Table 3. Endocrinologic values before and after treatments of LER and Kampo.

	Treatment Group	Serum level – mean (SD)			
		n	Baseline	6 months	*p* Value
Testosterone (ng/mL)	LER	18	3.28 (2.03)	3.44 (2.08)	0.51
	Kampo	14	3.60 (0.82)	3.80 (0.75)	0.17
Free testosterone (pg/mL)	LER	18	7.89 (3.73)	9.32 (6.08)	0.24
	Kampo	11	7.67 (3.28)	6.99 (3.38)	0.10
FSH (mIU/mL)	LER	17	13.0 (12.7)	12.8 (10.8)	0.66
	Kampo	15	11.0 (5.1)	10.9 (4.6)	0.77
LH (mIU/mL)	LER	16	5.41 (5.06)	5.31 (4.45)	0.85
	Kampo	11	6.70 (4.24)	6.28 (3.59)	0.48
PRL (ng/mL)	LER	13	7.56 (3.62)	8.10 (7.49)	0.83
	Kampo	12	4.85 (1.52)	4.93 (0.76)	0.89
E2 (pg/mL)	LER	5	36.0 (32.4)	32.8 (28.6)	0.18
	Kampo	10	15.8 (3.4)	16.4 (3.0)	0.44

The *p* values were calculated by paired *t*-test.
LER, LEOPIN ROYAL®; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; E2, estradiol.

analysis. The declines in the psychological sub-score and the total score in the AMS scale of the LER group were greater than those in the Kampo group, and the *p* values of G and G × M were less than 0.05. The IIEF-5 score increased in the LER group, and G and G × M showed significant differences. There was no inter-group difference in sexual sub-score in the AMS scale, SRQ-D and ADAM questionnaire. The serum levels of hormones showed no significant change in either group (Table 3).

Adverse events were noted in two cases of the LER group (epigastric discomfort and skin rash); no causal relationship of epigastric discomfort with LER was established, and that of skin rash with LER remains unclear.

Discussion

In the present study, somatic and psychological symptoms of aging in males showed greater improvement in the LER group than in the Kampo group. Further, only those subjects who were treated with LER reported an improvement in ED, as measured by the IIEF-5.

Kampo medications have been often used in male patients with LOH-related symptoms. Saikokaryukotsuboreito has been reported to be effective for eugonadal patients with LOH-related symptoms [10]. In the cases of severe nervousness, anxiety or irritation, Yokkansan (*Yi Gan San*) and keishikaryukotsuboreito (*Gui Zhi Jia Long Gu Mu Li Tang*) are used. Hachimijiogan, goshajinkigan and hochuekkito are used for ED. Kamishoyosan and hangekoubokuto are known to be medications for females, but these medicines are also used for males with somatic symptoms such as hot flashes and sweating abnormalities [11].

The LER formulation used in this study contains six crude extracts. AGE is one of the extracts that has several pharmacological actions. Kasuga et al. [1] have reported that AGE significantly enhanced sexual behaviors (mounting and intromission) and spermatogenesis in mice with testicular hypogonadism induced by warm water treatment. Nitric oxide (NO) is a trigger to increase blood flow into the corpus cavernous at erection. Morihara et al. [12] indicated that AGE temporarily increased NO production in the plasma of mice, and that the amelioration of NO induced by AGE was due to constitutive activation of NO synthases. Flow-mediated dilation (FMD) is known to reflect NO-mediated endothelial function. AGE was reported to increase the FMD in patients with coronary artery disease [13] and acute hyperhomocysteinemia induced by an oral methionine challenge in healthy subjects [14]. AGE also has effects on psychological stress. Kyo et al. [15] found a protective effect of AGE on damaged immune function caused by psychological stress induced by a communication box. Psychologically stressed mice showed decreased immune functions, which was restored by AGE. In addition, AGE has been reported to have anti-aging effects on the Senescence-accelerated mice (SAM); AGE extended survival and improved the learning and memory impairment of the SAM [16].

Panax ginseng is an important herbal medicine and is frequently blended in Kampo medications considered tonics. Jang et al. [2] performed a systematic review based on randomized clinical studies of red ginseng and provided evidence suggestive of the effectiveness of red ginseng in the treatment of ED. Ginseng has several pharmacological actions. Gillis [17] reported that the antioxidant and organ-protective actions of ginseng are linked to enhanced NO synthesis in the endothelium of the lung, heart, and kidney and in the corpus cavernosum, and that the enhanced NO synthesis could contribute to ginseng-associated vasodilation and perhaps also to an aphrodisiac action of the root.

Traditionally, the epimedium herb (Ikariso in Japanese, *Yinyanghuo* in Chinese), cuscuta seed (Toshishi, *Tusizi*) and velvet antler (Rokujo, *Lurong*) have been used as an aphrodisiac in East Asia [3]. Makarova et al. [18] reported that oral administration of a lipid-based suspension of *Epimedium koreanum* extract improved erectile function (intromissions and ejaculation) in aged rats. It is well known that icariin is an active ingredient in *Epimedium* sp. The pharmacological actions of icariin on erectile function have been reported to be mediated by the inhibition of cGMP-specific phosphodiesterase 5 (PDE5) *in vitro* [19], expression of NO synthase in castrated rats [20] and increment of serum testosterone levels in cyclophosphamide-treated rats [21].

The flavonoids extracted from the Semen Cuscutae have been reported to increase the weight of testis, epididymis, pituitary gland, stimulated testosterone and LH secretion in

male rats [22], and reversed the reduction of testosterone level and expression of androgen receptor gene in kidney-yang deficient mice [23]. Velvet antler has been reported to increase the weight of testis and prostate in castrated rats [24]. Oriental Bezoar (Goo, *Niuhuang*) is a dried gallstone of *Bos taurus domesticus* Gmelin, and is widely used in cardioactive over-the-counter (OTC) drugs in Japan [25].

There are two reports on the effect of LER, containing crude extracts of these natural products, on ED. Ushijima et al. [26] indicate a spermatogenesis-enhancing effect of LER on mice with testicular hypogonadism induced by warm water treatment, and Yaguchi et al. [27] reported that, of the 16 patients with unidentified complaints, one with ED showed a complete response to LER administration for 4 weeks.

In this study, even though the symptoms of aging in male were improved by LER administration, there was no change in the serum androgen level. As described above, the active ingredients in epimedium herb and cuscuta seed have been reported to have a serum testosterone increasing action in animal experiments. However, there is no report on the optimal doses of the action in human. As the dosage of the herbal medicines was not enough, a significant increase in serum testosterone may have not been observed during LER treatment. It has been reported that serum testosterone level does not correlate with erectile function and hypogonadism symptoms [28–31]. The effect of LER may be due to mechanisms other than androgen, for example, peripheral vasodilation by promoting NO synthesis.

Conclusion

Although this study is an open-labeled trial, our data indicate that LER is a safe medication and possibly superior to mainly kamishoyosan on the improvement of somatic and psychological symptoms of aging including ED for elderly male patients.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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Long-term results of radical prostatectomy with immediate adjuvant androgen deprivation therapy for pT3N0 prostate cancer

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Abstract

Background: Radical prostatectomy is used to treat patients with clinically localized prostate cancer, but there have been few reports of its use in locally advanced disease. We evaluated the long-term results of radical prostatectomy and immediate adjuvant androgen deprivation therapy in Japanese patients with pT3N0M0 prostate cancer.

Methods: We retrospectively reviewed 128 patients with pT3N0M0 prostate cancer who underwent radical prostatectomy at our institute from 2000 to 2006. All pT3N0 patients were treated with adjuvant androgen deprivation therapy shortly after radical prostatectomy. Immediate adjuvant androgen deprivation therapy was continued for at least 5 years. Twenty-three were excluded because of preoperative hormonal therapy, missing data, or others. Death from any cause, death from prostate cancer, clinical recurrence and hormone-refractory biochemical progression were analyzed by Kaplan-Meier graphs. Relative risks of progression were estimated using Cox proportional hazards models with 95% confidence intervals.

Results: The 10-year hormone-refractory biochemical progression-free survival rate was 88.3% and the cancer-specific survival rate was 96.3% after a median follow-up period of 8.2 years (range 25.6-155.6 months). Higher clinical stage ($p = 0.013$), higher Gleason score at biopsy ($p = 0.001$), seminal vesicle invasion ($p = 0.003$) and microlymphatic invasion ($p = 0.006$) were predictive factors for hormone-refractory biochemical progression by univariate analyses. Multivariate analyses identified Gleason score at biopsy ($p = 0.027$) and seminal vesicle invasion ($p = 0.030$) as independent prognostic factors for hormone-refractory biochemical progression. None of the patients with clinical T1 cancers ($n = 20$), negative surgical margin ($n = 12$), or negative perineural invasion ($n = 11$) experienced hormone-refractory biochemical progression.

Conclusions: Radical prostatectomy with immediate adjuvant androgen deprivation therapy may be a valid treatment option for patients with pT3N0M0 prostate cancer.

Keywords: Adjuvant androgen deprivation therapy, Pathological T3, Prognosis, Prognostic factor, Prostate cancer, Radical prostatectomy

Background

Tumor cell penetration of the prostatic capsule or invasion of the seminal vesicle is recognized as locally advanced prostate cancer of pathological T3N0. Patients with pT3N0 prostate cancers have the potential to suffer from disease relapse, and radical prostatectomy alone may fail to achieve a cure. The introduction of prostate specific antigen (PSA) assays means that more patients now undergo radical prostatectomy at earlier stages. However, pT3 disease still occurs in 25–58% of clinical T1 and T2 prostate cancer patients [1-4]. Although the management of patients with pT3 prostate cancer remains controversial, some reports recommend the use of adjuvant therapies in these patients [5-9]. Few studies have reported treatment outcomes of pT3 cancers, but some clinicopathologic factors, such as higher Gleason score, higher

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PSA level and seminal vesicle invasion are considered to be prognostic factors associated with poorer outcome [10-13]. However, to the best of our knowledge, there have been few reports of pT3N0 patients treated with adjuvant hormonal therapy, and more outcome data and accurate information are needed for these patients. We therefore analyzed clinical data from patients with pT3N0 prostate cancer to obtain detailed information and long-term outcome data. Importantly, a pathologic diagnosis of pT3N0M0 cancer is not necessarily accurate in patients who have undergone preoperative therapy, and this study therefore only included patients who had not received any preoperative treatment.

Methods

We retrospectively reviewed 128 patients with pT3N0M0 out of a total of 431 patients with prostate cancer who underwent radical prostatectomy at our hospital from January 2000 to December 2006. These patients were selected because immediate adjuvant hormonal therapy was applied in patients with pT3N0M0 prostate cancer during this period. Twenty-three patients were excluded from the analysis; four because of incomplete data, 12 because they had received preoperative hormonal therapy and seven because they had received bicalutamide only or estramustine as hormonal therapy. Data from the remaining 105 patients were analyzed. Bilateral obturator lymph nodes were dissected in all patients at radical prostatectomy. Immediate adjuvant androgen deprivation therapy was started within 12 weeks of radical prostatectomy. Undetectable PSA levels or PSA nadir were not required to be confirmed following radical prostatectomy. Clinical diagnosis was defined according to the 2009 TNM guidelines based on a digital rectal examination (DRE), transrectal ultrasonography, biopsy results, computed tomography scans and/or magnetic resonance imaging, and bone scintigraphy. All specimens were reviewed by a single pathologist. After radical prostatectomy, patients were followed-up at 1-month intervals for the first 3 months following surgery, then at 3-month intervals for 5 years, and finally at 3–6-month intervals thereafter. Follow-up examinations included measurement of PSA levels, and a DRE, computed tomography scan, magnetic resonance imaging or bone scintigraphy in the event of suspected disease recurrence. Immediate adjuvant therapy included surgical orchiectomy, administration of luteinizing hormone-releasing hormone (LHRH) analogs, and combined androgen blockade consisting of orchiectomy or an LHRH analog together with anti-androgens. Immediate adjuvant androgen deprivation therapy was continued for at least 5 years after radical prostatectomy. Salvage additive or altered hormonal therapies were initiated when the PSA level rose rapidly by >0.4 ng/ml, or when it rose consistently by >0.2 ng/ml for more than three consecutive visits. Salvage additive or altered hormonal therapy included: 1) combined androgen blockade consisting of orchiectomy or LHRH analog with bicalutamide; 2) suspension of bicalutamide to confirm the effects of anti-androgen withdrawal; 3) estramustine; and 4) dexamethasone.

Hormone-refractory biochemical progression was defined as a PSA level >0.2 ng/ml despite the above hormonal therapies. Clinical recurrence was defined as recognizable disease relapse on imaging examination. Clinical recurrence was treated by salvage radiation therapy. This study was approved by the Ethics Committee, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo.

The study end points were death from any cause, death from prostate cancer, clinical recurrence, and hormone-refractory biochemical progression. These end points were analyzed by plotting Kaplan-Meier graphs and comparing them according to each clinicopathologic factor using log-rank tests. Relative risks for hormone-refractory biochemical progression according to each clinicopathologic factor were estimated using the Cox proportional hazards models with 95% confidence intervals. All statistical analyses were performed using JMP version 9 (SAS Institute, Cary, NC, USA) and differences were considered statistically significant at $p < 0.05$. The following clinicopathologic factors were evaluated: age at radical prostatectomy, preoperative PSA level, preoperative T stage (clinical stage), Gleason score of the biopsy specimen, seminal vesicle invasion (representing stage pT3b), surgical margin of operation specimen, microlymphatic invasion, microvascular invasion, perineural invasion, and Gleason score.

Results

The clinical and pathological data for all 105 patients are shown in Table 1. The median age at surgery was 67.0 years and the median preoperative PSA level was 15.1 ng/ml (range 3.5–160.7, with lower and upper quartile values of 8.18 and 24.9). The median number of lymph nodes removed was 7.0 (range 2–19). A total of 43% of patients were underestimated preoperatively as having stage T1 (n = 20; 19.0%) or T2 (n = 25; 23.8%) tumors. Regarding the Gleason score at biopsy, 38 (36.2%) patients had a score of ≥8. Seminal vesicle invasion (pT3b) was detected in 42 patients (40.0%). Microlymphatic and microvascular invasions were detected in 33 (31.4%) and 51 (48.6%) patients, respectively. Immediate adjuvant androgen deprivation therapy consisted of androgen suppression with orchiectomy (n = 17), LHRH analog (n = 64), combined androgen blockade with orchiectomy or LHRH analog and bicalutamide or other anti-androgens (n = 24). Three patients changed their hormonal therapies during follow-up because of adverse events.

The median follow-up period was 98.7 months (range 25.6–155.6). During the follow-up period, eleven patients

(10.5%) experienced hormone-refractory biochemical progression. Seven patients experienced clinical recurrence and received salvage radiation therapy to clinically recurrent foci. Three (2.9%) patients died of prostate cancer and eight (7.6%) died of other causes. The 5- and 10-year cancer-specific survival rates were 98.1 and 96.3%, respectively. The 5- and 10-year hormone-refractory biochemical progression-free survival rates were 94.3 and 88.3%, with 5- and 10-year clinical recurrence-free survival rates of 96.0 and 93.0%, respectively. The 10-year estimated overall survival rate was 85.7%.

Table 2 shows the hormone-refractory biochemical progression-free survival rates calculated from Kaplan-Meier graphs, according to each clinicopathologic parameter. Univariate analyses using Cox proportional hazard models indicated that higher clinical stage ($p = 0.013$), higher Gleason score at biopsy ($p = 0.001$), seminal vesicle invasion ($p = 0.003$) and microlymphatic invasion ($p = 0.006$) were predictive factors for hormone-refractory biochemical progression (Table 2). Multivariate analyses identified Gleason score at biopsy and ($p = 0.027$) seminal vesicle invasion ($p = 0.030$) as independent prognostic factors for hormone-refractory biochemical progression (Table 2).

Table 1 Patient characteristics (n = 105)

Parameter		*n*	(%)
Age, years (median 67.0)	<65	37	35.2
	≥65	68	64.8
PSA, ng/ml [median 14.3 (2.4 – 160.7)]	<10	35	33.3
	10 ~ 20<	36	34.3
	20 ~ 50<	23	21.9
	≥50	11	10.5
Clinical stage	T1	20	19.0
	T2	25	23.8
	T3	59	56.2
	T4	1	1.0
Gleason score at biopsy	5 ~ 6	34	32.4
	7	33	31.4
	≥8	38	36.2
Seminal vesicle invasion	−	63	60.0
	+	42	40.0
Surgical margin	−	12	11.4
	+	93	88.6
Microlymphatic invasion	−	72	68.6
	+	33	31.4
Microvascular invasion	−	54	51.4
	+	51	48.6
Perineural invasion	−	11	10.5
	+	94	89.5
Gleason score at prostatectomy	5 ~ 6	16	15.2
	7	54	51.4
	≥8	35	33.3

Discussion

Despite widespread use of PSA measurement, pT3N0M0 prostate cancer still occurs in 25–58% of clinical T1 and T2 prostate cancer patients [1-4]. In the current study, about half of the patients (43%) were also understaged preoperatively as having organ-confined disease. Although pT3N0M0 prostate cancer is not rare, there have been few reports of treatment outcomes in these patients. The optimal postsurgical management for patients with such unfavorable pathological features remains questionable. We therefore analyzed clinical data from patients with pT3N0M0 prostate cancer to obtain detailed information and long-term outcome data.

The patients in this study achieved 5- and 10-year cancer-specific survival rates of 98.1 and 96.3%, respectively, and 5- and 10-year hormone-refractory biochemical progression-free survival rates of 94.3 and 88.3%, respectively. In a previous study, Inagaki et al. reported 1- and 3-year biochemical progression-free survival rates of 53.7 and 34.1% in 106 patients with pT3N0M0 prostate cancer treated with radical prostatectomy alone, after a mean follow-up of 1.5 years [10]. Delongchamps et al. and Briganti et al. reported 5-year biochemical progression-free survival rates of 48 and 45.0% in 147 and 500 patients with pT3N0M0 prostate cancer, respectively, treated with radical prostatectomy alone, after median follow-up periods of 5 and 3.9 years [11,12]. Thompson et al. reported 10-year metastasis-free survival rates and overall survival rates of 61 and 66% in 211 patients with pT3N0M0 prostate cancer treated with radical prostatectomy, even after salvage radiotherapy [5]. Single-modality therapy involving surgery alone might be of limited use in patients with stage pT3N0M0 prostate cancer, and a multimodal approach may be more beneficial. Three studies found that adjuvant radiotherapy after radical prostatectomy reduced the risk of subsequent biochemical recurrence in randomized clinical trials [5-7]. Thompson et al. reported a survival benefit of adjuvant radiotherapy, with a 10-year estimated survival rate of 74% in 214 patients with pT2-3 N0 prostate cancer treated with adjuvant radiotherapy, compared with 66% in 211 cases treated with surgery alone, after median follow-up period of 12.5 years [5,6]. This cohort included patients with pT3N0 and pT2N0 with positive surgical margins, and patients were allowed to receive salvage hormonal therapy during the follow-up period. Regarding the combination of radical prostatectomy and adjuvant hormonal therapy, Dorff et al. reported favorable outcomes in an interim report of a prospective randomized trial of 481

Table 2 Hormone refractory biochemical progression-free survival according to each clinicopathological parameter

Univariate and multivariate analyses for hormone refractory biochemical-progression free survival rates									
Parameter		Hormone refractory biochemical progression-fee survival rates		Univariate analysis			Multiivariate analyses		
		5 years	10 years	HR†	95% CI††	*p*	HR†	95% CI††	*p*
Age, years (median 67.0)	<65	94.6	89.9						
	≥65	94.1	87.6			0.687			
PSA, mg/ml [median 15.1 (3.5–160.7)]	<10	97.1	97.1	1					
	10-20<	88.9	80.8	6.18	1.1, 116.8	0.043			
	20-50<	100.0	100.0	0.0	0,.	0.315			
	≥50	90.9	60.6	12.9	1.9, 254.0	0.008			
Clinical stage	T1,2	100.0, 100.0	100.0, 88.9	1			1		
	T3~4	90.0	84.8	7.47	1.4, 137.1	0.013*	3.65	0.7, 68.6	0.161
Gleason score at biopsy	5~7	98.5	98.3	1			1		
	≥8	86.8	70.3	8.38	2.2, 55.0	0.001*	4.73	1.2, 31.7	0.027*
Seminal vesicle invasion	−	98.4	98.4	1			1		
	+	88.1	73.1	7.42	1.9, 48.7	0.003*	4.53	1.1, 30.1	0.030*
Surgical margin	−	100.0	100.0						
	+	93.6	86.7			0.183			
Microlymphatic invasion	−	97.2	95.0	1			1		
	+	87.9	74.4	5.33	1.5, 24.7	0.006*	2.18	0.9, 12.8	0.140
Microvascular invasion	−	96.3	90.5						
	+	92.2	85.6			0.468			
Perineural invasion	−	100.0	100.0						
	+	93.6	86.7			0.242			
Gleason score at prostatectomy	5–7	95.7	89.6						
	≥8	91.4	71.4			0.114			

†Hazard ratio by Cox proportional-hazard models
††Confidence interval

patients with pT2-3 N0-1 prostate cancer, including 61% of T3 and 16% of N1 patients [14]. Although longer observation periods are awaited, they reported 5-year biochemical progression-free and overall survival rates of 92.5 and 95.5%, respectively, in patients treated with adjuvant hormonal therapy consisting of goserelin and bicalutamide, after a median observation period of 4.4 years. Some studies reported encouraging results in more challenging patients with more severe pathological stages. Spahn et al. reported on 173 patients with pT3N0-1 tumors, including 43.3% of N1, who had undergone prostatectomy [15]. They reported an 8-year cancer-specific survival rate of 86.3% and an overall survival rate of 77.3% after a median observation period of 5.7 years in patients treated with adjuvant hormonal therapy comprising an LHRH analog with or without flutamide. Siddiqui et al. reported an advantage of adjuvant hormonal therapy with an LHRH analog, bilateral orchiectomy, or anti-androgens in a retrospective study of 191 pT3bN0M0 prostate cancer patients [9]. They found that, although the overall survival rate was similar to that in the matched control cohort, the biochemical progression-free and cancer specific survival rates were improved, with 10-year biochemical progression-free and cancer-specific survival rates of 60 and 94%, respectively, after a median follow-up of 10 years [9]. In accordance with this previous report, subgroup analyses of pT3bN0 patients in the current study demonstrated excellent outcomes, with 5- and 10-year cancer-specific survival rates of 95.1 and 90.8%, respectively. These results indicate that the combination of radical prostatectomy and adjuvant androgen deprivation therapy may produce excellent outcomes in patients with pT3N0M0 prostate cancer.

The current study achieved a 10-year cancer-specific survival rate of 96.3% and a 10-year estimated overall survival rate of 85.7% after a median follow-up period of 8.2 years. These survival rates were higher than those in previous reports, which may require an explanation. Immediate commencement of adjuvant androgen deprivation therapy after radical prostatectomy, and its comparatively long duration (at least 5 years), may have contributed to the beneficial effect. Supportive treatment strategies, such as prompt adjustment or alteration of hormonal therapy

in the event of a slight increase in PSA levels, may also have improved the treatment efficacy. It is also possible that Japanese men are more sensitive than other ethnic groups to hormonal therapy after radical prostatectomy. Akaza et al. reported 5- and 10-year cancer-specific survival rates of 90 and 69%, respectively, in 68 Japanese patients with clinical T3N0M0 tumors who were treated with hormonal therapy alone [16]. However, Ueno et al. reported 5- and 8-year progression-free survival rates of 59.8 and 48.1%, respectively, in 245 Japanese patients with clinical T3N0M0 cancers treated with combined androgen blockade (63.5%) or castration [17].

The Asia Consensus Statement 2013 in the NCCN Clinical Practice Guidelines in Prostate Cancer states that androgen deprivation is a candidate treatment option for post-radical prostatectomy recurrence in Asian patients negative for distant metastasis. The results of the current study suggest that a treatment strategy consisting of radical prostatectomy and immediate adjuvant androgen deprivation therapy may offer favorable cancer control in Japanese patients with pT3N0M0 prostate cancer. This strategy was also feasible and well tolerated. Immediate adjuvant androgen deprivation therapy thus represents an attractive option for patients with pT3N0M0 prostate cancer.

Few studies have reported on prognostic factors in patients with pT3 prostate cancer. The current study found that higher clinical stage, higher Gleason score at biopsy, and seminal vesicle and microlymphatic invasion were unfavorable factors, and multivariate analyses identified seminal vesicle invasion and Gleason score at biopsy as independent prognostic factors for hormone-refractory biochemical progression. Interestingly, no patients with clinical T1 tumors (n = 20), negative surgical margin (n = 12), or negative perineural invasion (n = 11) experienced hormone-refractory biochemical progression. In partial agreement with our results, previous studies identified Gleason score, PSA, seminal vesicle invasion and lymphovascular invasion as prognostic predictors in patients with pT3N0 stage prostate cancer undergoing radical prostatectomy [10-13].

The limitations of this study included its retrospective nature and the relatively small sample size. Further investigations, including prospective studies, are needed to compare the additive effects of multimodal therapies in patients with pT3N0, to allow the better selection of patient populations most likely to benefit from treatment. The current study indicated a significant hazard ratio for seminal vesicle invasion or with higher Gleason score at biopsy, suggesting that patients with pT3b or with higher Gleason score may be the leading candidates for such studies.

These findings were based on pathologic results. The majority of the patients included in the study were considered to have lower grade and stage at diagnosis, and T3N0 was only diagnosed after radical prostatectomy. These results suggest that radical prostatectomy is a reasonable option for the initial treatment of prostate cancer, and allow for the better selection of patients who will require additional therapies.

Conclusions

Radical prostatectomy with immediate adjuvant androgen deprivation therapy may be a valid treatment option for patients with pT3N0 prostate cancer.

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

YTS made substantial contributions to conception and design, analysis and interpretation of data and was involved in drafting the manuscript. HF made substantial contributions to conception and design, analysis and interpretation of data and was involved in revising it critically for important intellectual content. MS, TF, TN and HN made substantial contributions to acquisition of data. HK made substantial contributions to conception and design and helped to draft the manuscript. TM and MF evaluated the pathological specimens in a manner blinded to the clinical information. YH conceived and supervised the study, helped to draft the manuscript and was involved in revising it critically for important intellectual content. All authors read and approved the final manuscript.

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UROLOGY - ORIGINAL PAPER

Risk factors for septic shock in acute obstructive pyelonephritis requiring emergency drainage of the upper urinary tract

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Abstract
Purpose To assess the risk factors for septic shock in patients with acute obstructive pyelonephritis requiring emergency drainage of the upper urinary tract.
Methods We retrospectively reviewed the records of 48 patients who underwent emergency drainage of the upper urinary tract for sepsis associated with acute obstructive pyelonephritis at our institute. Univariate and multivariate analyses were performed to identify the risk factors.
Results Among 54 events of sepsis, we identified 20 events of septic shock requiring vasopressor therapy. Cases with shock were more likely than those without shock to have ureteral stone (70 vs 38 %, $p = 0.024$) and positive blood culture results (81 vs 28 %, $p = 0.006$). They received drainage significantly earlier than those without shock (1.0 vs 3.5 days, $p < 0.001$). Univariate analysis demonstrated that acute obstructive pyelonephritis by ureteral stone, rapid progression (the occurrence of symptoms to drainage $\leq$1 day), positive blood culture, leukocytopenia (<4,000/mm^3), thrombocytopenia (<120,000/mm^3), and prothrombin time international normalized ratio $\geq$1.20 were correlated with septic shock. Multivariate logistic regression analysis identified thrombocytopenia ($p = 0.005$) and positive blood culture ($p = 0.040$) as independent risk factors for septic shock.
Conclusions Thrombocytopenia and positive blood culture were independent risk factors for septic shock in acute obstructive pyelonephritis requiring emergency drainage. Thrombocytopenia would be practically useful as a predictor of septic shock.

Keywords Acute obstructive pyelonephritis · Septic shock · Thrombocytopenia · Ureteral drainage

Introduction

Acute obstructive pyelonephritis sometimes requires emergency drainages of the upper urinary tract by percutaneous nephrostomy or retrograde ureteral stenting [1]; however, septic shock may develop despite appropriate emergency drainage.

The Surviving Sepsis Campaign Guidelines, first published in 2004 and updated in 2008 [2, 3], are now regarded as the international standard for treatment of severe sepsis including urosepsis. Although the guidelines recommend emergency drainage for acute obstructive pyelonephritis, little information is known for the risk for developing septic shock in spite of drainage. Recently, Yamamoto et al. reported that age and presence of paralysis were independent risk factors for septic shock in patients receiving emergency drainage for acute pyelonephritis with ureteral calculi. However, in this study, they analyzed limited cases only with ureteral calculi [4], and for cases with other causes, the risk factor remains uncertain. Thus, in this study, we have attempted to identify the risk factors for septic shock in cases with other causes as well as ureteral calculi.

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Methods

We retrospectively reviewed the records of patients who underwent emergency drainage of the upper urinary tract for sepsis associated with acute obstructive pyelonephritis at our institute from April 2006 to September 2011. The diagnosis of sepsis was made by the criteria for systemic inflammatory response syndrome (SIRS), which included two or more of the following conditions: (1) body temperature greater than 38 °C or less than 36 °C, (2) heart rate greater than 90 beats per minute, (3) tachypnea, as manifested by a respiratory rate greater than 20 breaths per minute or hyperventilation, as indicated by a partial CO_2 pressure less than 32 mmHg, and (4) white blood cell count greater than 12,000/mm^3 or less than 4,000/mm^3, or more than 10 % immature neutrophils [5, 6]. Patients' age, sex, underlying comorbidities, performance status, the side of infected kidney, type of drainage, cause of obstruction, and the time interval between the occurrence of symptoms of pyelonephritis and drainage were recorded. We also evaluated SIRS score, the results of blood and urine culture before antibiotic treatment, and laboratory examinations including blood leukocyte count, blood thrombocyte count at nadir, C-reactive protein (CRP), serum creatinine and total bilirubin level, and prothrombin time international normalized ratio (PT-INR) prior to drainage.

Septic shock was defined as sepsis with a systolic blood pressure less than 90 mmHg despite adequate fluid replacement or using vasopressors for at least 1 h [7, 8]. By comparing patients who did or did not progress to septic shock, we identified risk factors for septic shock.

Chi-square test, Student's t test, and Mann–Whitney U test were used for univariate analysis to compare variables between cases with septic shock and without. All variables that were significant on univariate analysis were entered into multivariate analysis, and logistic regression analysis was used for multivariate analysis. Analyses were performed by JMP 9 (SAS institute Inc., Tokyo, Japan). P values less than 0.05 were considered significant. This study was approved by the institutional ethical committee (3,124).

Results

We identified 48 patients who met SIRS criteria and required emergency ureteral drainage for acute obstructive pyelonephritis. A total of 54 emergency drainage procedures were undertaken, with once in 44, twice in two, and three times in two patients. The patients' clinical characteristics are shown in Table 1. The cause of obstruction was ureteral stone in 27, malignant neoplasm in 14, obstruction of ureteral stent in six, anastomotic stenosis of ileal conduit in three, adhesion after lower abdominal operation in two, ureteropelvic obstruction in one, and unknown in one. The patients' laboratory data prior to drainage is shown in Table 2. Culture samples prior to treatment were taken from blood and urine in 34 events (63 %) and 53 events (99 %), respectively. Organisms isolated from blood and/or urine culture were *Escherichia coli* ($n = 22$), *Pseudomonas aeruginosa* ($n = 5$), and other gram-negative bacilli ($n = 12$).

Septic shock occurred in 20 events of drainage. No patients without septic shock died, but one female 74-year-old patient died of septic shock in 8 days after drainage. She had end-stage bladder cancer and poor performance status but no other complications. The other 19 cases with septic shock finally recovered from septic shock with intensive care and the median length of hospital stay was 15 days (5–75). Patients with septic shock were treated with antibiotics, vasopressors in all cases, blood infusion in 10 cases, gamma globulin in 10 cases, antithrombin III agents in 6 cases, blood purification therapy in 4 cases, and recombinant human soluble thrombomodulin in 3 cases (Table 3).

Cases with shock were more likely than those without shock to have ureteral stone ($p = 0.024$) and positive blood culture results ($p = 0.006$). They received emergency drainage significantly earlier than those without shock ($p < 0.001$).

Univariate analysis demonstrated that septic shock was significantly associated with ureteral stone ($p = 0.024$), rapid progression (the occurrence of symptoms to drainage $\leq$1 day, $p = 0.01$), positive blood culture ($p = 0.006$), leukocytopenia (<4,000/mm^3) ($p = 0.01$), thrombocytopenia (<120,000/mm^3) ($p < 0.001$), and coagulopathy (PT-INR $\geq$ 1.20) prior to drainage ($p = 0.004$). Multivariate logistic regression analysis identified thrombocytopenia [odds ratio (OR) 23.90; 95 % CI 2.64–216.18; $p = 0.005$] and positive blood culture [OR 9.11; 95 % CI 1.11–74.79; $p = 0.040$] as independent risk factors for septic shock.

Discussion

We have found that thrombocytopenia and positive blood culture were significantly associated with septic shock independently in acute obstructive pyelonephritis requiring emergency ureteral drainage.

Previous studies documented coagulopathy and thrombocytopenia as the predictors of progression of sepsis [9, 10]. This would be natural in considering the process of multiple organ dysfunction and septic shock [9–11]. Septic conditions stimulate the release of local and systemic proinflammatory mediators, which would result in low systemic vascular resistance and hypotension [12, 13].

Table 1 Characteristics of patients

	Septic shock (+) ($n = 20$)	Septic shock (−) ($n = 34$)	p value
Sex			
Male	5	11	0.568[†]
Female	15	23	
Age			
Mean (range)	60 (40–81)[a]	64 (38–84)[a]	0.200[‡]
Underlying comorbidities			
Diabetes mellitus	5	10	0.972[†]
Malignancy	5	16	0.188[†]
Steroid use	3	7	0.883[†]
Laterality			
Right	12	19	0.768[†]
Left	8	15	
Performance status			
0	13	20	0.157[†]
1	4	8	
2	1	6	
3	2	0	
Drainage			
Percutaneous nephrostomy	5	15	0.160[†]
Retrograde ureteral stent	15	19	
Cause of obstruction			
Urinary stone	14	13	0.024[†]
Others	6	21	
SIRS score			
2	4	11	0.388[†]
3	8	15	
4	8	8	
Interval between onset and drainage (days)			
Median (range)	1.0 (0–3)[b]	3.5 (0–20)[b]	<0.001[‖]
≤1 day	13	7	0.001[†]
≥2 days	7	27	

[a] Mean (range), [†] chi-square test, [‡] Student's t test, [b] Median (range), [‖] Mann–Whitney U test

These mediators also activate coagulation cascade and promote fibrin clot formation and platelet activation, inducing coagulopathy and thrombocytopenia [12–14]. Our univariate analysis also demonstrated that thrombocytopenia and prolonged PT-INR were risk factors, although the latter did not remain as a significant factor in multivariate analysis. Thrombocytopenia may be clinically useful as a predictor of septic shock, since blood platelet count is a quick test.

Positive blood culture was marginally associated with septic shock in our study. Hsu et al. [15] reported that patients with complicated acute pyelonephritis with positive blood culture were likely to present severe sepsis or shock. However, the results of blood culture need at least 8 h, which may weaken the utility of blood culture in emergency situation [16, 17]. Moreover, in our study, blood culture was not taken correctly in 20 patients; in 14 cases, antibiotic therapy had been already begun before the referral to our institute, which might lead potential selection bias because these cases might have been under serious condition.

Yamamoto et al. reported that the interval from the occurrence of symptoms to drainage was significantly shorter in patients with septic shock in univariate analysis [14], which is similar to our results. Thus, the rapid progression may be an important risk factor but multivariate analysis in our study failed to show the significance.

Serum creatinine and bilirubin were not significant factors in our study, although they are included in the sequential organ failure assessment score [18], which was commonly used to evaluate the organ damage or predict outcomes in septic patients [18, 19]. This may be because serum creatinine level in patients with acute obstructive pyelonephritis, sometimes elevated by obstructive uropathy

Table 2 Laboratory data of patients prior to drainage

	Septic shock (+) (n = 20)	Septic shock (−) (n = 34)	p value
White blood cell count (/μL)	11,200 (1,200–47,900)[a]	11,650 (4,000–35,200)[a]	<0.001[†]
Leukocytopenia (<4,000/μL %)	5	0	0.01[‡]
Thrombocyte count nadir (×10,000/μL)	8.15 (0.80–41.90)[a]	22.0 (6.6–54.9)[a]	<0.001[†]
Thrombocytopenia (<1.2 × 10^5/μL %)	16	3	<0.001[‡]
PT-INR	1.31 (0.92–3.51)[a]	1.13 (0.90–1.49)[a]	0.005[†]
Elongation of PT-INR (≥1.20 %)	16	12	0.004[‡]
CRP (mg/dL)	13.69 (4.33–29.74)[a]	12.84 (0.88–37.68)[a]	0.425[†]
Serum creatinine level (mg/dL)	2.26 (0.93–6.50)[a]	2.13 (0.45–19.79)[a]	0.802[†]
Serum total bilirubin level (mg/dL)	0.80 (0.20–1.70)[a]	0.60 (0.20–2.40)[a]	0.914[†]
Blood culture			
Positive	13	5	0.006[‡]
Negative	3	13	
Urine culture			
Positive	19	23	0.064[‡]
Negative	1	10	

[a] Median (range), [†] Mann–Whitney U test, [‡] chi-square test

Table 3 Risk factors for septic shock

Variables	Number	Multivariate analysis	
		OR (95 %CI)	p value
Cause of obstruction			
Ureteral stone	27	Reference	0.951
Other	27	1.81 (0.13–23.75)	
Blood culture			
Positive	16	Reference	0.040
Negative	18	9.11 (1.11–74.79)	
Leukocytopenia (<4,000/μL)			
No	49	Reference	0.999
Yes	5	>50 (0.00 to >200.0)	
Thrombocytopenia (<1.2 × 10^5/μL)			
No	35	Reference	0.005
Yes	19	23.90 (2.64–216.18)	
PT-INR			
<1.20	26	Reference	0.266
≥1.20	28	3.24 (0.41–25.75)	
Onset to drainage			
≥2 days	34	3.25 (0.37–28.65)	0.288
≤1 day	20	Reference	

rather than SIRS, may not reflect the severity of sepsis. Serum bilirubin level was elevated in only 5 patients with shock in our study.

Comorbidities such as malignancy, diabetes mellitus, and steroid use were known to be associated with infectious diseases [20], although they were not necessarily risk factors for septic shock in acute obstructive pyelonephritis. Yoshimura et al. [21] reported that diabetes mellitus and immune suppression status were not associated with septic shock by examining 473 patients with urosepsis associated with upper urinary tract calculi. Similarly, Lee et al. [22] found that diabetes mellitus and malignancy were not

related to septic shock in 208 bacteremic acute pyelonephritis patients. However, the reason for the lack of association was not discussed in these papers.

The weakness of our study is a retrospective analysis of a single institution with a limited number of cases. Confirmatory studies with a larger population may be required.

Conclusion

Our study revealed thrombocytopenia would be clinically more useful as a predictor of septic shock in the emergency room.

Conflict of interest None declared.

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Thrombocyte count nadir (×10,000/μL)	8.15 (0.80–41.90)[a]	22.0 (6.6–54.9)[a]	<0.001[†]
Thrombocytopenia (<1.2 × 10^5/μL %)	16	3	<0.001[‡]
PT-INR	1.31 (0.92–3.51)[a]	1.13 (0.90–1.49)[a]	0.005[†]
Elongation of PT-INR (≥1.20 %)	16	12	0.004[‡]
CRP (mg/dL)	13.69 (4.33–29.74)[a]	12.84 (0.88–37.68)[a]	0.425[†]
Serum creatinine level (mg/dL)	2.26 (0.93–6.50)[a]	2.13 (0.45–19.79)[a]	0.802[†]
Serum total bilirubin level (mg/dL)	0.80 (0.20–1.70)[a]	0.60 (0.20–2.40)[a]	0.914[†]
Blood culture			
Positive	13	5	0.006[‡]
Negative	3	13	
Urine culture			
Positive	19	23	0.064[‡]
Negative	1	10	

[a] Median (range), [†] Mann–Whitney U test, [‡] chi-square test

Table 3 Risk factors for septic shock

Variables	Number	Multivariate analysis	
		OR (95 %CI)	p value
Cause of obstruction			
Ureteral stone	27	Reference	0.951
Other	27	1.81 (0.13–23.75)	
Blood culture			
Positive	16	Reference	0.040
Negative	18	9.11 (1.11–74.79)	
Leukocytopenia (<4,000/μL)			
No	49	Reference	0.999
Yes	5	>50 (0.00 to >200.0)	
Thrombocytopenia (<1.2 × 10^5/μL)			
No	35	Reference	0.005
Yes	19	23.90 (2.64–216.18)	
PT-INR			
<1.20	26	Reference	0.266
≥1.20	28	3.24 (0.41–25.75)	
Onset to drainage			
≥2 days	34	3.25 (0.37–28.65)	0.288
≤1 day	20	Reference	

rather than SIRS, may not reflect the severity of sepsis. Serum bilirubin level was elevated in only 5 patients with shock in our study.

Comorbidities such as malignancy, diabetes mellitus, and steroid use were known to be associated with infectious diseases [20], although they were not necessarily risk factors for septic shock in acute obstructive pyelonephritis. Yoshimura et al. [21] reported that diabetes mellitus and immune suppression status were not associated with septic shock by examining 473 patients with urosepsis associated with upper urinary tract calculi. Similarly, Lee et al. [22] found that diabetes mellitus and malignancy were not

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Guest editorial: immunotherapy for hematological malignancies: the quest to overcome tolerogenic drive

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How easily a healthy body can defend itself against ordinary pathogens! If only it was possible to do the same against self-derived malignancies. Unfortunately, however, the physiology of the immune system discourages such a persistent fight by establishing the overwhelming hurdle of "tolerance". For this reason, tumor immunotherapy stands as an ultimate challenge to the fundamental basis of immunology. Will we ever win this battle? Recent remarkable advances in related fields are finally beginning to make tumor immunologists confident enough to answer in the affirmative.

In fact, it has been hematologists who have led the field of tumor immunotherapy through their work in allogeneic hematopoietic stem cell transplantation, which exploits the power of graft-versus-tumor effects. However, this is inevitably accompanied by graft-versus-host disease, which precludes elderly patients, who represent a major population suffering from hematological malignancies, from receiving allogeneic transplantation. Clearly, we must develop novel therapies for such patients.

In contrast to the tremendous power of allogeneic reaction, autologous antitumor responses are usually feeble. Heightening them to a clinically meaningful level is a daunting task in the face of the driving force of tolerance. However, rational strategies for overcoming each step in the series of tolerogenic mechanisms have gradually brought immunotherapy into the arena of cancer therapy.

Positive and negative components that, respectively, enhance and suppress immune responses are present in the immune system, as in other physiological systems, and are responsible for maintaining homeostasis. The primordial positive component that triggers virtually any type of immune response is innate immunity. The main reason why the immune system efficiently combats pathogens is that innate immune cells express an abundant array of receptors that recognize various molecules found in microbes. Such recognition provokes intense inflammatory responses and the activation of dendritic cells (DCs), the most potent antigen-presenting cells for T cells. Inflammation and activated DCs subsequently initiate effective antigen-specific immune responses, that is, adaptive immunity. This "innate immunity-DC-adaptive immunity" axis is the essence of the positive immune component.

To prevent immune responses to autologous or innocuous antigens, and to avoid excessive responses to noxious antigens, the immune system has several layers of negative (suppressive) components. First of these is the induction of central tolerance in the thymus, through which high-affinity self-reactive T cells are eliminated. To assure tolerance to innocuous antigens in the periphery, peripheral tolerance is maintained by several mechanisms, among which regulatory T cells play a key role. Furthermore, negative signals transmitted to T cells through inhibitory receptors, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1), represent crucial molecular mechanisms for the timely termination of T cell responses [1]. In addition, tumor tissues co-opt certain immunosuppressive components, including regulatory T cells, PD-1 ligands, and myeloid-derived suppressor cells, thus creating a microenvironment hostile to tumor-reactive T cells.

These positive and negative components provide targets for provoking antitumor immune responses. In this issue, Dr. Bocchia reviews peptide vaccines derived from antigens that are preferentially expressed by tumor cells, thus

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circumventing self-tolerance at least in part. In addition, developing appropriate adjuvants that are combined with peptide vaccines will be important for improving efficacy by triggering innate immune responses [2]. Dr. Kitawaki reviews DC vaccines that exploit the power of the most potent T cell stimulator. Dr. Fujiwara and Dr. Turtle review adoptive immunotherapy using T cells modified with T-cell receptor or chimeric antigen receptor genes, which bypasses tolerance induced in vivo in cancer patients by transferring tumor-reactive T cells cultured ex vivo. Theoretically, all of these methods can be combined with blockade of the negative immune components to enhance efficacy. In particular, blocking CTLA-4 and/or PD-1 signals, which have been shown to induce remarkable clinical effects by themselves [3], are immediate candidates. The anti-CCR4 monoclonal antibody, which eliminates regulatory T cells, also represents a promising enhancer of antitumor immunity. Such blockade of "immune checkpoints" will constitute an integral component of tumor immunotherapy [1].

Although immunotherapy is generally considered to be "safe", we need to be careful about side effects as efficacy increases. For example, targeting antigens that are expressed by normal cells can cause severe adverse events [4]. Also, blocking inhibitory pathways hardwired into the immune system, which are crucial for maintaining self-tolerance and modulating the amplitude of physiological immune responses, cause collateral tissue damage [1]. We need to take care to minimize these side effects to an acceptable level.

In any event, tumor immunotherapy has certainly begun to come of age. We can expect to observe remarkable advances in the translation of basic findings to clinical applications. In the field of hematological malignancies, immunotherapy will be applied mainly to elderly patients ineligible for allogeneic transplantation. Furthermore, if "autologous" immunotherapy turns out to be truly effective, it may replace part of "allogeneic" immunotherapy as we pursue safer cancer therapies.

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ORIGINAL ARTICLE

The critical role of cyclin D2 in cell cycle progression and tumorigenicity of glioblastoma stem cells

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Cancer stem cells are believed to be responsible for tumor initiation and development. Much current research on human brain tumors is focused on the stem-like properties of glioblastoma stem cells (GSCs). However, little is known about the molecular mechanisms of cell cycle regulation that discriminate between GSCs and differentiated glioblastoma cells. Here we show that cyclin D2 is the cyclin that is predominantly expressed in GSCs and suppression of its expression by RNA interference causes G1 arrest *in vitro* and growth retardation of GSCs xenografted into immunocompromised mice *in vivo*. We also demonstrate that the expression of *cyclin D2* is suppressed upon serum-induced differentiation similar to what was observed for the cancer stem cell marker *CD133*. Taken together, our results demonstrate that cyclin D2 has a critical role in cell cycle progression and the tumorigenicity of GSCs.

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Keywords: cancer stem cells; cell cycle; cyclin D2; glioblastoma

INTRODUCTION

Glioblastoma is the most common primary brain tumor in adults.[1] Patients diagnosed with glioblastoma have a median survival of <1 year with generally poor responses to all therapeutic modalities. The existence of cancer stem cells responsible for tumor initiation and development has been demonstrated in a variety of tumors.[2] The discovery of glioblastoma stem cells (GSCs) was made by applying techniques for cell culture and analysis of normal neural stem cells (NSCs) to brain tumor cell populations.[3] Glioblastoma cells cultured in serum-free media supplemented with epidermal growth factor and basic fibroblast growth factor form spheres and maintain stem cell-like properties and tumorigenicity, but when grown under serum-containing culture conditions, glioblastoma cells undergo irreversible differentiation and lose their tumorigenicity.[4] Therefore, the mechanisms of maintaining the stem-like properties of GSCs have been studied extensively.

D-type cyclins are known to have critical roles in cell cycle progression.[5] Three D-type cyclins, cyclin D1, D2 and D3, are encoded by distinct genes, but show significant amino-acid similarity. Among these, cyclin D1 was first discovered and has been studied most extensively. D-type cyclins associate with partner cyclin-dependent kinases, CDK4 and CDK6, and promote phosphorylation and subsequent inactivation of the retinoblastoma tumor suppressor gene product, RB and RB-related proteins. This causes the release or de-repression of the E2F transcription factors and allows cells to enter the S phase. Dysregulation of the G1/S transition appears to be a common event in tumorigenesis.[6] Indeed, alterations in important components of the RB pathway are frequently observed in a variety of tumors, including glioblastoma.[6–8] In this study, we investigate the role of cyclin D2 in cell cycle progression and the tumorigenicity of GSCs.

RESULTS

Predominant expression of cyclin D2 in GSCs

We cultured GSCs isolated from four patients, GB1–3 and 5, under serum-free conditions that favor NSC growth.[4] Our DNA array analysis revealed that GB1 and GB2, 3 and 5 belong to the mesenchymal and proneural groups, respectively[9] (Supplementary Figure 1A). *In vitro* differentiation was induced in medium containing fetal bovine serum.[4] Differentiated GB1–3 cells proliferated more rapidly than their parental undifferentiated cells did (data not shown). By contrast, proliferation of GB5 cells was inhibited in medium containing serum (Supplementary Figure 2). Consistent with these properties, immunoblotting analysis using anti-RB antibodies revealed that RB was highly phosphorylated in differentiated GB1–3 cells and undifferentiated GB5 cells (Figure 1a). These differences may reflect the properties associated with the primary tumors from which they were derived.

To study the molecular mechanisms of cell cycle regulation in GSCs, lysates from glioblastoma cells were subjected to immunoblotting analysis with antibodies to various cyclins. We found that cyclin D2 was abundantly expressed in undifferentiated GB2, 3 and 5 cells, but was barely expressed in differentiated GB2, 3 and 5 cells, which had been cultured in serum-containing medium (Figure 1a). By contrast, cyclin D1 expression was higher in differentiated than in undifferentiated glioblastoma cells. The expression levels of cyclin D3 did not change significantly. In GB1 cells, cyclin D2 expression was not detected in either

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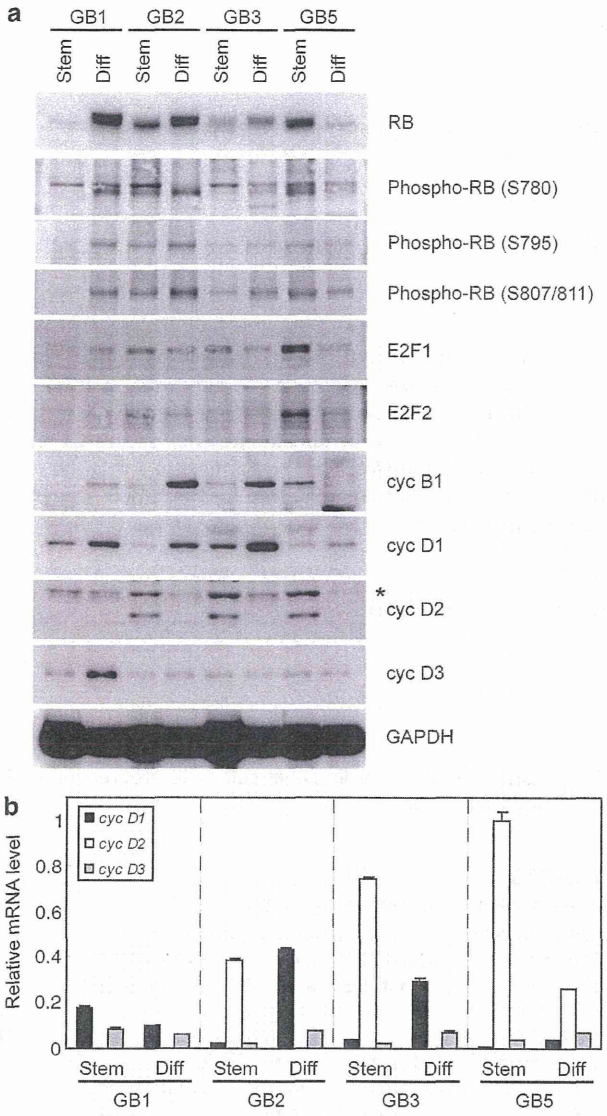

Figure 1. Predominant expression of cyclin D2 in GSCs. (**a**) Lysates from undifferentiated (stem) or differentiated (diff) GB1–3 and 5 cells were immunoblotted with antibodies to the indicated proteins. The asterisk indicates a non-specific band. (**b**) The mRNA levels of D-type cyclins in undifferentiated (stem) or differentiated (diff) GB1–3 and 5 cells were evaluated by quantitative RT–PCR. Error bars represent the s.d. ($n = 3$).

the undifferentiated or differentiated state. This may be a common feature in the mesenchymal subtype of glioblastoma (Supplementary Figure 1B). Reverse transcription–polymerase chain reaction (RT–PCR) analysis also revealed that *cyclin D2* messenger RNA (mRNA) was predominantly expressed in undifferentiated GB2, 3 and 5 cells (Figure 1b).

GSC-specific expression of cyclin D2

To further investigate the expression pattern of cyclin D2, we focused our analyses on GB2, as cyclin D2 is highly expressed and its expression is dramatically reduced by serum-induced differentiation in these cells. Time-course experiments showed that following serum addition, undifferentiated GB2 cells became attached to the bottom of the dish and formed a monolayer (Supplementary Figure 3A), as reported previously.[4] The expression levels of *cyclin D2* was significantly decreased within one day after serum addition and was completely downregulated in late-passage cells (>10 passages), similar to what was observed for the cancer stem cell marker *CD133* (Mizrak *et al.*[10]) and the NSC marker *nestin*[11] (Figure 2a, left panel). By contrast, *cyclin D1* mRNA was induced upon serum stimulation and highly expressed in late-passage cells. However, when differentiated GB2 cells (>10 passages) were cultured in serum-free conditions, cells started to form nonadherent, multicellular spheres indistinguishable from undifferentiated GSCs (Supplementary Figure 3B). Nevertheless, the expression levels of *cyclin D2* as well as *CD133* and *nestin* were not fully restored by serum deprivation (Figure 2a, right panel).

We also investigated the expression profiles of D-type cyclins in patient glioblastomas, commonly used glioma cell lines and normal human NSCs taken from a public microarray database (Figure 2b). As expected, almost all patient glioblastomas expressed substantial levels of *cyclin D2*, whereas no glioma cell line did. *Cyclin D1* was highly expressed in the glioma cell lines. Moreover, the expression of *cyclin D2* as well as *CD133* and *nestin* was not restored by serum withdrawal in the glioma cell line T98G, U251 or U87 (Figure 2c). Furthermore, either overexpression of p21 or depletion of E2F1 and/or E2F2 did not lead to the restoration of *cyclin D2* expression (Supplementary Figures 4A–D). These data are compatible with the notion that these cell lines have become differentiated under serum-containing conditions. Interestingly, *cyclin D2* and *D3*, but not *cyclin D1*, were found to be expressed in human NSCs. *Cyclin D3* and *D1* showed a reciprocal expression pattern in the glioma cell lines.

We next investigated the expression profiles of D-type cyclins in various histological grades of gliomas. The expression levels of *cyclin D2*, but not of *cyclin D1* or *D3*, were found to be significantly upregulated in glioblastomas (grade IV) compared with astrocytomas (grade II or III) and non-tumor tissues (Supplementary Figure 5 and Supplementary Table 1). Taken together, these data raise the possibility that cyclin D2 expression may be a common feature of GSCs.

Important role of cyclin D2 in cell cycle progression of GSCs

We next examined the role of cyclin D2 in cell cycle progression of GSCs using small interference RNA (siRNA). Flow-cytometric analyses of DNA content in undifferentiated GB2 cells showed that knockdown of cyclin D2, but not of cyclin D1 and/or D3, resulted in a significant increase in the fraction of cells in the G1 phase (Figure 3a, left panel). Consistent with this result, knockdown of cyclin D2 led to an increase in the amount of the hypophosphorylated form of RB, suggesting that the activity of CDK4 and/or CDK6 was suppressed and cells were arrested in the G1 phase of the cell cycle (Figure 3b, left panel). Silencing of cyclin D2 expression also caused a reduction in the level of cyclin B1, the expression of which is known to be low in the G1 phase (Figure 3b, left panel). In addition, knockdown of cyclin D2 resulted in a slight decrease in the expression levels of E2F1 and E2F2. We also observed that ectopic expression of cyclin D1 or D3 partially rescued the reduction in RB expression and phosphorylation caused by knockdown of cyclin D2. Thus, a certain amount of D-type cyclins may be required for cell cycle progression of undifferentiated GB2 cells (Supplementary Figure 6). By contrast, knockdown of cyclin D1, but not of cyclin D2 or D3, induced G1 arrest of differentiated GB2 cells cultured in serum-containing medium (Figures 3a and b, right panels). In addition, although amplification of the *cdk4* locus has been detected at a higher frequency than that of the *cdk6* locus,[7] we found that both CDK4 and CDK6 are responsible for phosphorylation of RB in undifferentiated GB2 cells (Supplementary Figures 7A and B). These results suggest that the cyclin D2–CDK4/6 complexes have an important role in cell cycle progression of undifferentiated, but not of differentiated, GSCs.

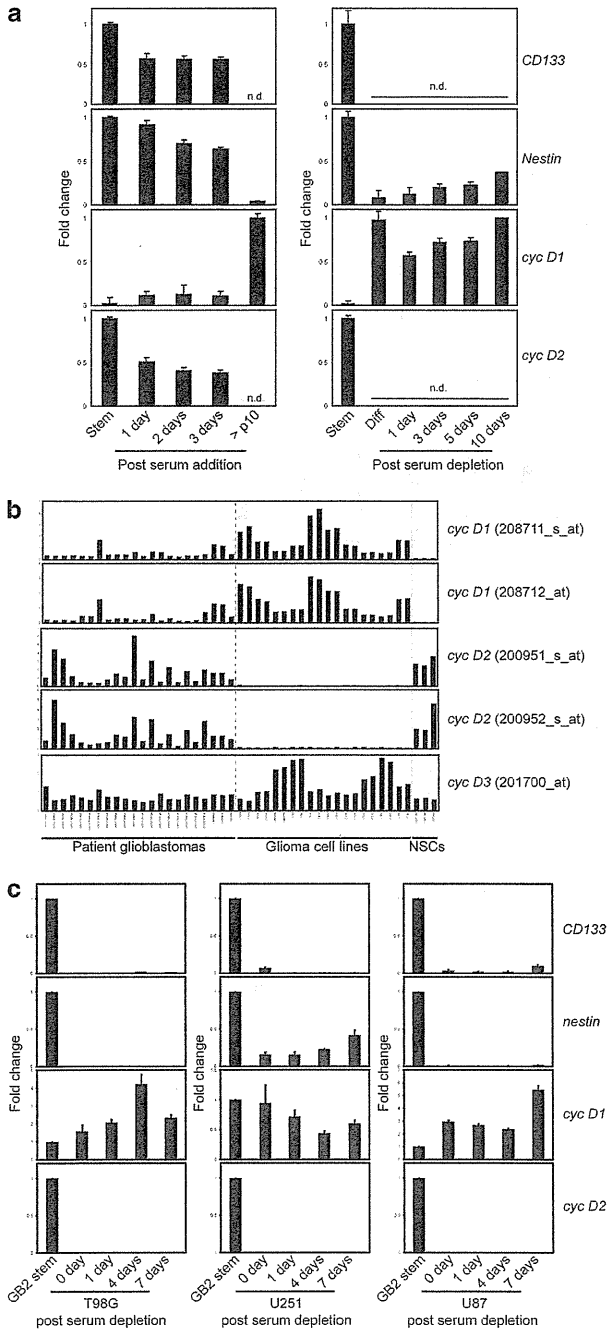

Figure 2. GSC-specific expression of *cyclin D2*. (**a**) Undifferentiated (stem) GB2 cells were cultured in medium containing serum for the indicated times (left). Differentiated (diff) GB2 cells were cultured in serum-free medium for the indicated times (right). The mRNA levels of the indicated genes were evaluated by quantitative RT–PCR and shown as fold change over mRNA levels in GB2 stem cells. Error bars represent the s.d. ($n = 3$). ND means not detected. (**b**) Gene expression profiles of D-type cyclins taken from a public microarray database (GSE4536) (Lee *et al.*[4]). Data of two independent probes for *cyclin D1* and *D2*, and one probe for *cyclin D3* are shown. (**c**) The glioma cell lines T98G, U251 and U87 were cultured in serum-free medium for the indicated times. The mRNA levels of the indicated genes were evaluated by quantitative RT–PCR and shown as fold change over mRNA levels in GB2 stem cells.

Critical role of cyclin D2 in the tumorigenicity of GSCs

To clarify the significance of cyclin D2 expression in the tumorigenicity of GSCs, we transplanted GB2 cells into the frontal lobe of immunocompromised mice. As reported previously,[4] all mice transplanted with undifferentiated GB2 cells developed tumors and died within 2 months, whereas mice transplanted with differentiated GB2 cells survived over 5 months (Figure 4a). Histopathological analyses of tumor xenografts demonstrated that undifferentiated GB2 cells formed a highly invasive tumor spreading across the hemispheres, which represents an important feature of human glioblastoma (Figure 4b). RT–PCR analysis using human-specific primers demonstrated that the xenograft tumor still maintained the predominant expression of *cyclin D2* (Figure 4c and Supplementary Figure 8A). Consistent with this observation, when mice were transplanted with undifferentiated GB2 cells in which cyclin D2 expression was stably repressed by lentivirus-delivered short hairpin RNAs (shRNAs) (Supplementary Figures 8B and C), they survived significantly longer than those transplanted with undifferentiated GB2 cells infected with control lentivirus (Figure 4a). By contrast, overexpression of cyclin D2 did not restore *CD133* and *nestin* expression, as well as tumorigenicity of differentiated GB2 cells (Supplementary Figures 9A-C). These results suggest that cyclin D2 has a critical role in the tumorigenicity of GSCs.

DISCUSSION

A number of molecular studies have identified critical genetic events in glioblastoma, including the following: dysregulation of growth factor signaling; activation of the phosphatidylinositol-3-OH kinase pathway; and inactivation of the p53 and RB pathways.[7,8] Among those three core pathways, the RB pathway is obviously the most important for the regulation of G1/S progression.[6] Actually, 78% of glioblastomas are shown to harbor RB pathway aberrations, such as deletion of the *cdkn2a/cdkn2b* locus, amplification of the *cdk4* locus and deletion or inactivating mutations in *RB1* (Cancer Genome Atlas Research Network[7]). Importantly, amplification of the *cyclin D2* locus is also reported.[7] In this study, we have shown that cyclin D2 is the most abundantly expressed cyclin in GSCs among the three D-type cyclins. Moreover, suppression of cyclin D2 expression by RNA interference caused G1 arrest *in vitro* and growth retardation of GSCs xenografted into immunocompromised mice *in vivo*. Altogether, these data suggest the critical role of cyclin D2 in cell cycle progression and the tumorigenicity of GSCs.

Tumor cells in culture are valuable for studying the mechanisms of tumorigenesis. Growth media containing serum have been used for maintaining a variety of cancer cells, including glioblastoma. However, it has been shown that serum causes irreversible differentiation of GSCs.[4] Differentiated GSCs have gene expression profiles that are different from those of their parental GSCs and NSCs, and are neither clonogenic nor tumorigenic.[4] Our study shows that cyclin D2 expression is silenced when GSCs are cultured in the presence of serum. We also found that cyclin D1 expression is enhanced during serum-induced differentiation of GSCs. These results explain why commonly used glioblastoma cell lines abundantly express cyclin D1, but not cyclin D2.

It has been shown that GSCs and NSCs share similar properties such as the potential for self-renewal and differentiation.[12] Intriguingly, cyclin D2 has been reported to be the only D-type cyclin expressed in adult mouse NSCs.[13,14] Thus, it is interesting to speculate that the predominant expression of cyclin D2 in GSCs may be the reflection of the property associated with adult NSCs. It is possible that transcription factors that are important for the maintenance of the stem cell state may also be involved in cyclin D2 expression. It may also be possible that DNA

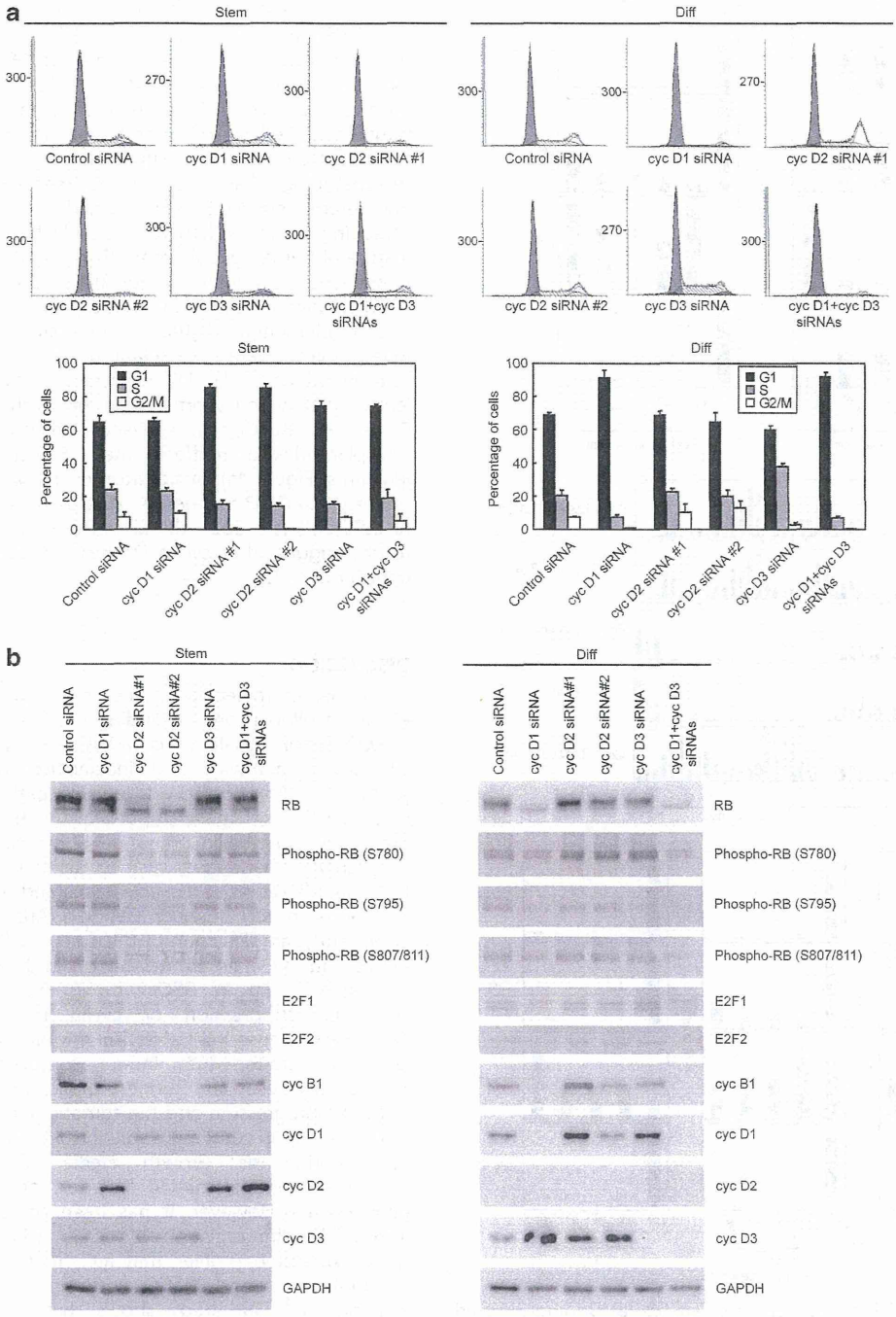

Figure 3. Important role of cyclin D2 in cell cycle progression of GSCs. (**a**) Undifferentiated (stem) (left) or differentiated (diff) (right) GB2 cells were transfected with siRNAs targeting D-type cyclins. After 72 h, cells were fixed, stained with propidium iodide and analyzed by flow cytometry for DNA content. The *x* axis represents DNA content and the *y* axis the number of cells (upper). The bar graph represents the percentages of cells in G1, S and G2/M (lower). Error bars represent the s.d. ($n = 3$). (**b**) Undifferentiated (stem) (left) or differentiated (diff) (right) GB2 cells were transfected with siRNAs targeting D-type cyclins. After 72 h, lysates were immunoblotted with antibodies to the indicated proteins.

methylation and/or mRNA stabilization by alternative cleavage- and polyadenylation-mediated shortening of 3′-UTR are involved in the alteration in cyclin D2 expression.[15]

It is important to define reliable markers that are expressed in cancer stem cells. Overexpression of cyclin D1 has been implicated in the pathogenesis of various human cancers.[5,6,16] However, our results raise the possibility that cyclin D2, rather than cyclin D1, could be a novel prognostic marker for glioblastoma. Hence, it is intriguing to perform univariate and multivariate analyses to compare the expression levels of cyclin D2 with tumorigenic capacity and tumor invasiveness.

As cancer stem cells are considered to be responsible for tumor initiation and development, GSCs may be promising targets for the therapy of glioblastoma. We therefore speculate that inhibitors