 ng/mL TRAIL. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD. Data was analyzed using Student's *t*-test. $^*P < 0.05$. OF, treatment of transfection reagent oligofectamine only.

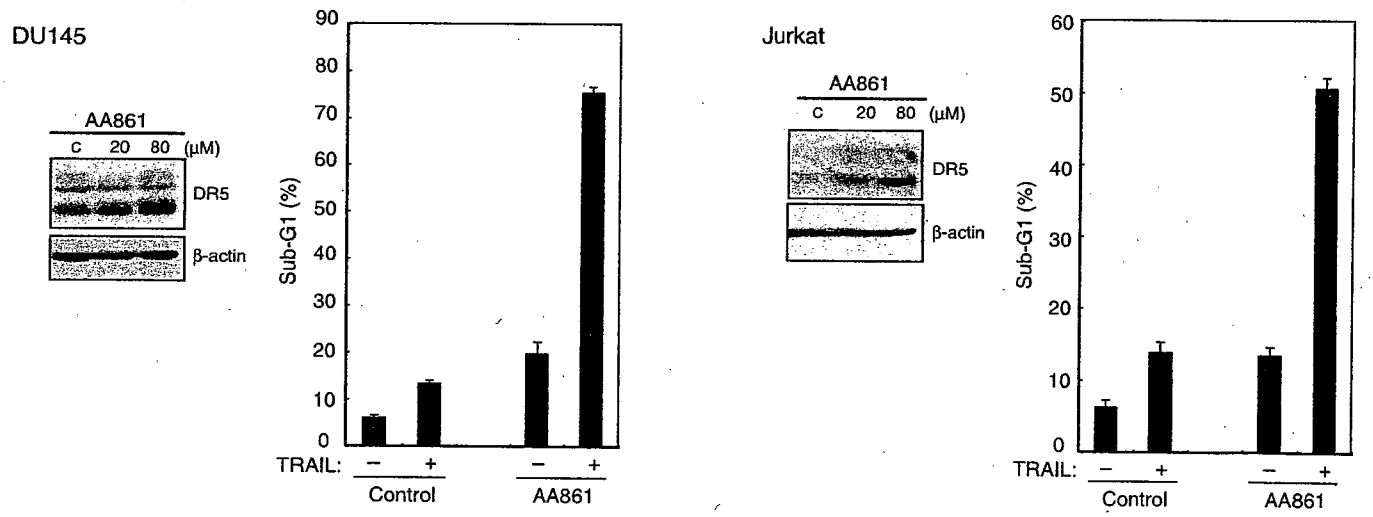

Fig. 6. AA861 up-regulates death receptor 5 (DR5) expression and sensitizes DU145 and Jurkat cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). DU145 and Jurkat cells were treated with AA861 (20 or 80 μM) for 24 h and the cell lysates were analyzed by western blotting of DR5. β-Actin is shown as a loading control. DU145 and Jurkat cells were treated with 80 μM AA861 and/or 50 ng/mL TRAIL. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD. C or −, treated with dimethylsulfoxide (DMSO).

The authors provide useful information on combined treatment with NDGA and TRAIL. First, TRAIL is a promising antitumor agent; however, some tumor cells, including Jurkat cells, remain resistant to TRAIL.[31,32] Thus, it is necessary to overcome TRAIL resistance in malignant tumor cells. It was demonstrated that NDGA and AA861 are able to enhance the ability of TRAIL in apoptosis of malignant tumors.

Lipoxygenases overexpress and increase the metastatic potential in prostate cancer cells.[2,5] In particular, 5-lipoxygenase plays a critical role in prostate cancer cell growth treated with arachidnic acid.[34] Therefore, lipoxygenase inhibitors, such as NDGA and AA861, have been considered to be effective in prostate cancer treatment. Moreover, prostate cancer is one of the most common malignancies and the second leading cause of male cancer-related death in the USA. Hence, new strategies are needed to improve prostate cancer therapy. The authors showed here that lipoxygenase inhibitors drastically induce apoptosis in prostate cancer DU145 cells together with TRAIL, suggesting that the

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combination of lipoxygenase inhibitors and TRAIL may be useful for new prostate cancer treatment.

Jurkat, DU145 and SW480 cells carry mutations in the tumor suppressor *p53* gene. Mutations of the *p53* gene exist in one-half of malignant tumors and confer resistance to conventional anti-tumor agents.[35,36] The present results indicate that the combined treatment of NDGA and TRAIL is effective even in malignant tumors harboring *p53* mutation. In addition, DR5 is a target gene of p53.[26,28,29] The present results indicate that NDGA induces DR5 expression in a p53-independent manner. NDGA did not increase DR5 promoter activity, suggesting that an enhancer regulated by NDGA locates in a region except for the DR5-upstream promoter that was examined or that NDGA increases DR5 mRNA stability. Moreover, 80 μM NDGA increased DR5 protein but not mRNA compared with 40 μM NDGA treatment. As the reason for the difference between DR5 mRNA and protein induced by NDGA, 80 μM NDGA might increase DR5 protein synthesis or stability.

The most attractive feature of TRAIL is its tumor-selective killing, with little or no toxicity in normal cells.[19–21] If NDGA sensitizes TRAIL-induced apoptosis in normal cells, the combination would not be valuable. Using PBMC as a model of normal cells, the authors show that NDGA do not sensitize to TRAIL-induced apoptosis in normal cells, suggesting that the combination of NDGA and TRAIL provides tumor-selective cell death.

In conclusion, the authors have shown that lipoxygenase inhibitors act as sensitizers of TRAIL-induced apoptosis through the up-regulation of DR5 in a p53-independent manner. The present results suggest that lipoxygenase inhibitors may be useful to increase the efficacy of TRAIL in the treatment of malignant tumors.

Acknowledgments

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Oncologic Outcome of Hand-Assisted Laparoscopic Radical Nephrectomy

Akihiro Kawauchi, Kimihiko Yoneda, Akira Fujito, Koji Okihara, Jintetsu Soh, Yasuyuki Naitoh, Yoichi Mizutani, and Tsuneharu Miki

OBJECTIVES To evaluate and compare the oncologic outcome of hand-assisted retroperitoneoscopic radical nephrectomy (HALS) with that of open radical nephrectomy.

METHODS The clinical and follow-up data of 123 patients with localized renal cell carcinoma who underwent HALS were retrospectively compared with those of 70 patients who underwent open radical nephrectomy.

RESULTS No significant differences were found in operating time, complication rates, or transfusion rates between the HALS and open groups. The estimated blood loss was significantly less in the HALS group. The median follow-up period was 41.0 months for the HALS group, significantly shorter than that for the open group (74.5 months). The 3 and 5-year disease-free survival rate for the HALS and open groups was 94% and 92% and 93% and 91%, respectively. The 3 and 5-year cancer-specific survival rate for the HALS and open groups was 96% and 92% and 98% and 94%, respectively. No significant differences were found in the disease-free and cancer-specific survival rates between the two groups. In the HALS group, no significant differences were found in the disease-free survival rate between those undergoing surgery by less-experienced surgeons who had performed laparoscopic nephrectomy on 10 cases or less and those undergoing surgery by more experienced surgeons.

CONCLUSIONS The oncologic outcome of HALS did not differ much from that of the open approach. Also, the experience of the surgeon did not affect the oncologic outcome. However, extended follow-up is necessary to assess the true oncologic efficacy of HALS. UROLOGY 69: 53–56, 2007. © 2007 Elsevier Inc.

Since the initial report by Clayman *et al.*,[1] laparoscopic nephrectomy has gained popularity and is becoming a standard procedure in the treatment of renal lesions. For renal cell carcinoma, laparoscopic radical nephrectomy is one of the first-choice treatments at many hospitals worldwide because of its advantages compared with open surgery, including decreased blood loss, improved cosmetics, and faster convalescence.[2]

Hand-assisted techniques for laparoscopic nephrectomy have been reported since 1994.[3,4] Although the advantages and disadvantages of hand assistance have been much discussed, this technique has been established for the surgeon as an alternative choice.[5–7] The exact choice for an individual patient might be different for every surgeon, but hand assistance is useful when intact specimen removal is required, the surgeon has limited experience, the situation is expected to be difficult, or the patient's other medical comorbidities mandate a rapid procedure.[8]

Recently, the mid-long-term prognosis of patients treated by laparoscopic radical nephrectomy was reported.[9–11] In these reports, the prognosis did not differ from that for patients treated by the open procedure. In the hand-assisted approach, the reported oncologic outcomes with relatively short follow-up showed no cancer-specific death in 48 patients and only two recurrences in 95 patients.[12,13]

In this report, we evaluated the oncologic outcome of hand-assisted retroperitoneal laparoscopic radical nephrectomy (HALS) and compared it to that of open radical nephrectomy.

MATERIAL AND METHODS

A total of 123 patients with Stage T1N0M0 or T2N0M0 renal cell carcinoma underwent HALS at 22 hospitals from November 1999 to December 2004. The clinical and follow-up data for these patients were retrospectively compared with those of 70 patients who underwent open radical nephrectomy from October 1996 to November 2003.

The operative techniques have been previously reported.[14] In brief, the patients were placed in the lateral position and a 7.0

From the Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Reprint requests: Akihiro Kawauchi, M.D., Department of Urology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kyoto 602-8566, Japan. E-mail: kawauchi@koto.kpu-m.ac.jp

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Table 1. Patient characteristics

Characteristic	HALS-Nx	Open-Nx	P-Value
Patients (n)	123	70	
Period	11/1999–12/2004	10/1996–11/2003	
Age (yr)	61.5 ± 12.4	62.1 ± 12.3	0.7407
BMI (kg/m^2)	23.4 ± 3.4	22.7 ± 3.1	0.1375
Sex (n)			0.3528
Male	82	42	
Female	41	28	
Laterality (n)			0.1416
Right	55	39	
Left	68	31	
Tumor size (mm)	43.5 ± 19.9	43.8 ± 19.6	0.8981

HALS-Nx = hand-assisted laparoscopic radical nephrectomy; Open-Nx = open radical nephrectomy; BMI = body mass index.

to 7.5-cm incision was made beside the umbilicus. The anterior and posterior fascias of the rectus muscle were incised, and the retroperitoneal space was dissected bluntly with fingers, forceps, and retractors. The LAP DISC (Hakko Shoji, Tokyo, Japan) was placed at the incision. Three 12-mm ports were used. The assistant's hand, the left for the right kidney and the right for the left kidney, was used for hand assistance. Hand assistance was mainly used for palpation of the renal artery to identify it quickly, for retraction of the kidney, and for blunt dissection around the kidney. The other techniques corresponded to those for the standard retroperitoneal laparoscopic procedure. The specimen was extracted intact through the LAP DISC.

HALS was performed by 25 surgeons and one and/or two of us (A.K. and/or A.F.) supervised all procedures. Of the 25 surgeons, 2 had performed more than 10 cases of laparoscopic nephrectomy and 23 surgeons had performed fewer than 10. Of the 123 patients in the HALS group, 87 underwent surgery by the less-experienced surgeons, 35 underwent surgery by the more experienced surgeons, and 1 patient required conversion to an open procedure.

From 1996 to 1999, all patients underwent open nephrectomy. Thereafter, the operation approach, open or laparoscopic, was chosen by patients after informed consent had been obtained. With regard to the laparoscopic procedures, the indication for HALS in all cases was Stage T1-T2N0 from November 1999 to December 2001. Thereafter, it was chosen when the surgeon's experience with laparoscopic nephrectomy was 10 cases or fewer or in difficult situations such as previous retroperitoneal or abdominal surgery or a large specimen was expected.

Statistical analyses were performed using commercially available software. Categorical and continuous data were compared with the chi-square test and Student's *t* test, respectively. Survival analyses were performed using the Kaplan-Meier analysis, with the log-rank test.

RESULTS

No significant differences were found in the background data between the two groups (Table 1). The mean operating time of 207 ± 51 minutes in the HALS group did not differ from that of 211 ± 65 minutes in the open surgery group. The mean estimated blood loss of 173 ± 185 mL in the HALS group was significantly less than that of the open surgery group at 448 ± 444 mL ($P < 0.0001$).

One conversion to an open procedure was necessary because of renal vein injury during HALS nephrectomy

Table 2. Histologic findings

Finding	HALS-Nx (n)	Open-Nx (n)
Stage		
pT1	105	52
pT2	8	9
pT3a	8	8
pT3b	1	1
Grade		
1	22	16
2	85	46
3–4	15	8

Abbreviations as in Table 1.

(0.81%), and this case was excluded from the analysis of survival. The complication rate was 8.9% (11 of 123) in the HALS group and 10.0% (7 of 70) in the open surgery group ($P = 0.8082$). In the HALS group, a small injury of the ileum in 1 case was closed by an open midline incision 5 days after HALS and a port site hernia was repaired surgically. Additional complications included 1 case each of pancreatic injury treated conservatively, pneumothorax, pulmonary embolus, abscess in the retroperitoneal space, and wound infection, as well as 2 cases of hematoma in the port site. Transfusion was performed in only 1 patient (0.81%) in the HALS group and 3 patients (4.3%) in the open surgery group ($P = 0.1056$).

Histologic examination revealed renal cell carcinoma in all patients in both groups (Table 2). No significant differences were found in the pathologic findings between the two groups.

The median follow-up period of 41.0 months in the HALS group was significantly shorter than that of 74.5 months in the open surgery group. Metastases were found in 9 patients in the HALS group, including seven lung, two bone, one lymph node, one liver, one pancreas, and one brain metastasis. No port site, incision site, or local recurrence has been found. The recurrence rate was 7.4% (9 of 122). Seven patients died of metastatic disease. The 3 and 5-year disease-free survival rate was 94% and 92% in the HALS group, respectively. The 3 and 5-year cancer-specific survival rate was 96% and 92%, respectively.

In the open surgery group, metastases were found in 7 patients, and the recurrence rate was 10% (7 of 70). Six patients died of metastatic disease. The 3 and 5-year

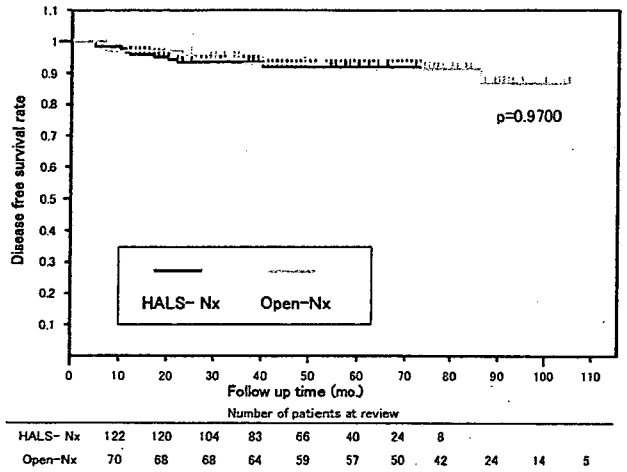

Figure 1. Kaplan-Meier disease-free survival curve.

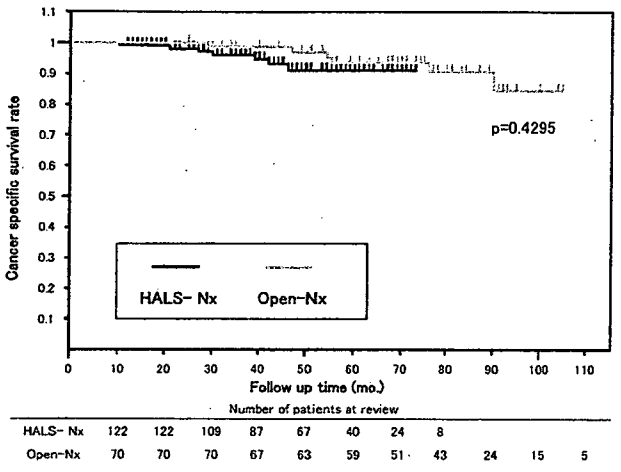

Figure 2. Kaplan-Meier cancer-specific survival curve.

disease-free survival rate was 93% and 91%, respectively. The 3 and 5-year cancer-specific survival rate was 98% and 94%, respectively.

No significant differences were found in the disease-free and cancer-specific survival rates between the HALS and open groups (Figs. 1 and 2). In the analysis of the subgroups in relation to tumor stage and tumor grade, the disease-free survival rates did not differ between the two groups. In the HALS group, no significant differences were found in the disease-free survival rates between the 87 cases performed by the less-experienced surgeons (10 or fewer laparoscopic nephrectomies) and the 35 cases performed by the more-experienced surgeons. Comparing the histologic findings in both groups, 7 of 9 patients with metastasis in the HALS group and 4 of 7 in the open group had histologic findings of pT3a, grade 3-4, and/or venous invasion for which a poor prognosis would be expected.

COMMENT

The oncologic effectiveness of radical nephrectomy can be evaluated on the basis of four criteria in three areas: the immediate adequacy of the procedure, the ill effects specific to the procedure such as seeding or development of metastatic disease, and long-term patient survival.[10] In the first area, the earliest surrogate endpoints to assess the adequacy of laparoscopic radical nephrectomy compared with open radical nephrectomy are tumor size, specimen weight, and margin status. In the present study, the tumor size and weight of the specimen were similar in both groups. Additionally, no patients had positive surgical margins. Accordingly, both procedures were thought to have the same capacity to allow for removal of the kidney, and HALS was superior to the open procedure in terms of blood loss and cosmetics.

Regarding the ill effects, we observed no tumor seeding in the renal bed or wound in either group, although in previous studies, the local and port site recurrence rate was reported as 0.73% and 0.09% to 0.18%, respectively.[15,16] In terms of the development of metastatic disease, the disease-free survival rate did not differ in either group in the present study. Regarding the patient characteristics of those with metastatic disease, 7 of 9 patients with metastasis in the HALS group and 4 of 7 in the open group had histologic findings of pT3a, grade 3-4, and/or venous invasion from which a poor prognosis would be expected. Thus, the development of metastasis was connected to the tumor characteristics rather than the operation itself.

With regard to patient survival, the cancer-specific survival rate in the HALS group did not differ from that in the open group. One of the disadvantages of the hand-assisted approach is that less working space is available secondary to the hand in the abdominal cavity. In particular, with the retroperitoneal approach, the space is smaller than that with the transperitoneal approach. In such a narrow space, the use of the hand might be suspected to affect the oncologic outcome. However, the prognosis of the patients treated by HALS was similar to that for patients treated by the open approach. The other concern was whether surgeon experience would affect patient prognosis, because hand assistance is indicated for educational purposes or when the surgeon has had limited experience. In such cases, some disadvantages, such as a longer operating time, could be expected. It was reported that the insufflation time for those performing four or fewer cases was significantly longer than that for those performing 16 or more cases.[17] The present study, however, showed that surgeon experience did not affect the oncologic outcome. Thus, patient survival did not differ between the two groups. Similarly, no significant differences were found in patient survival between the laparoscopic group and the open group in the previous reports.[9-11] The 5-year disease-free and cancer-specific survival rates for the standard laparoscopic procedure were reported to be 91% to 95% and 94% to 98%, respectively.[9-11] The corresponding rates of 92% and 92% in the HALS group in the present study are comparable to the previous results.

CONCLUSIONS

The results of our study have shown that the oncologic outcome after HALS did not differ from that in the open approach. Surgeon experience also did not affect the oncologic outcome. However, extended follow-up is necessary to assess the true oncologic efficacy of this procedure.

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Original Article: Clinical Investigation

Paclitaxel, ifosfamide, and nedaplatin (TIN) salvage chemotherapy for patients with advanced germ cell tumors

Norio Nonomura,[1] Daizo Oka,[1] Kazuo Nishimura,[1] Masashi Nakayama,[1] Hitoshi Inoue,[1] Yoichi Mizutani,[2] Tsuneharu Miki[2] and Akihiko Okuyama[1]

[1]Department of Urology, Graduate School of Medicine, Osaka University, Osaka, and [2]Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Background: The paclitaxel, ifosfamide, and cisplatin regimen has been used to treat metastatic testicular cancer with successful results. We investigated the usefulness of a paclitaxel, ifosfamide, and nedaplatin (TIN) regimen as salvage therapy for patients with advanced testicular germ cell tumors (GCTs).

Methods: Eight patients with advanced GCTs were treated with TIN. The treatment was performed as salvage therapy for cases refractory to therapies, such as bleomycin, etoposide and cisplatin, and irinotecan with nedaplatin. The TIN regimen consisted of paclitaxel (200 mg/m^2) by 24-h infusion on day 1, followed by ifosfamide (1.2 g/m^2) infusions over 2 h on days 2–6, and nedaplatin (100 mg/m^2) given over 2 h on day 2.

Results: Seven out of eight patients achieved a disease-free status after chemotherapy, followed by surgical resection of the residual tumor. Six of the seven patients have continued to show no evidence of disease after salvage therapy, with a median follow-up period of 27 months, but one patient developed a 'growing teratoma syndrome' in the mediastinum 31 months after TIN chemotherapy. All patients developed grade 4 leukocytopenia. However, it could be managed by using granulocyte colony-stimulating factor. Only one patient developed grade 2 sensory neuropathy and no patient developed nephrotoxicity.

Conclusion: The TIN regimen was efficacious and well-tolerated as salvage chemotherapy for Japanese patients with advanced GCTs.

Key words: germ cell tumor, ifosfamide, nedaplatin, paclitaxel, salvage chemotherapy.

Introduction

Germ cell tumors (GCTs) of the testis are relatively rare, representing ≈ 1% of all malignancies in men.[1] However, they constitute the most common solid tumors in young males aged 20–35 years. Since the introduction of cisplatin combination chemotherapy, it has become possible to cure ≈ 70–80% of patients with advanced GCTs.[2] Despite this considerably high cure rate, 20–30% of patients do not respond completely to first-line chemotherapy or relapse after completion of this therapy. For patients with refractory or relapsed GCTs, the current standard salvage chemotherapy with cisplatin, ifosfamide plus etoposide (VIP) or vinblastine (VeIP) can achieve a complete response in 50% of patients. However, only approximately one-half of these responses remain durable, resulting in a long-term disease-free-survival rate of 10–25%.[3–5] One of the other treatments that has been tested for patients with GCTs resistant to the cisplatin-based standard chemotherapy regimen consists of high-dose chemotherapy (HDCT) followed by autologous stem cell support. However, according to preliminary data on 280 patients who relapsed after first-line cisplatin-based chemotherapy and were randomized to receive either three cycles of conventional-dose chemotherapy plus HDCT or four cycles of standard conventional dose chemotherapy, no statistically significant differences were observed between the treatment groups in terms of event-free and overall survival.[6] In some recent studies, other agents, such as gemcitabine, irinotecan, and temozolomide, have been used as salvage chemotherapy for these GCTs. In a recent report, paclitaxel, ifosfamide and cisplatin (TIP) achieved a complete response in 23 of 30 (77%) relapsed patients with testicular GCTs.[7] These results suggest that there might be some advantage in the use of TIP as salvage chemotherapy for patients with unfavorable prognostic features, such as a refractory tumor or late relapse, and also in its use as part of induction chemotherapy for poor-risk patients with an incomplete response to first-line chemotherapy. In Japan, Kawai *et al.* reported that five of eight patients (62%) achieved a disease-free status after TIP chemotherapy and surgical resection of the residual tumor.[8] However, this regimen has not been clinically available in Japan, as paclitaxel, the key drug of the regimen, has not been approved for testicular cancer by the Japanese Ministry of Health, Labor and Welfare. The clinical use of cisplatin is sometimes limited due to its nephrotoxicity in advanced GCT patients. The purpose of the present study was to test the usefulness of a paclitaxel, ifosfamide, and nedaplatin (TIN) regimen in Japanese patients with advanced GCTs and to investigate the usefulness of nedaplatin instead of cisplatin in this regimen for preventing renal failure.

Patients and methods

Patients' characteristics and prior treatment

Eight male patients with advanced GCTs were registered for TIN chemotherapy at Osaka University Hospital between April 2001 and March 2005. In Osaka University Hospital, the use of paclitaxel for patients with GCTs was reviewed and approved by the Committee of the University Hospital Investigative Fund. Written informed consent was obtained from each patient. The patients' characteristics are

Correspondence: Norio Nonomura MD, PhD, Department of Urology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita-city, Osaka 565-0871, Japan. Email: nono@uro.med.osaka-u.ac.jp

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Table 1 Patients' characteristics and prior therapies

Case	Age (years)	Histology	Stage	IGCCC	First-line chemotherapy (maximal response)	Second-line chemotherapy (maximal response)	Further therapy
1	27	S, IT, Ch	aB	Intermediate	PVB × 4 (PR^{m-})	VIP × 2 (PD)	CPT-11 + N × 5, VP-16 + N × 1, RPLND cancer (+), CPT-11 + N × 2, HD-VIP × 4
2	30	S, IT	IIIA	Intermediate	BEP × 2, EP × 2 (NC)	CPT-11 + N × 2 (PR^{m-})	RPLND cancer (+), CPT-11 + N × 1
3	25	Y, T	IIIA	Intermediate	BEP × 2 (NC)	CPT-11 + N × 1 (NC)	–
4	41	E, T	IIIC	Poor	BEP × 3, EP × 1 (PR^{m-})	CPT-11 + N × 1 (PD)	RPLND cancer (+), CPT-11 + N × 2
5	27	E, Y, Ch, T	IIIB2	Intermediate	BEP × 3, EP × 2 (PR^{m+})	CPT-11 + N × 2 (PR^{m-})	–
6	40	Ch	IIIC	Poor	BEP × 3, EP × 2 (PR^{m+})	HD-VIP × 1 (NC)	–
7	27	E	IIIC	Poor	BEP × 6 (PR^{m-})	CPT-11 + N × 2 (NC)	–
8†	31	Y	IIIB2	Intermediate	BEP × 2 (PR^{m+})	VIP × 3 (CR)	–

†Relapsed case. BEP, bleomycin + etoposide + cisplatin; Ch, choriocarcinoma; CPT-11 + N, irinotecan + nedaplatin; CR, complete response; E, embryonal carcinoma; EP, etoposide + cisplatin; HD, high-dose; IGCCC, International Germ Cell Consensus Classification. IT, immature teratoma; LN, lymph node; $^{m+}$, marker positive; $^{m-}$, marker negative; NC, no change; PD, progression of disease; PR, partial response; PVB, cisplatin + vinblastine + bleomycin; RPLN, retroperitoneal lymph node; S, seminoma; T, mature teratoma; VIP, etoposide + ifosfamide +vcisplatin; Y, yolk sack tumor.

summarized in Table 1. The median age at the time of treatment was 28.5 years (range = 25–41 years). All patients had a histologically confirmed GCT with measurable disease and elevated serum tumor markers. According to the International Germ Cell Consensus Classification (IGCCC),[9] five cases were 'intermediate' and three were 'poor'. The pretreatment evaluation included a history and physical examination, chest X-ray, serum tumor markers, and routine blood chemistry. Further requirements were an adequate renal function defined by a creatinine clearance of 50 mL/min, adequate bone marrow function, defined as a leukocyte count of 3000/μL, a thrombocyte level of 100 000/μL, and no other major organ dysfunction. Depending on the sites of metastatic disease, all patients underwent helical computed tomography (CT) of the chest, abdomen, and pelvis. The prior chemotherapy treatments and the maximal responses also are described in Table 1. Seven cases were treated with bleomycin, etoposide, and cisplatin (BEP) and one with cisplatin, vinblastine, and bleomycin as the induction chemotherapy.

High-dose chemotherapy with peripheral blood stem cell transplantation was performed for two patients (cases 1 and 6). Case 1 received retroperitoneal lymph node dissection (RPLND) after obtaining consolidation with fourth-line chemotherapy. The pathological examination revealed viable cancer cells in the resected specimens. Then, he received two cycles of adjuvant chemotherapy with irinotecan plus nedaplatin (CPT-11 + N). As right hydronephrosis appeared during this adjuvant therapy, caused by paracaval lymph node metastasis, with a slight increase of serum human chorionic gonadotropin (HCG) and lactate dehydrogenase (LDH), he was treated by four cycles of HDCT using VIP. During the HDCT, the tumor markers were normalized once. However, because the tumor markers rose again just after the HDCT, he was subjected to TIN chemotherapy. Case 2 received CPT-11 + N regimen as second-line chemotherapy and achieved a negative partial response (PR^{m-}. As the RPLND showed a major proportion of viable cancer cells in the resected specimen, he received CPT-11 + N as adjuvant chemotherapy. As bronchopulmonary lymph node metastasis appeared during the adjuvant chemotherapy, the TIN regimen was given to him. Cases 3, 5, 6, and 7 received this regimen as the third-line chemotherapy because they showed progression after second-line chemotherapy. Case 4 showed a PR^{m-} after receiving three cycles of BEP and one cycle of etoposide and bleomycin (lung metastasis: complete response [CR]; RPLN: PR). As the RPLND showed viable cancer cells, he received adjuvant chemotherapy of two cycles of CPT-11 + N. However, during the CPT-11 + N chemotherapy, liver metasatsis appeared. Then, he received TIN. Case 8 was the recurrent case. He achieved CR (lung metastases) after receiving two cycles of BEP and three cycles of VIP and maintained his disease-free status for 1 year. As lung metastasis appeared again 1 year after obtaining CR, he received TIN.

Chemotherapy program

Chemotherapy consisted of paclitaxel (200 mg/m^2) by 24-h infusion on day 1, followed by ifosfamide (1.2 g/m^2) infusion over 2 h on days 2–6, and nedaplatin (100 mg/m^2) given over 2 h on day 2. This cycle was repeated every 4 weeks. The dosages and schedule were chosen by referring to the reported TIP regimen,[7] but the dose of paclitaxel was fixed at 200 mg/m^2 in the present study and cisplatin (100 mg/m^2) was changed to nedaplatin (100 mg/m^2). As paclitaxel is not an approved chemotherapeutic agent in Japan, we tried to avoid serious complications with the conventional TIP regimen; thus, we extended one cycle to four weeks. There is no evidence whether the dosing of nedaplatin compensates the dose intensity reduction of paclitaxel and ifosfamide. Mesna (400 mg) was administered intravenously with/after ifosfamide infusion every 4 h thereafter for a total of three doses per day. All patients received prophylactic premedication of 6 mg of dexamethasone 6 h before paclitaxel, intravenous ranitidine, and oral diphenhydramine (50 mg of each) 30 min prior to the paclitaxel administration. If the white blood cell (WBC) count was <1000/μL, patients received granulocyte colony-stimulating factor (G-CSF) daily by subcutaneous injection until the WBC count >3000/μL.

Table 2 Treatment outcomes

Case	Relapse/residual sites and their maximal diameter (cm)	Marker status before TIN†	No. of cycles	Marker normalization	Size reduction (%)	Clinical response/ pathological response	Outcome (duration: months)
1	Residual tumors: RPLN (1.0), liver (1.0)	HCG = 16 LDH = 499	4 4	Yes	96	PR^{m-}/pCR	NED (45+)
2	Relapse: bronchopulmonary LN (1.0)	Negative	2	–	100	CR/–	NED (38+)
3	Residual tumors: RPLN (5.0), mediastinal LN (1.0)	AFP = 44	3	Yes	–	NC/teratoma	NED (31+)
4	Relapse: liver (1.5)	LDH = 285	4	Yes	100	CR/–	NED (16+)
5	Residual tumors: RPLN (1.0), mediastinal LN (1.0)	AFP = 6 LDH = 341	2	Yes	94	PR^{m-}/pCR	NED (15+)
6	Residual tumors: mediastinal LN (0.9) lung (3.6), brain (0.6)	HCG = 6	3	No	–	PD	DOD (12)
7	Residual tumors: RPLN (1.0), lung (3.5)	Negative	4	–	91	PR^{m-}/pCR	NED (9+)
8	Residual tumor: lung (4.0)	AFP = 14	4	Yes	86	PR^{m-}/pCR	NED (45+)

†Normal levels of tumor markers: AFP < 5.0 ng/mL; HCG = 0–5 mIU/L; LDH = 103–229 U/L. AFP, α-fetoprotein; CR, complete response; DOD, died of disease; HCG, human chorionic gonadotropin; LDH, lactate dehydrogenase; LN, lymph node; $^{m+}$, marker positive; $^{m-}$, marker negative; NC, no change; NED, no evidence of disease; pCR, pathological complete response; PD, progression of disease; PR, partial response; RPLN, retroperitoneal lymph node.

Evaluation of response and toxicities

Evaluations were performed approximately every 1 month after the first day of the cycle of chemotherapy and included a physical examination, complete blood count, comprehensive chemistry panel, α-fetoprotein, HCG measurements, and a helical CT scan of the chest, abdomen, and pelvis, as required for the assessment of tumor response. After the completion of two cycles of chemotherapy and radiographic and tumor marker assessment, surgical resection of all residual masses was considered. The response to the therapy was categorized according to the General Rules for Clinical and Pathological Studies on Testicular Tumors.[10] The survival duration was measured from the date of the completion of TIN. Evaluation of the toxicities was performed according to the National Cancer Institute Common Toxicity Criteria.[11]

Results

Patient outcomes

Two to four cycles of TIN (median = 3.5 cycles) per patient were administered. Seven patients received the full-dose regimen, while for one patient (case 8), the fourth cycle was performed with 70% of the full dose of all chemotherapeutic agents. The response was assessable in all patients (Table 2). Seven patients achieved normalized serum tumor markers by a series of chemotherapy treatments. The responses of measurable disease were defined as CR in two patients (cases 2 and 4) and progression of disease (PD) in one patient (case 6). Four patients (cases 1, 5, 7, and 8) achieved PR^{m-} and underwent postchemotherapy surgery. The surgery included a thoracotomy in one patient (case 8), RPLND in three patients (cases 1, 5 and 7), and RPLND plus resection of a mediastinal tumor (case 3). The pathological examinations of cases 1, 5, 7, and 8 revealed necrosis and fibrosis in the resected tissues. The pathological diagnosis of case 3 was mature teratoma. One patient (case 6) achieved a transient disappearance of metastases in the liver, kidney, skin, and stomach and a PR of multiple lung metastases after two cycles of TIN, but the brain metastases revealed no change. We then offered the patient gamma-knife radiosurgery for the brain metastases, but he refused this therapy. During the third cycle of TIN and continued irinotecan plus nedaplatin regimen, disease progression of the lung and brain metastases was observed and he died of the disease just 1 year after the completion of TIN therapy. Therefore, seven of the eight patients achieved a disease-free status after TIN and subsequent surgical resection of the residual tumor mass. Of them, six patients are currently alive without evidence of disease at a median follow-up duration of 27 months (range = 9–45 months). The one remaining patient (case 3), who relapsed at 31 months after TIN therapy, underwent the resection of the mediastinal metastasis. The pathological diagnosis of the resected tissue was also mature teratoma. Therefore, this case was defined as 'growing teratoma syndrome'.

Toxicities

The toxicity profile is described in Table 3. All patients showed grade 4 leukocytopenia, which could be managed by using G-CSF. Only one case (case 1) developed grade 2 sensory neuropathy of both hands; while no other motor neuropathy was observed in any case. Grade 2 nausea, gastritis, and anorexia were observed in all patients. Seven patients developed grade 2 fatigue and one patient developed grade 2 alopecia. No remarkable renal toxicity was observed in any patient.

Discussion

Paclitaxel is a member of the taxoid family and has broad antitumor activity. It binds to the β-subunit of tubulin in cell microtubules, promoting the polymerization of tubulin into stable microtubules, hence

Table 3 Summary of adverse events

Adverse event	Grade				
	1	2	3	4	5
Hematological					
Anemia	0	8	0	0	0
Leukopenia	0	0	0	8	0
Thrombocytopenia	0	8	0	0	0
Non-hematological					
Fatigue	1	7	0	0	0
Alopecia	7	1	–	–	–
Anorexia	0	8	0	0	0
Gastritis	0	8	0	0	0
Nausea	0	8	0	0	0
Neuropathy: sensory	7	1	0	0	0

inhibiting the formation of stable microtubule bundles and leading to cellular death. Paclitaxel showed antitumor activity with response rates of 11–25% in refractory or relapsed GCT patients in several single-agent phase II studies.[12,13] Paclitaxel also showed synergy with cisplatin and alkylating agents against cisplatin-resistant teratocarcinoma cell lines *in vitro*.[14] These encouraging results led to the further evaluation of paclitaxel as part of salvage regimens. Therefore, paclitaxel has been increasingly used in different combination programs for the treatment of relapsed and refractory GCTs.[15,16] More specifically, the combination of paclitaxel with ifosfamide and cisplatin (TIP) was examined in the phase I and II trials conducted by the Memorial Sloan Kettering Cancer Center.[15] The study recruited 30 patients in the phase I trial and 24 in the phase II trial; all of them had relapsed GCTs and received TIP as the first-line salvage regimen. Paclitaxel was administered at escalating doses of 175, 215 or 250 mg/m^2 during phase I and at 250 mg/m^2 during phase II, along with ifosfamide at 5 g/m^2 and cisplatin at 100 mg/m^2 every 21 days for a total of four cycles. The overall response and complete response rates were 80% and 77%, respectively, while 73% of cases achieved long-term disease-free survival. The main toxicity was myelosuppression and two of 30 patients experienced grade 3 peripheral neuropathy.

Most patients need hydration in order to prevent renal dysfunction caused by cisplatin. Nedaplatin is a second-generation platinum derivative with reduced nephrotoxicity and peripheral neuropathy that exerts antitumor activity against various cancers, including testicular, gynecological, and lung carcinomas.[17,18] The official indications in Japan are head and neck, testicular, lung, esophageal, ovarian, and cervical cancers. A randomized clinical trial comparing nedaplatin to cisplatin, both in combination with vinblastine, showed no advantage over cisplatin in the objective response and overall survival, with less toxicity. More thrombocytopenia was observed, but nedaplatin caused less leucopenia, nephrotoxicity, and gastrointestinal toxicity.[19]

In this study, we treated patients with the TIN regimen, in which nedaplatin was used instead of cisplatin, and tested the usefulness of TIN as a salvage treatment for patients with unfavorable prognostic features of GCTs. In fact, in our study, there were no cases of renal dysfunction occurring during the TIN regimens. In the refractory and relapsed cases, a group of heavily pretreated patients, TIN was well-tolerated without grade 3 or higher non-hematological toxicities. Although all patients developed grade 4 leukocytopenia, they could be managed with G-CSF. Sensory neuropathy was seen in only one patient and no patient experienced higher than grade 2 neurotoxicity. With respect to these side-effects, TIN could be more useful than TIP in our series. These favorable toxicity profiles might be related to the extended treatment regimen to 4 weeks per one cycle compared to the original TIP regimen. We have no evidence whether the nedaplatin dosing compensates the dose intensity reduction of paclitaxel and ifosfamide. Based on our previous report, nedaplatin exerted the equivalent antitumor effect as cisplatin against advanced GCTs, at least in combination with irinotecan.[20] As a possible synergistic neurotoxic effect was suggested for the combination of cisplatin derivatives and paclitaxel,[21] close symptom assessment and neurological examination are recommended when using TIN, especially when using it as salvage therapy. In our series, seven of the eight patients with refractory or relapsed disease achieved a disease-free status after TIN. Although one patient relapsed at 31 months after TIN therapy, the other six patients have continuously remained in disease-free status. The TIN regimen induced a durable response with no relapses of GCTs in two patients with liver metastases. It was better tolerated as the salvage chemotherapy for poor-risk patients than our prior regimen with irinotecan plus nedaplatin.[20]

The European Organization for Research and Treatment of Cancer (EORTC)-designed phase II/III randomized studies of BEP compared to paclitaxel plus BEP in patients with intermediate-prognosis GCTs and the studies are now under way.

In summary, TIN was an effective salvage chemotherapy for relapsed and selected refractory GCTs, like the TIP regimen. Furthermore, TIN therapy caused less non-hematological toxicities than the TIP therapy. However, larger and longer-term follow-up studies are needed to examine various TIN protocols as salvage chemotherapy for advanced GCTs.

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Clinical efficacy of alternative antiandrogen therapy in Japanese men with relapsed prostate cancer after first-line hormonal therapy

Koji Okihara, Osamu Ukimura, Noriyuki Kanemitsu, Yoichi Mizutani, Akihiro Kawauchi, Tsuneharu Miki and Kyoto Prefectural University of Medicine Prostate Cancer Research Group

Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Background: To confirm the effectiveness of alternative antiandrogen therapy (AAT) in Japanese patients with prostate cancer relapse after first-line hormonal therapy.

Methods: A total of 80 patients who had successive serum prostate-specific antigen (PSA) progression after first-line hormonal therapy (luteinizing hormone-releasing hormone agonist alone: 21 cases; combined antiandrogen blockade therapy: 59 cases) were enrolled. We evaluated the positive ratio of antiandrogen withdrawal syndrome (AWS), the PSA responses with second- and third-line AAT, and cause-specific survival in terms of the effectiveness of AAT.

Results: The overall positive AWS ratio after first-line therapy was 33%, while that after second-line therapy was 7%. There was no correlation between the first-line PSA response and the positive AWS. Of the 10 positive and the 20 negative AWS cases, secondary antiandrogen administration was effective in 50% and 60% of cases, respectively. The positive PSA responders at second- and third-line therapy were 51% and 13%, respectively. For second-line therapy, the effective rates from steroidal to non-steroidal, from non-steroidal to non-steroidal antiandrogen, and from non-steroidal to steroidal were 83%, 43%, and 14%, respectively. The cause-specific survival of the second-line responders was significantly better than that of the non-responders.

Conclusion: There was a substantial number of patients who found second-line AAT to be modestly effective. Flutamide was effective as an alternative antiandrogen for the patients' relapse treatment with bicalutamide in Japanese men.

Key words: alternative antiandrogen, antiandrogen withdrawal syndrome, prostate cancer, relapse.

Introduction

The incidence, as well as the mortality of prostate cancer in Japan, is still lower than those in Western countries.[1] However, prostate cancer is becoming a major public health concern in Japan because the age-adjusted incidence of this malignancy rapidly increased 6.5-fold between 1975 and 1998.[2] The age-adjusted mortality rate also increased 4.3-fold between 1980 and 2000. In addition to the increasing incidence and mortality rate, 40% of all registered Japanese prostate cancer cases ($n = 4529$) in 2000[3] were diagnosed at >75-years-old and ≈ 20% of the men were newly diagnosed with metastatic disease. Considering the high incidence and mortality specifically in elderly Japanese men and the substantial number with metastatic disease, antiandrogen therapy still plays a major role in treating prostate cancer. However, most patients with locally advanced or metastatic disease relapse after initial treatment with castration or combined androgen blockade (CAB) therapy. In 1997, Scher *et al.* reported that 38.5% of patients with progressive disease who relapsed after treatment with flutamide responded to alternative antiandrogen therapy (AAT).[4] Thereafter, Kojima *et al.* reported the clinical efficacy of AAT in 70 Japanese patients with prostate cancer.[5]

The aim of this study is to confirm the efficacy of AAT and to compare effectiveness in terms of steroidal and non-steroidal antiandrogen administration.

Correspondence: Koji Okihara MD, Department of Urology, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kyoto 602-8566, Japan. Email: okihara@koto.kpu-m.ac.jp

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Materials and methods

A total of 80 Japanese patients with histologically proven prostate cancer were enrolled from January 1999 to December 2004. The patients' age ranged from 52–86 years (mean ± SD: 71.7 ± 8.4 years). The median prostate-specific antigen (PSA) ranged from 7.7–8710 ng/mL (mean ± SD: 868 ± 1741 ng/mL). The follow-up time was 21–150 months (median: 42 months). All the patients were treated with hormonal therapy and had disease progression after first-line hormonal therapy (luteinizing hormone-releasing hormone [LH-RH] agonist alone: 21 cases, CAB therapy: 59 cases). Of the 59 cases with CAB, 53 cases received non-steroidal antiandrogen (flutamide [FLT], 375 mg daily: 22 cases; bicalutamide [BCL], 80 mg daily: 31 cases). The remaining six cases received steroidal antiandrogen (chlormadinone acetate [CMA], 100 mg daily).

We obtained institutional review board approval with the aim of retrospectively reviewing the patients' medical records. No patient had received prior therapy, including irradiation and cytotoxic therapies. The relevant patient characteristics are listed in Table 1. To analyse the responses to AAT in comparison with an equivalent group of Japanese men, we applied similar evaluative criteria to those in the report by Kojima *et al.*[5]

Definition of serum prostate-specific antigen responses at 3 months after first-line androgen deprivation therapy

1 A complete response (CR): normalization of PSA level (<4.0 ng/mL).
2 A partial response (PR): >50% decrease in the PSA level compared to the initial PSA level but >4.0 ng/mL.

Table 1 Clinical characteristics of the enrolled patients

Characteristic	N (%)
Gleason score:	
5–7	45 (56)
8–10	35 (44)
T category:	
T2	6 (8)
T3	54 (67)
T4	20 (25)
N category:	
N0	49 (61)
N1	31 (39)
M category:	
M0	30 (38)
M1	50 (62)
Clinical stage:	
C	26 (32)
D1	8 (10)
D2	46 (58)

3 Progression of disease (PD): >25% increase in the PSA level compared with the initial level.
4 No change: defined as between PR and PD.

Definition of prostate-specific antigen progression and antiandrogen withdrawal syndrome

Prostate-specific antigen progression was defined as three successive PSA level increases. After PSA progression, primary antiandrogen administration was discontinued in all the 59 cases with CAB (>4 weeks for FLT and CMA, >8 weeks for BCL), while the LH-RH analog agonist continued. The positive antiandrogen withdrawal syndrome (AWS) was defined as >50% decrease of the PSA level compared with that at the time of discontinuing the primary antiandrogen.

Prostate-specific antigen evaluation after alternative antiandrogen therapy

The PSA responders at second- and third-line therapy were classified as those who had a 50% decrease of the PSA level at the end of first- and second-line antiandrogen therapy, respectively.

Definition of response duration

Response duration was defined as the time from the start of hormonal therapy until progression.

Statistical analyses

For the statistical analysis of data, the Student's *t*-test, χ^2 test or Fisher's exact test were applied using StatView software (SAS Institute, Cary, NC, USA). The statistical significance was defined as $P < 0.05$. To compare the cause-specific survival rates, Kaplan-Meier curves were constructed.

Results

Of the enrolled 80 patients, 35 cases (43.7%) had CR of the PSA level at 3 months after first-line therapy (47.6%, 10/21) with the LH-RH agonist alone, compared with 42.3% (25/59) with CAB therapy ($P = 0.79$). Of the 21 cases with the LH-RH agonist alone and the 59 cases with CAB therapy, 95.2% (20/21) and 94.9% (56/59) reached CR or PR, respectively. Of the 76 patients with CR or PR of the PSA level, there was no significant difference in the duration of response for first-line hormonal therapy between the LH-RH agonist-alone group (22.3 ± 23.0 months) and the CAB therapy group (10.7 ± 11.9 months). In addition, there was no significant difference in the duration of the response based on steroidal or non-steroidal antiandrogen administration (CAB using BCL: 7.6 ± 7.8 months, CAB using FLT: 13.9 ± 14.7 months, CAB using CMA: 17.0 ± 17.5 months).

Relationship between first-line prostate-specific antigen response and positive antiandrogen withdrawal syndrome rate after primary antiandrogen and second alternative therapy

A total of 30 cases (51%, 30/59) could be evaluated for the AWS rate after primary antiandrogen therapy. The remaining 29 cases were excluded because of the shortage of observation for the AWS. Secondary antiandrogen (FLT or CMA) was started <8 weeks after the discontinuing of BCL in 27 (93%) of the 29 cases.

We compared the positive AWS rate after primary antiandrogen and the second alternative therapy in terms of the PSA response. Of the 30 cases that were evaluated for the AWS rate, the PSA decreased to PR or CR (>50%) in 10 cases (33%, response of duration mean ± SD: 6.7 ± 4.0 months). Of the 15 patients, one patient (7%) responded to the second-line antiandrogen withdrawal and the positive AWS was not observed in any of the three cases after the third-line hormonal therapy was discontinued. Comparing the CR cases with the PR cases, there were no significant differences in the positive AWS rate (CR: 32%, 6/19; PR: 36%, 4/11) or in the duration of the antiandrogen withdrawal response (CR: 6.6 ± 4.7 months; PR: 8.5 ± 4.4 months). The positive AWS rates in men treated with CMA, FLT, and BCL were 40% (2/5), 33% (2/6), and 32% (6/19), respectively. There were no significant differences between the AWS responses and the antiandrogens.

Of the 10 positive and the 20 negative AWS cases, secondary antiandrogen was effective in five (50%) and 12 (60%) cases, respectively. There was no significant difference between the AWS response and the effect of subsequent hormonal therapy. In our series, the AWS response could not predict the effect of subsequent hormonal therapy.

Efficacy of second-line and third-line antiandrogen therapy

To compare our series with the previous report by Kojima *et al.*[5] simultaneously, we set the two figures (Fig. 1a,b) based on the efficacy of AAT against relapsed prostate cancer. The effective rate in men who were given additional steroidal or non-steroidal antiandrogen after androgen suppression monotherapy was 71% in our series. In our series, the effective rates from CMA to non-steroidal antiandrogen (FLT or BCL) and from non-steroidal antiandrogen to CMA were 83% (5/6) and 14% (1/7), respectively. The rates from FLT to BCL and from BCL to FLT were 53% (9/17) and 38% (11/29), respectively. The change in antiandrogen from second-line to third-line is shown in Figure 1b. Of the 15 cases, the effective rate was 13% in our series,

a)

First-line		Second-Line	Effectiveness (%)	Duration of response* (mos.)
AS	→	MAB with CMA	6/10 (60%)	15.8±5.6
	↗	MAB with FLT	4/5 (80%)	10.7±6.0
	↗	MAB with BCL	5/6 (83%)	29.2±6.4.
MAB with CMA	→	MAB with FLT	3/3 (100%)	12.0±7.1
	↗	MAB with BCL	2/3 (67%)	4.3±2.1
MAB with FLT	→	MAB with CMA	1/5 (20%)	4.5
	↗	MAB with BCL	9/17 (53%)	5.5±4.9
MAB with BCL	→	MAB with FLT	11/29 (38%)	5.1±5.2
		MAB with CMA	0/2 (0%)	
			41/80 (51%)	8.6±4.6

b)

Second-Line		Third-Line	Effectiveness (%)	Duration of response** (mos.)
MAB with CMA	→	MAB with FLT	0/1 (0%)	
	↗	MAB with BCL	1/3 (33%)	5.2
MAB with FLT	→	MAB with BCL	1/3 (33%)	4.8
	↗	MAB with CMA	0/6 (0%)	
MAB with BCL	→	MAB with CMA	0/1 (0%)	
	↗	MAB with FLT	0/1 (0%)	
		Total	2/15 (13%)	5.0

Fig. 1 Efficacy of alternative antiandrogen therapy in the enrolled patients. (a) Change of antiandrogen between first- and second-line therapy. *, duration of the response to second-line therapy (months). (b) Change of antiandrogen between second- and third-line therapy. **, duration of response to third-line therapy (months).

while that in Kojima *et al.* was 29.4% (5/17).[5] In our series, no responder was treated with CMA as third-line therapy (0/7).

Cause-specific survival in terms of the response to second-line therapy

Similar to the results in the previous report,[5] the survival of second-line responders in all cases (stages C, D1, and D2) was significantly better than that of the non-responders (5-year survival rates = 91.7% and 62.2%, respectively; $P = 0.002$) (Fig. 2a). In the cases with stages D1 and D2 alone, there was also a significant difference between the responders and non-responders (5-year survival rates = 80.0% and 53.0%, respectively; $P = 0.012$) (Fig. 2b). Comparing the PSA response during first-line therapy between the responders and non-responders, the proportion of responders in second-line therapy who had achieved PSA CR (PSA response at 3 months starting after first-line therapy; 18/26) was statistically higher than those without CR (12/40, $P = 0.004$).

Discussion

In the previous studies, a substantial amount of AWS responses has been reported in men with advanced prostate cancer prior to the starting of AAT.[5,6] However, the positive response rates were widely ranged based on the dosage of the primary or secondary antiandrogen administration. Kojima *et al.* speculated that a low daily dose of FLT in Japan induced a lower positive AWS response.[5] As described, all the subjects in this study were Japanese and daily doses for FLT, BCL, and CMA were very similar to those in the previous study.[5] Furthermore, in terms of the characteristics of the enrolled patients, there were no significant

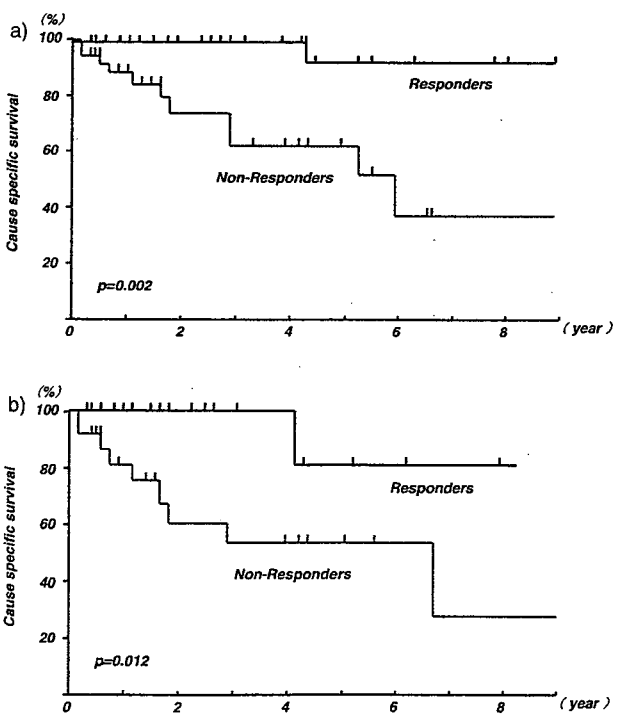

Fig. 2 Cause-specific survival in terms of the response to second-line therapy. (a) All patients whose stages were C, D1, and D2. The survival was evaluated from the time of progression of the first-line therapy. (b) Patients with stage D1 and D2 alone. The survival was evaluated from the time of progression of the first-line therapy.

differences in the distribution of age, initial PSA range, biopsy Gleason score, TNM categories,[7] and clinical stage between the two studies.[5] We speculate the homogeneity of patient background in the two retrospective studies explains the similarity of results for the PSA response at 3 months after the initiation of hormonal therapy (overall CR or PR rates in our study and the previous study were 95.0% and 95.7%, respectively), as well as the duration of response (months) based on antiandrogen administration (BCL: 7.6 ± 7.8 months [our study] versus 9.3 ± 6.0 months [Kojima *et al.*[5]]; FLT: 13.9 ± 14.7 months [our study] versus 14.6 ± 10.3 months [Kojima *et al.*[5]]; CMA: 17.0 ± 17.5 months [our study] versus 29.4 ± 38.3 months [Kojima *et al.*[5]]).

The similarity of patients' backgrounds also resulted in a similar positive AWS rate after CAB as the first-line hormonal therapy (33.3% [10/30] in this study compared to 35.8% [19/53] in Kojima *et al.*[5]). Interestingly, the positive AWS rates after second-line therapy also were similar (7% [1/15] in this study versus 8.0% [2/25] in Kojima *et al.*[5]). Similarly, the results of first-line hormonal therapy (CR and PR) did not significantly affect the AWS response rate (31.5% versus 36.3% in this study, 30.0% versus 42.9% in Kojima *et al.*[5]). Our results enhanced the evidence that the primary PSA response could not predict the AWS response.

In the results regarding the change of antiandrogens between first- and second-line therapy, the overall effective rate in our study was ≈ 12% higher than that in the previous study (51% versus 39.6%, $P = 0.52$). The reason for the higher rate in our study might originate from the difference in the effective rate concerning cases in which antiandrogen administration was added in second-line therapy (71% in our study versus 46% in Kojima *et al.*[5]). In our series, the effective rates from CMA to non-steroidal antiandrogen (FLT or BCL) and from non-steroidal antiandrogen to CMA were 83% (5/6) and 14% (1/7), respectively, while those in Kojima *et al.* were 36% (8/22) and 0% (0/4), respectively (Fig. 1).[5] Interestingly, the effectiveness in the change from CMA (steroidal) to non-steroidal antiandrogen (BCL or FLT) revealed a higher rate (46%, 13/28, when combining the two studies[5]) compared with the rate from non-steroidal to CMA (9%, 1/11, when combining the two studies[5]). Furthermore, no case where the patient went from the non-steroidal antiandrogen to CMA was effective in the change of antiandrogen from second- to third-line therapy (0%, 0/7) in our study or Kojima *et al.*[5] Combining the two sets of results, we speculate that the change to CMA might be less effective compared with the change from CMA to a non-steroidal antiandrogen. However, in the change in first- and second-line therapy, the effective rates between our study and Kojima *et al.*'s study revealed similar results in the change from non-steroidal to other non-steroidal antiandrogens (43% [20/46] in our study and 50% [7/14] in the Kojima *et al.*[5]). The PSA response rates in the change from FLT to BCL were previously reported as being from 38.5–42.9%.[4,8] Considering other results, second-line AAT, from non-steroidal to non-steroidal, was effective in a substantial number of men with advanced prostate cancer, regardless of differences in their race. In 2006, Lam *et al.* demonstrated that there was no report of responses of FLT following BCL therapy.[9] However, Kojima *et al.* already have reported that FLT was effective as an alternative antiandrogen for relapse treatment with BCL in Japanese men.[5] Combining this report's results with the results from our study, we also found that FLT was effective after relapse with BCL.

As is well-known, androgen receptor (AR) mutation might play a key role in AWS.[10,11] Suzuki reported that AR hyper-activated mutation might cause so-called anti-AWS.[12] In addition to the occurrence of AWS, AR mutation, such as the codons 877 and 741, might influence the effectiveness of AAT.[11,13] Primary non-steroidal antiandrogen administration in time might select for mutant AR, which can be stimulated by this agent but inhibited by the alternative non-steroidal antiandrogen.[8] The results of this study revealed that previous antiandrogen treatment altered the response to subsequent hormonal therapy.

In this study, the cause-specific survival rate of second-line responders in all cases, as well as the cases with stage D disease, was significantly better than that of the non-responders. Kojima *et al.*'s study also revealed significant differences between the responders and non-responders.[5] These two studies might lead to the speculation that other options, such as chemotherapy and experimental trials, need to be examined in non-responders without choosing third-line hormonal therapy. Furthermore, it is very important to predict the response of second-line therapy. Similar to Kojima *et al.*'s results,[5] pretreatment parameters, such as age, clinical stage, and pretreatment PSA value, could not predict the response of second-line therapy (data not shown). However, our result showed a certain correlation between first-line responsiveness (PSA CR) and second-line responsiveness. The PSA response after first-line therapy might be a possible parameter in predicting the response of second-line therapy, combining the data from Kojima *et al.*[5] with our results. Based on their data, the proportion of responders in second-line therapy who had achieved PSA CR (14/21) also was higher than those without CR (14/33, $P = 0.08$).

To clarify whether the PSA response can be a critical factor prior to second-line AAT, further analysis with a larger number of men will be necessary.

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ARTICLE

A Differential Ligand-mediated Response of Green Fluorescent Protein-tagged Androgen Receptor in Living Prostate Cancer and Non-prostate Cancer Cell Lines

Hiroo Nakauchi, Ken-ichi Matsuda, Ikuo Ochiai, Akihiro Kawauchi, Yoichi Mizutani, Tsuneharu Miki, and Mitsuhiro Kawata

Department of Urology (HN,AK,YM,TM) and Department of Anatomy and Neurobiology (KM,IO,MK), Kyoto Prefectural University of Medicine, Kyoto, Japan

SUMMARY Androgen has been shown to promote the proliferation of prostate cancer through the action of the androgen receptor (AR). Mutation (T877A) of the AR gene found in an androgen-sensitive prostate cancer cell line, LNCaP, has been postulated to be involved in hypersensitivity and loss of specificity for androgen. In the present study, trafficking of AR and AR (T877A) in living prostate and non-prostate cancer cell lines under high and low concentrations of androgen and antiandrogen was investigated by tagging green fluorescent protein (GFP) to the receptors. In the presence of a high concentration of androgen, AR-GFP localized in the nucleus by forming discrete clusters in all cell lines. AR (T877A)-GFP was also translocated to the nucleus in LNCaP and COS-1 cells by the addition of a high concentration of androgen. In contrast, in the presence of a low concentration of androgen, the translocation of AR-GFP and AR (T877A)-GFP was observed in LNCaP cells, but not in COS-1 cells. Upon the addition of antiandrogen, AR-GFP was translocated to the nucleus but did not form subnuclear foci in both COS-1 and LNCaP cells, whereas AR (T877A)-GFP in both cells was translocated to the nucleus with subnuclear foci. The present study demonstrates the differential response of nuclear trafficking of AR and its mutant in prostate cancer cell lines and COS cells, and the subcellular and subnuclear compartmentalization provide important information on the sensitivity of the AR mutation.

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KEY WORDS
androgen receptor
prostate cancer
GFP
live cell imaging
hormone sensitivity

THE ANDROGEN RECEPTOR (AR) belongs to the steroid/nuclear receptor superfamily that functions as ligand-dependent transcription factors to regulate expression of target genes by binding to specific hormone-responsive elements in their promoters and enhancers. The AR gene maps to band Xq11–q12 and encodes a 110-kDa protein composed of 919 amino acids in humans (Chang et al. 1985; Tenbaum and Baniahmad 1997). AR protein is composed of four distinct domains: N-terminal transcription–activation function-1 (AF-1) domain, the central DNA-binding domain (DBD), hinge region, and the C-terminal ligand-binding domain (LBD) (Simental et al. 1992; MacLean et al. 1997). Steroid/nuclear receptors can be divided into three categories based on their unliganded distribution: those primarily in the nucleus (estrogen receptor and thyroid hormone receptor), those in the cytoplasm (glucocorticoid receptor and retinoic acid receptor), and those with a mixed distribution in both the cytoplasm and nucleus (mineralocorticoid receptor, progesterone receptor, and vitamin D receptor). AR without binding ligands is primarily located in the cytoplasm (Zhou et al. 1994; Georget et al. 1997) and is thought to be in an inactive state in which it is bound to chaperones, including heat-shock proteins (hsp56, hsp90, and hsp70) (Picard and Yamamoto 1987; Yeh et al. 1999; Pratt et al. 2004). It is well conceived that androgen binding induces conformational changes in AR, but whether the same complex responsible for

Correspondence to: Mitsuhiro Kawata, Department of Anatomy and Neurobiology, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan. E-mail: mkawata@koto.kpu-m.ac.jp

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unliganded AR undergoes nuclear translocation is not clear (Kumar et al. 2006). The most commonly utilized and best-characterized nuclear import process is mediated by the recognition of clusters of basic amino acids referred to as the nuclear localization signal (NLS). The NLS, once exposed, can be recognized by importin α with importin β, which mediates AR translocation from the cytoplasm to nucleus (Kumar et al. 2006). Within the nucleus, AR forms, homodimer, and the molecular basis by which AR mediates assembly of the transcription complex have been emerging recently (Zhou et al. 1994; Tomura et al. 2001; Black and Paschal 2004).

Hormone architectonics of the tissue and cell have evolved from autoradiographic techniques. Tritium-labeled steroids were systemically administered, and uptake cells were visualized by immersion of the emulsion (Eisenfeld 1975). Although it was useful for the demonstration of ligand distributions, it did not provide information on the receptor itself or precise subcellular distributions due to its limited resolution (Stumpf and Roth 1966; Stumpf 1983). Purification of steroid/nuclear receptors and the development of specific antibodies in conjunction with immunocytochemistry enabled us to investigate their distribution at the cellular level (Press and Greene 1988; Walsh et al. 1990). There is still controversy regarding the distribution of the receptors because sections were required for fixation and permeabilization. Recent studies have used green fluorescent protein (GFP), a 27-kDa protein from the jellyfish, *Aequorea victoria* (Shimomura et al. 1962), allowing us to directly detect its chimera protein without fixing and staining the cells. It also permits the real-time imaging of the subcellular localization of the chimera protein in live cells (Nordeen et al. 2001). This GFP-tagging method has clearly shown that steroid/nuclear receptors labeled with GFP retain their normal transcriptional activity and ligand-binding specificity (Hager et al. 2000; Kawata et al. 2001). Studies including ours on the subcellular localization of AR in living cells using GFP have been reported (Georget et al. 1997; Tyagi et al. 2000; Ochiai et al. 2004). After agonist binding, GFP-tagged ARs translocated from the cytoplasm to the nucleus and concentrated transiently in a subnuclear compartment that has the appearance of foci in a boundary region between euchromatin and heterochromatin (Tomura et al. 2001).

AR mutations with the substitution of amino acids in each domain have been implicated in the pathogenesis of a number of clinical disorders including prostate cancer (Heinlein and Chang 2004). The presence of AR mutations is generally found to increase with cancer stage and may contribute to the progression of prostate cancer and the failure of endocrine therapy by allowing AR transcriptional activation in response to antiandrogens or other endogenous hormones. In prostate cancer cells, replacement of threonine with alanine at the position of 877 of AR in the ligand-binding domain has been shown to be involved in hypersensitivity and the loss of specificity for androgen (Gaddipati et al. 1994; Suzuki et al. 1996). The molecular basis of AR function underlying these disorders remains unknown, and there are few reports on the trafficking of AR mutations in prostate cancer cell lines using GFP imaging analysis.

In the present study, real-time imaging of wild-type AR-GFP and mutated AR (T877A)-GFP was examined by comparing prostate cancer cells, which include LNCaP cells (androgen-sensitive prostate cancer cell line), DU 145 cells, PC-3 cells (hormone-refractory prostate cancer cell lines) and non-prostate cancer cells, and COS-1 cells at different concentrations of androgen and antiandrogen, with special emphasis on the trafficking in subcellular and subnuclear compartmentalization. Here we present the differential ligand-mediated response of GFP-labeled AR and its mutation in living cells.

Materials and Methods

Cell Culture

LNCaP, DU 145, and PC-3 cells were originally obtained from the American Type Culture Collection (Manassas, VA). LNCaP and PC-3 cells were maintained in RPMI 1640 (Nacalai Tesque; Kyoto, Japan) with 6% and 10% fetal bovine serum, respectively. DU 145 and COS-1 cells were maintained in DMEM (Invitrogen; Carlsbad, CA) with 10% fetal bovine serum.

Western Blot Analysis

For Western blot analysis, COS-1 cells were transiently transfected with pAR-GFP (see Plasmids and Transfection). The cells were solubilized in lysis buffer. Proteins were separated with SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immunobilon-P; Millipore, Billerica, MA) using a semi-dry blotting apparatus (Transblot-SD; Bio-Rad Laboratories, Hercules, CA). Immunoblot was performed with anti-AR antibody (PG-21; provided by Dr. G. Prins) diluted 1:1000 using chemiluminescent detection (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Characterization of the anti-AR antibody has been published elsewhere (Prins et al. 1991; Lu et al. 1998). Polyclonal rabbit antibodies were raised against amino acids 1–21 of the rat AR, and this PG-21 recognized both unliganded and liganded AR.

Immunocytochemistry and Immunofluorescence

Cultured cells were fixed for 15 min at 37C in 4% paraformaldehyde in PBS. Fixed cells were incubated with anti-AR antibody for 24 hr at 4C. Cultured cells were then reacted with biotinylated goat anti-rabbit IgG antibody for 1 hr at room temperature. Cultures were reacted with the streptavidin–biotin peroxidase complex [Histofine SAB-PO (R) kit; Nichirei, Tokyo, Japan] for 1 hr at room temperature. Cells were then visualized with 0.02% 3,3-diaminobenzidine (Sigma; St Louis, MO) and 0.006% H_2O_2 in Tris–HCl-buffered saline (pH 7.6).

For immunofluorescence detection, Alexa Fluor 488-linked anti-rabbit IgG antibody (1:1000 dilution; Molecular Probes, Eugene, OR) was used as a secondary antibody.

Plasmids and Transfection

Vectors expressing AR-GFP and AR (T877A)-GFP were provided by Dr. H. Nawata (Taplin et al. 1995; Tomura et al. 2001). The N terminus of the GFP sequence was fused to the C terminus of the human AR sequence. Both nucleotide sequencing and Western blot confirmed the validity of the plasmid constructs. Cells were maintained overnight in a poly-L-lysine-coated 35-mm dish (Falcon; Piscataway, NJ) in medium. Cells were transfected using Effectene Transfection Reagent (Qiagen; Hilden, Germany) with the plasmid of AR-GFP or AR (T877A)-GFP. The precise procedure of transfection was carried out according to the manufacturer's protocol. For ligand stimulation, cells were treated with 10^{-6} M testosterone, 10^{-9} M dihydrotestosterone (DHT), or 10^{-5} M bicalutamide (AstraZeneca; Alderley Park, UK) at 37C.

Confocal Laser-scanning Microscopy for Real-time Imaging of Living Cells

Cells transfected with the GFP chimera construct were plated on a poly-L-lysine-coated 35-mm glass-bottom dish (Matsunami Glass Inc.; Osaka, Japan), and the medium was replaced with Opti-MEM (Invitrogen) for the absence of serum and testosterone for 18 hr before observation. Cells were observed with a ×63 oil immersion objective lens (Plan-Apochromat; Carl Zeiss, Oberkochen, Germany). Fluorescence images of a single Z-section from a cell were collected with a confocal laser-scanning microscope (pinhole = 1.0 Airy Units) (LSM 510META; Carl Zeiss) every 10 min after the treatment of ligands. GFP fluorescence was viewed using a 488-nm argon laser, 505-nm long-pass filter, and a 488-nm dichroic mirror. The same experiments were carried out at least three times, and typical images are shown in the figures.

Results

Characterization and Localization of Endogenous AR of Prostate Cancer Cell Lines

Because the presence of endogenous AR in prostate cancer cells has been controversial (Tilley et al. 1995; Alimirah et al. 2006), we investigated whether prostate cancer cell lines that include DU 145, PC-3, and LNCaP cells had endogenous AR by Western blot analysis using anti-AR antibody (Figure 1). AR protein was not expressed in DU 145 or in PC-3 cells but was expressed in LNCaP cells at the same level of expression as in COS-1 cells transfected with rAR.

Immunocytochemistry following the streptavidin–biotin peroxidase method with anti-AR antibody showed that AR immunoreactivity was observed only in LNCaP cells and not in DU 145 or PC-3 cells in prostate cancer cell lines. The result was consistent with Western blot analysis (Figure 2). Brown-colored reaction products

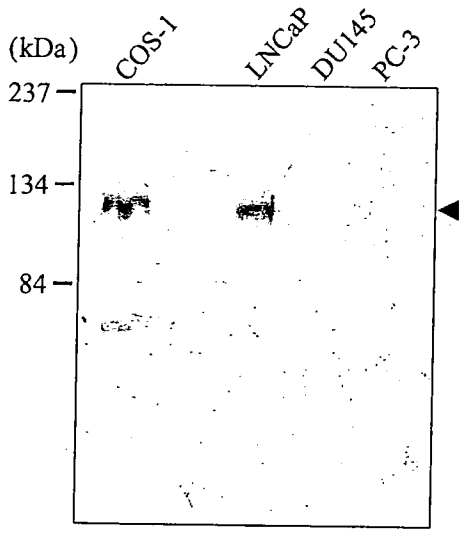

Figure 1 Expression of androgen receptor (AR) in prostate cancer cell lines. Cell lysates of prostate cancer cell lines, LNCaP, DU145, and PC-3, and COS-1 transfected with an expression plasmid of AR (COS-1) were applied to SDS-PAGE and blotted with anti-AR antibody. Specific bands were detected at the predicted molecular mass of 110 kDa in COS-1 and LNCaP lanes (arrowhead).

showing endogenous AR were detected in both the cytoplasm and nucleus of LNCaP cells without ligand, and it was translocated completely to the nucleus in the presence of 10^{-6} M testosterone. As a control, we examined AR immunoreactivity in COS-1 cells transfected with plasmid vector expressing rAR. Immunoperoxidase reaction products showing AR were observed in the cytoplasm of COS-1 cells in the absence of testosterone, whereas AR immunoreactivity was seen in the nucleus after the addition of 10^{-6} M testosterone. We also performed a fluorescent method using Alexa 468-labeled IgG as a secondary antibody. The same result as with the immunoperoxidase method was observed: fluorescence was observed in both the cytoplasm and nucleus of LNCaP cells in the absence of testosterone, but bright immunofluorescence was seen only in the nucleus after the addition of 10^{-6} M testosterone. In addition, immunofluorescence of ligand-activated AR was observed as a discrete non-uniform pattern in comparison with a diffuse distribution in the absence of ligand.

Subcellular Localization and Trafficking of AR-GFP With High Concentration of Androgen

We transfected plasmids expressing AR-GFP to COS-1, LNCaP, DU 145, and PC-3 cells, and fluorescent images were scanned of single living cells by confocal laser microscopy after the addition of 10^{-6} M testosterone (Figure 3). In cell lines of COS-1, DU 145, and PC-3, the fusion protein of AR-GFP was detected in the cytoplasm of these cells in the absence of andro-