TABLE 2
Multivariate Logistic Regression Analysis of Gene Methylations in the Series of PC and BPH

Variable	Coefficient	SE	Chi-square	P^a	HR[b]	95% CI[c]	log HR[d]
APC	2.056	0.525	15.338	< 0.001	7.813	2.792–21.859	2.056 (0.067)
GSTP1	1.773	0.644	7.586	0.006	5.891	1.667–20.814	1.773 (0.057)
MDR1	1.151	0.514	5.009	0.025	3.162	1.154–8.664	1.151 (0.037)

All data are adjusted by age. PC: prostate carcinoma; BPH: benign prostatic hyperplasia.
[a] *APC*, *GSTP1*, and *MDR1* methylation was a significant dependent predictor of pathology.
[b] Individual gene hazard ratios for pathogenesis were different from one another.
[c] 95% CI: 95% confidence interval.
[d] M-score was determined as the sum of the corresponding log hazard ratio for pathology.

edu/~urolab/methprimer) developed in our laboratory.[28] Primers used for MSP and USP analysis are as follows: universal primers: 5′-TAATTTTTTTGTTTGTT-GGGGATT-3′ (sense), 5′-ACTACACCAATACAACCA-CATATC-3′ (antisense); MSP primers: 5′-TATTGCG-GAGTGCGGGTC-3′ (sense), 5′-TCGACGAACTCCC-GACGA-3′ (antisense); USP primers: 5′-GTGTTTTATTGTGGAGTGTGGGTT-3′ (sense), 5′-CCAATCAACAAACTCCCAACAA-3′ (antisense). An initial polymerase chain reaction (PCR) product was amplified with universal primers, which have no CpG sites in either forward or reverse primers, followed by a second nested PCR with primers specific for MSP or USP. For semiquantitative analysis, a preliminary suitable number of PCR cycles for each MSP and USP were carried out to determine the linear range of the reaction. To ensure this, at least one initial PCR was performed using 32 cycles each for MSP and USP. Then, a suitable PCR cycle was chosen for each sample. The annealing temperature and PCR cycles used for MSP and USP primers was 64 °C and 32 cycles. The sequences of primers for *GSTP1* and *MDR1* and their PCR conditions have been described previously.[20,21] In each assay, absence of DNA template served as negative control. The obtained MSP and USP products were analyzed by electrophoresis in 3% agarose gels and stained with ethidium bromide. With ImageJ software (http://rsb.info.nih.gov/ij), relative methylation levels (%) were calculated by using the area under the curve corresponding to each band (MSP and USP).[20,21] For methylation analysis of *APC*, we used 5.3 % methylation as a cut-off value, which was the average percentage of methylation in 69 BPH samples. Using these criteria, MSP positivity for *APC* was defined as those PCs with a percentage of methylation of more than 5.3%, and negative methylation was less than 5.3%. The criteria for MSP positivity of *GSTP1* and *MDR1* were described previously.[20,21]

Bisulfite DNA Sequencing Analysis

Bisulfite-modified DNA (1 μL) was amplified using a pair of universal primers in a total volume of 20 μL. Direct bisulfite DNA sequencing of the PCR products using either forward universal primer or reverse universal primer was performed according to the manufacturer's instructions (Applied BioSystems, Foster City, CA).

Statistical Analysis

To compare patients' background or methylation frequency for each gene, chi-square test was employed. Using a previously reported analytical technique,[23] we calculated the M-score for each sample, defined as the sum of the corresponding log hazard ratio (HR) coefficients, which were derived from multivariate logistic regression analysis of each methylated gene in the BPH and PC samples (Table 2). For each clinicopathologic finding, the association with PSA failure-free probability was determined using Kaplan–Meier curves with a log-rank analysis. The relation between M-score and clinicopathologic findings, except smoking status, was analyzed by either the Mann–Whitney *U* test, Kruskal–Wallis test, or Spearman rank correlation test. The relation between M-score and smoking status was analyzed by the Bonferroni-adjusted Mann–Whiteney *U* test. For this comparison test among the three groups of smoking statuses, the nonadjusted statistical levels of significance of $P < 0.05$ corresponds to a Bonferroni-adjusted statistical significance of $P < 0.0167$.

RESULTS

Methylation Status of the *APC* Promoter in Clinical Samples

Representative results of MSP and USP assays for *APC* in PCs and BPHs are shown in Figure 1A. MSP-positive bands were present in the majority of PCs, and less so in BPH samples. The result of the methylation study

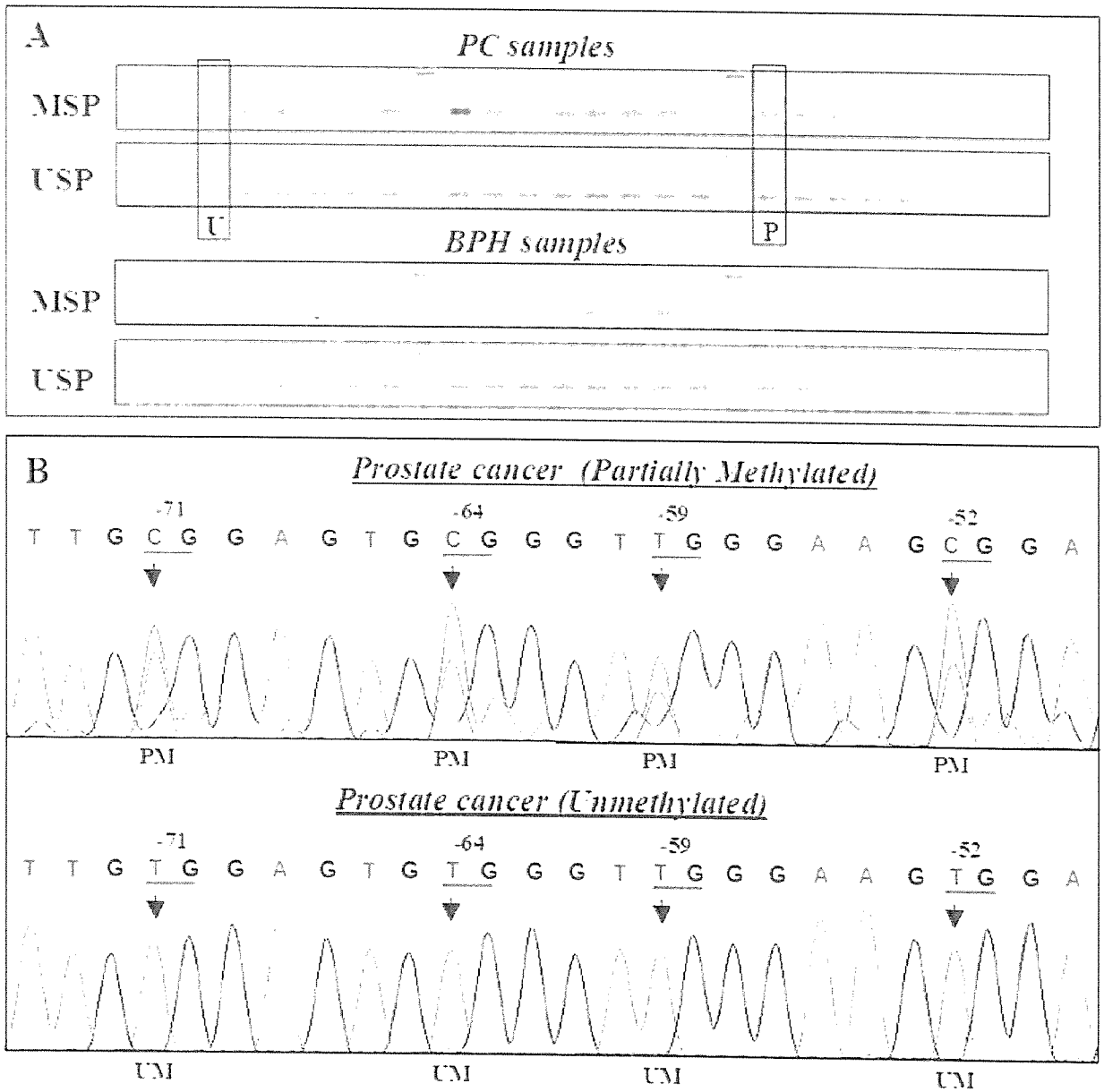

FIGURE 1. Methylation status of the *APC* promoter in clinical samples. (A) MSP and USP bands for 24 samples of BPH and PC are shown. Lanes 'P' and 'U' represent samples depicting partial methylation and unmethylion, respectively. (B) Bisulfite DNA sequencing of partially methylated (top), unmethylated (bottom) samples correspond to lanes 'P' and 'U', respectively. In partially methylated samples, there was a "T" peak along with a "C" peak at the CpG sites. In unmethylated samples, every CpG site was unmethylated. PM and UM correspond to partial methylation and unmethylation, respectively.

was also confirmed by bisulfite DNA sequencing. Figure 1B shows results of a typical bisulfite DNA sequencing in a PC sample. In sample 'P' (corresponding to Fig. 1A, lane 'P') with both MSP and USP bands, there was a "T" peak along with a "C" peak at the CpG sites, indicating partial-methylation (Fig. 1B, top). In sample 'U' (corresponding to Fig. 1A, lane 'U'), where no MSP band was observed, the CpG sites were completely unmethylated (Fig. 1B, bottom).

Evaluation of M-Score; Multigene Methylation Analysis with *APC*, *GSTP1*, and *MDR1* to Distinguish PC from BPH

APC methylation analysis showed positive MSP bands in 104 of 164 (63.4%) PCs and 6 of 69 (8.7%) BPHs (Fig. 2). As we have reported previously, positive MSP bands for *GSTP1* methylation analysis were found solely in 87 of 164 (53.0%) PCs and in 4 of 69 (5.8%) BPHs, whereas, for *MDR1* methylation analysis, positive MSP bands were found in 89 of 164 (48.7%) PCs

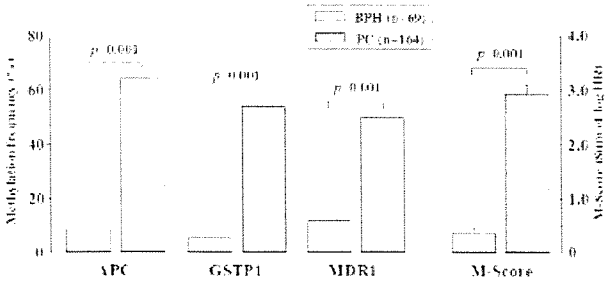

FIGURE 2. Correlation of gene methylation frequency with pathology (BPH vs. PC) and methylation score (M-score). The methylation frequency in PC samples was significantly higher than that in BPH samples for *APC, GSTP1* and *MDR1* genes (64.1% vs. 8.7%, 54.0% vs. 5.8%, and 55.3% vs. 11.6%, respectively). Right side: M-score, determined as the sum of the corresponding log hazard ratio for pathology (BPH vs. PC) (Table 2), was also significantly higher in PC samples than in BPH samples.

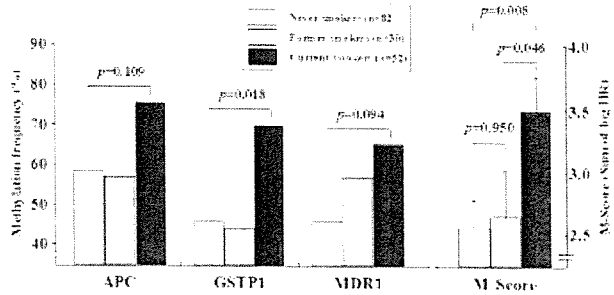

FIGURE 3. Correlation of methylation frequency with smoking status and methylation score (M-score). The methylation frequency in current smokers was significantly higher than that in former or never smokers for *GSTP1* (69.2%, 43.3%, and 46.3%, respectively). There was no significant difference in *APC* or *MDR1* methylation frequency among smokers (75.0%, 56.7%, and 58.5%, respectively for *APC*; 65.4%, 56.7%, and 46.3%, respectively for *MDR1*). Right side: When methylation data was combined (M-score), there was a significant difference in M-score between current and never smokers (3.52 ± 0.27 vs. 2.56 ± 0.23, $P = 0.008$ by Bonferroni-adjusted test). However, there was only a trend to significance in M-score between current and former smokers (3.52 ± 0.27 vs. 2.59 ± 0.37, $P = 0.046$ by Bonferroni-adjusted test).

and in 8 of 69 (11.6%) BPHs.[20,21] A significant difference in the methylation status of each gene was found between the series of PCs and BPHs (Fig. 2). As shown in Table 2, multivariate logistic regression analysis revealed that *APC, GSTP1,* and *MDR1* methylation was a significant dependent predictor of pathology (BPH vs. PC) ($P < 0.001$, $P = 0.006$, and $P = 0.025$, respectively). The individual gene hazard ratios for pathogenesis (PC vs. BPH) were different from one another. For instance, cases with *APC* methylation are 7.813 times more likely to have PC than those with negative methylation, whereas the HR for *MDR1* is 3.162. The M-score determined by the sum of log HR was significantly higher in PC than in BPH (Fig. 2). All statistics were age adjusted to eliminate potential influence caused by age.

Correlation between Aberrant Methylation and Smoking Status

Looking at PC samples by smoking status, positive MSP bands for *APC, GSTP1,* and *MDR1* methylation analysis were found: in 48 (58.5%), 38 (46.3%), and 38 (46.3%) of never smokers,[82] respectively; in 17 (56.7%), 13 (43.3%), and 17 (56.7%) of former smokers,[30] respectively; and in 39 (75.0%), 36 (69.2%), and 34 (65.4%) of current smokers,[52] respectively (Fig. 3). There was significant difference between smoking status in *GSTP1* methylation status ($P = 0.018$), whereas no significant difference was found in *APC* or *MDR1* methylation status ($P = 0.109$ and $P = 0.094$, respectively) (Fig. 3). When we employed the M-score that combined the methylation analysis for three genes, the difference in M-score between current and never smokers was statistically significant ($P = 0.008$) (Fig. 3, right). Conversely, the difference in M-score was not significant: between current and former smokers ($P = 0.046$) or between former and never smokers ($P = 0.950$) (Fig. 3, right). Spearman rank correlation test revealed significant correlation between pack-years smoked and M-score in PCs ($P = 0.039$). There were no relations between methylation status and other smoking variables, such as duration of smoking and age at starting smoking.

Correlation of M-Score with Clinicopathologic Findings

Among PCs, the M-score showed a significant stepwise increase with advancing pathologic stage (1.34 ± 0.26 in pT2a, 2.97 ± 0.24 in pT2b, 3.64 ± 0.29 in pT3a, 4.07 ± 0.56 in pT3b, and 4.98 ± 0 in pT4) ($P < 0.001$) (Fig. 4A). Similarly the M-score increased as the Gleason sum (GS) increased (2.20 ± 0.23 in GS < 7, 3.44 ± 0.26 in GS = 7, and 3.74 ± 0.31 in GS > 7) ($P < 0.001$) (Fig. 4B). For preoperative PSA levels, the M-score was higher in PSA > 10 ng/mL (3.27 ± 0.26) than in PSA ≤10 ng/mL (2.61 ± 0.20) ($P = 0.041$) (Fig. 4C). Moreover, the M-score was higher in advancing pathologic features as follows: in capsular invasion (Cap) (positive [3.65 ± 0.25] vs. negative [2.48 ± 0.20] [$P < 0.001$]), in seminal vesicle involvement (SV) (positive [4.71 ± 0.19] vs. negative [2.74 ± 0.17] [$P = 0.002$]), in pelvic lymph lymph node metastasis (pN) (positive [4.48 ± 0.37] vs. negative [2.55 ± 0.19] [$P = 0.002$]), in venous involvement (v) (positive [3.79 ± 0.24] vs. negative [2.44 ± 0.20] [$P < 0.001$]), in lymphatic vessel involvement (ly) (positive [3.70

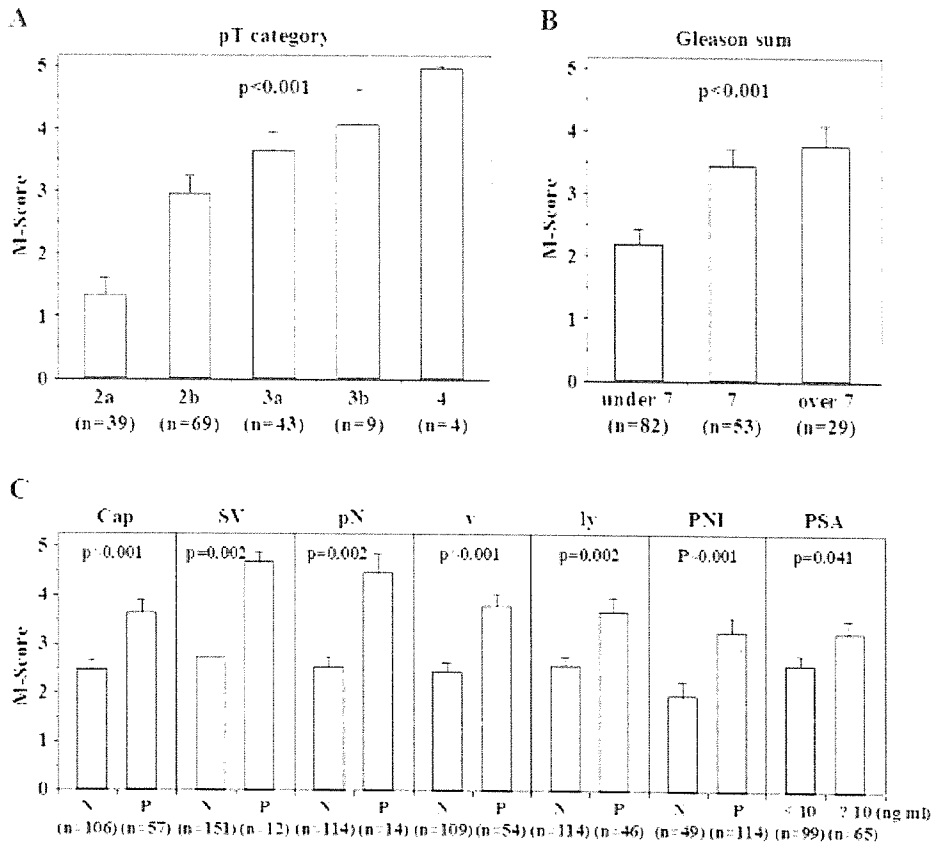

FIGURE 4. Correlation of M-score with clinicopathologic features. Among the overall PC patients: (A) Higher stage of disease (1.34 ± 0.26 in pT2a, 2.92 ± 0.24 in pT2b, 3.84 ± 0.26 in pT3a, 4.21 ± 0.62 in pT3b, and 4.98 ± 0 in pT4); (B) Higher Gleason sum (2.20 ± 0.23 in GS <7, 3.58 ± 0.25 in GS = 7, and 3.819 ± 0.29 in GS > 7; and (C) Higher preoperative PSA levels (3.35 ± 0.25 in PSA > 10 ng/mL, 2.64 ± 0.20 in PSA ≤10 ng/mL) correlated with an increased methylation score (M-score). There was significant correlation between M-score and worse clinicopathologic findings. Cap: capsular invasion; SV: seminal vesicle involvement; pN: lymph lymph node invasion; ly: lymphatic vessel invasion; and PNI: perineural invasion. N and P correspond to negative and positive, respectively.

± 0.29] vs. negative [2.57 ± 0.19] [$P = 0.002$]), and in perineural invasion (PNI) (positive [3.29 ± 0.18] vs. negative [1.95 ± 0.29] [$P < 0.001$]) (Fig. 4C). We also observed that the M-score was age related in the total group of BPHs ($P = 0.001$) but not in the total group of PCs ($P = 0.067$).

Prognostic Features

We analyzed PSA failure-free probability as disease-free survival. Of the clinicopathologic features considered, only Gleason sum was significantly associated with poor outcome in univariate analyses ($P = 0.020$) (Fig. 5).

DISCUSSION

Our results indicate that the M-score was significantly higher in current smokers than in never smokers in PCs (Fig. 3, right). Spearman rank correlation test also revealed significant correlation between pack-years smoked and M-score in PCs ($P = 0.039$). Moreover, there was significant correlation of M-score with advanced pathologic features such as pT category and Gleason sum (Fig. 4). These results suggest that smoking status may influence tumor progression through CpG hypermethylation of related genes dose-dependently. Hickey et al. reviewed 23 prospective cohort studies, 5 nested case–control studies, 1 retrospective cohort study, and 36 case–control studies addressing smoking and PC.[4] Although most of the prospective cohort studies and all of the nested case–control studies that used incident PC as the outcome found no association between current smoking and PC, 33% of 15 population-based case–control studies showed a significant association between smoking and PC risk. They concluded these conflicting results depend on the research design used and how well the study controlled for possible confounding factors. Furthermore, the majority of prospective studies (62% of 13) that used PC death as the outcome noted a positive association between current smoking and PC, supporting our findings where current smoking and advanced PC are associated through CpG hypermethylation of related genes. Therefore, the present study elucidates novel mechanisms whereby smoking may increase PC risk.

Interestingly in lung cancers, some investigators have previously reported that starting smoking during adolescence was the only significant parameter associated with *RASSF1A* gene methylation, and no association was observed with pack-years smoked.[11,12] In

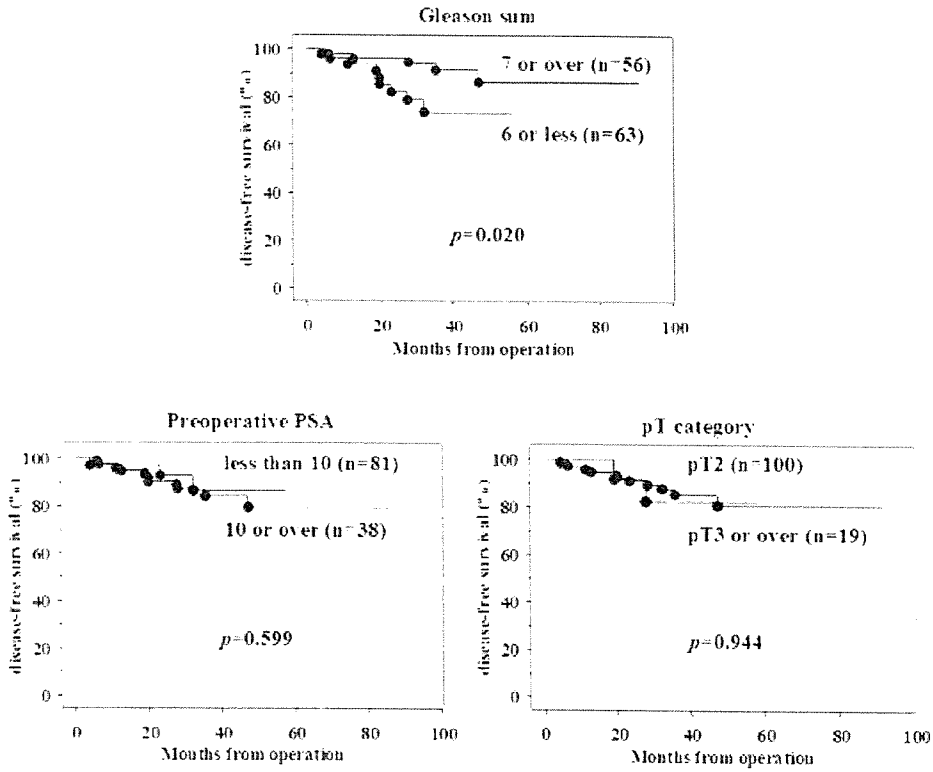

FIGURE 5. Kaplan–Meier PSA failure-free survival curves of PC patients after radical prostatectomy are grouped according to evaluated variables: Gleason sum, preoperative PSA level, and pT category. Follow-up ranged from 0.7 to 91.4 months, with a median of 33.9 months. Circles represent censored data points.

contrast, our result indicated that pack-years smoked was the only parameter associated with methylation status in PCs. Also, methylation status in PC was influenced by smoke exposure in a dose-dependent manner. These findings suggest that methylation induced by smoke exposure may depend on tumor type or gene and that it may be one of the reasons why the mean age of patients with lung carcinoma is younger than that of patients with PC.

We also analyzed PSA failure-free probability as disease-free survival. Of the clinicopathologic features considered, only Gleason sum was significantly associated with poor outcome (Fig. 5). The significant association of the M-score with Gleason sum (Fig. 4) suggests that smoking may influence PC prognosis through CpG hypermethylation of these genes. However, our median follow-up time was probably too short for thorough analysis. We also found there was a trend toward a decreased M-score among former smokers compared with current smokers ($P = 0.046$ by Bonferroni-adjusted test) (Fig. 3, right), suggesting that demethylation of these genes may be occurring in patients who had quit smoking for more than 12 months. Therefore, patients could decrease their risk for advanced PC by becoming nonsmokers.

Other investigators have used multigene methylation analysis in their studies of various cancers.[7,8,10] However, their methods of multigene methylation analysis are the sum of the number of genes methylated. Studies have shown that when multigenes are analyzed in the same samples, interpretation of results should be carefully considered because each gene or other clinical factors, including age, may influence one another.[22,23] In the current study, we attempted to integrate the methylation status of multigenes by using a M-score that is the sum of the log HR analyzed by multivariate logistic regression analysis for pathology (BPH vs. PC). This analysis provides automatically adjusted statistical data,[22] with each HR directly related to gene methylation in PC samples compared with BPH (methylation-negative) samples (Table 2). By adding the log HR of each gene in a multigene analysis, it is therefore possible to predict the risk of PC in individual patients. Similarly, Ray et al. employed multivariate Cox proportional hazards models for their multigene methylation analysis in medulloblastoma, and they used the sum of the log HR as a risk score for each patient.[23] In our study, we found no significant correlation between smoking status and methylation frequency of the *APC* or *MDR1* gene individually. However, by employing a combined analysis (M-score) of the methylation status of the three genes used in this study, differences in methylation status between categories of smokers became appar-

ent (Fig. 3, right). Furthermore, among PCs, the M-score showed a significant stepwise increase with advancing PT category, increasing Gleason score, higher PSA levels, and advancing pathologic features (Fig. 4). Thus, when examining the methylation status of multigenes in PC, the M-score is a reliable, superior, analytical tool for diagnosis and outcome prediction.

In conclusion, this is the first study to demonstrate significant correlation of methylation status of multigenes with smoking status in PC. Smoking status may influence both progression and prognosis of PC through CpG hypermethylation of related genes.

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EUROPEAN UROLOGY XXX (2007) XXX–XXX

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Phase-II Study of Docetaxel, Estramustine Phosphate, and Carboplatin in Patients with Hormone-Refractory Prostate Cancer

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Article info

Article history:
Accepted December 13, 2006
Published online ahead of print on December 26, 2006

Keywords:
Docetaxel
Estramustine phosphate
Carboplatin
Hormone-refractory prostate cancer

Abstract

Objectives: To determine the safety and efficacy of combination chemotherapy with docetaxel (DTX), estramustine phosphate (EMP), and carboplatin (CBDCA) in patients with hormone-refractory prostate cancer (HRPC).

Methods: This study included a total of 40 HRPC patients. We evaluated the activity of the following schedule: weekly DTX 30 mg/m^2 iv, daily EMP 10 mg/kg po, and CBDCA AUC = 6 iv on day 1 of a every 4-wk cycle. Treatment was continued until disease progression or excessive toxicity.

Results: All patients were assessable for response. A median of six consecutive cycles was administered per patient. Levels of prostate-specific antigen decreased by more than 50% in 95.0% of the patients. Consumption of medication for cancer-induced pain was reduced in 84.6%. Partial response was attained in 66.7% of measurable lesions. Of patients with bone metastasis, 8.3% demonstrated partial response. With a median follow-up of 11.4 mo, the median time to progression was 12.0 mo, and the median overall survival time was 26.6 mo. The predominant toxicities were grade-3 or -4 anemia in 32.5% of the patients, leukopenia in 20.0%, and thrombocytopenia in 17.5%. However, all toxicity was temporary and reversible with dose reduction or temporary cessation of chemotherapeutic agents. There were no therapy-related deaths.

Conclusions: Combination chemotherapy with DTX/EMP/CBDCA was found to have significant clinical activity with an acceptable toxicity profile in HRPC patients. More suitable selection of patients may be beneficial in terms of improved overall survival in the future.

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0302-2838/$ – see back matter © 2007 Published by Elsevier B.V. on behalf of European Association of Urology. doi:10.1016/j.eururo.2006.12.030

Please cite this article in press as: Kikuno N, et al., Phase-II Study of Docetaxel, Estramustine Phosphate, and Carboplatin in Patients with Hormone-Refractory Prostate Cancer, Eur Urol (2007), doi:10.1016/j.eururo.2006.12.030

1. Introduction

Hormone-refractory prostate cancer (HRPC) is clinically defined as a metastatic or locally advanced prostate carcinoma that has become refractory because of androgen ablation therapy. Although the survival benefits of docetaxel (DTX)-based therapy have been demonstrated in patients with metastatic HRPC in the Southwest Oncology Group (SWOG) 9916 [1] and TAX 327 [2] studies, management of this incurable disease remains a significant challenge to clinicians, because it is unknown whether DTX-based multicomponent regimens represent any ultimate impact on the survival of HRPC patients.

Such taxanes as paclitaxel (PTX) and DTX bind to different sites on B tubulin and arrest cells at different phases of the cell cycle (S vs. G2M) [3]. DTX disrupts the cellular microtubular network, promoting the assembly of stable microtubules and inhibiting disassembly approximately twice as effectively as PTX [4,5]. In addition, tissue culture studies suggest that the dose required for 50% inhibition of growth by DTX in combination with EMP is 60-fold lower than PTX plus EMP [6].

Estramustine phosphate (EMP) is rapidly ephosphorylated in vivo to estramustine, which dysregulates normal microtubule assembly, resulting in cell growth inhibition in human prostate cancer cell lines [7,8]. Although single-agent PTX, DTX, or EMP therapies offer limited efficacy, many studies have demonstrated that EMP activity may be synergistic against HRPC with other antimitotic agents, such as taxanes [9,10].

Carboplatin (CBDCA) has a distinct toxicity profile from cisplatin that allows for a potentially equivalent antitumor effect with less toxicity in many tumor types. CBDCA has been studied as single agents and in multidrug regimens for HRPC [11–15]. Oh et al [16] reported excellent results from a phase 2 trial of EMP (240 mg three times per day for 5 d), DTX (70 mg/m^2), and CBDCA (AUC; 5) with granulocyte colony-stimulating factor (G-CSF) support given on an every-3-wk schedule [16] and from a phase 1 study of weekly doses of DTX (20, 25, 30, 36, or 43 mg/m^2), daily EMP 140 mg, and monthly CBDCA (AUC; 5 or 6) [17] in patients with progressive HRPC. We also previously conducted a pilot study of combination chemotherapy consisting of weekly DTX 30 mg/m^2, daily EMP10 mg/kg, and monthly CBDCA (AUC; 6) and confirmed that this regimen was comparatively active and safe as a second-line treatment for heavily pretreated patients with PTX-resistant HRPC [18]. Therefore, we investigated a prospective phase 2 trial to evaluate the efficacy and toxicity of this combination chemotherapy with DTX/EMP/CBDCA for Japanese patients with HRPC.

2. Methods

2.1. Patient selection

Eligible patients had histologically confirmed prostate cancer and showed progression despite discontinuation of antiandrogen (androgen withdrawal). Ongoing androgen deprivation with a luteinizing hormone-releasing hormone (LH-RH) analogue was required in patients who had not undergone bilateral orchiectomy. Pretreatment evaluation included medical history, a physical examination, a complete blood count, a chemistry profile, serum prostate-specific antigen (PSA), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) levels, 24-h creatinine clearance, a 12-lead electrocardiogram, a chest X-ray, a bone scan, computerized tomography (CT), and magnetic resonance imaging (MRI). Other inclusion criteria included an Eastern Cooperative Oncology Group (ECOG) performance status (PS) score of 0–3, a baseline leukocyte count greater than 3000/μl, hemoglobin 8.0 g/dl or greater, a platelet count exceeding 100,000/μl, adequate renal function defined as serum creatinine 1.5 times or less than the upper limit of normal (ULN), adequate liver function defined as bilirubin less than ULN and aspartate transaminase less than 1.5 times ULN, adequate cardiac function, and a life expectancy of more than 3 mo. More than 8 wk must also have elapsed since any major surgery, radiotherapy, or any prior chemotherapy. Written informed consent was obtained from all patients as well as the institutional review board approval.

2.2. Treatment regimen

In consideration of the synergistic antitumor activity of the DTX/EMP/CBDCA combination, the treatment regimen was conducted under the same conditions as combination chemotherapy with PTX/EMP/CBDCA that we previously reported [19]. In brief, DTX 30 mg/m^2 was administered intravenously (iv) for 1 h weekly (days 1, 8, 15, and 22). Premedication consisting of dexamethasone 8 mg was administered iv for 30 min before each DTX infusion. Patients received oral EMP 10 mg/kg/d, divided into three daily doses throughout the treatment protocol (days 1–28), 1 h before or 2 h after ingesting food. CBDCA was administered iv for 1 h on day 1 every 4 wk, dosed to an AUC of 6. The treatment schedule was repeated every 4 wk for a minimum of four cycles. Patients who demonstrated response to the therapy continued treatment until disease progression, complete response, the development of treatment-limiting toxicity, or withdrawal of consent. The administration of G-CSF was permitted as needed to prevent severe leukopenia according to the American Society of Clinical Oncology guidelines. Furthermore, blood transfusions were performed for severe symptoms related to thrombocytopenia and anemia. Patients did not routinely receive concomitant anticoagulants, because the incidence of cardiovascular toxicity in Japanese patients is generally lower than that in Western patients. Toxicity was graded according to the National Cancer Institute common

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toxicity criteria (Version 2.0). If grade 3–4 toxicity occurred, all subsequent cycles of therapy were interrupted until the toxicity resolved to less than grade 2 and hematological parameters had recovered to at least meet entry criteria. If a patient's toxicity was higher than grade 3, subsequent doses of DTX and CBDCA were reduced by 25% for the next cycle. The EMP dose was reduced by 25% in the case of severe gastrointestinal toxicity.

2.3. *Evaluation*

Systematic sextant biopsy of the prostate under transrectal ultrasound (TRUS) guidance was undertaken. Measurable soft tissue disease was defined as any lesion 1 cm × 1 cm or greater in bidimensional measurements. Patients were evaluated for response by imaging studies and systematic sextant biopsies at least every 8 wk for four cycles. Pain status was evaluated with the use of the Wong-Baker face scale, and intake of nonsteroidal anti-inflammatory drugs (NSAIDs) and narcotics was monitored. PSA levels were determined every 4 wk. Since changes in the intensity or size of osseous lesions using bone scans were difficult to interpret, the appearance of one or more new osseous lesions was required in a bone scan to identify disease progression. Likewise, the disappearance of one or more lesions was required to identify improvement. The PSA level at the baseline had to be greater than 4 ng/ml to assess post-therapy changes. A post-therapy change in the PSA level was defined on the basis of the degree of change from the baseline. Progression of the PSA level was defined as three consecutive increases in PSA of 50% over the nadir value at a minimum of 4 ng/ml. Systematic sextant biopsies, which were evaluated for antitumor therapeutic effect and reduction in the number of positive biopsy specimens, were classified as "improvement," "no change," or "progression" by a pathologist. Our combined response criteria were defined on the basis of the consensus criteria in phase 2 trials published in 1999 [20]. Time to progression (TTP) was measured from the first day of treatment to the off-study data or progressive disease (PD). Overall survival time (OST) was measured from the initiation of therapy to death or the last follow-up.

2.4. *Statistical analysis*

The Kaplan-Meier product limit estimator was used to estimate TTP and OST.

3. Results

3.1. *Patient characteristics*

A total of 40 patients with HRPC were enrolled in this study. Their pretreatment characteristics are summarized in Table 1. The median patient age was 73.0 yr (range: 52–86 yr). ECOG PS was 0–1 in 72.5% of patients and 2–3 in 27.5%. The median hemoglobin, PSA, ALP, and LDH levels at baseline were 12.2·g/dl, 71.3 ng/ml, 113 U/l, and 182 U/l, respectively. Bidimensionally measurable extraosseous disease was

Table 1 – Patient characteristics

Pt age (yr)	
Median	73.0
Range	52–86
No. ECOG performance status (%)	
0	13 (32.5)
1	16 (40.0)
2	6 (15.0)
3	5 (12.5)
Gleason score	
Median	8
Range	5–9
PSA at diagnosis (ng/ml)	
Median	96.4
Range	0.8–6113.9
PSA nadir at lion none therapy (ng/ml)	
Median	0.8
Range	0.01–19.1
Duration of initial hormone therapy (mo)	
Median	19.7
Range	2.1–134.0
PSA doubling time after diagnosis of HRPC (mo)	
Median	0.85
Range	0.38–35
Laboratory data at baseline	
Hemoglobin (g/dl)	
Median	12.2
Range	8.9–13.5
PSA (ng/ml)	
Median	71.3
Range	4.1–3584.1
Alkaline phosphatase (U/l)	
Median	113
Range	97–387
Lactate dehydrogenase (U/l)	
Median	182
Range	173–256
No. measurable extraosseous disease (%)	
Neg.	23 (57.5)
Pos.	17 (42.5)
Lymph nodes	13
Lung	5
Liver	3
No. osseous disease (%)	
Neg.	4 (10.0)
Pos.	36 (90.0)
No. prostate (%)	
Neg.	1 (2.5)
Pos.	39 (97.5)
Neg. biopsy (no viable cancer cells)	7
Pos. biopsy (viable cancer cells)	32
No. cancer pain (%)	
Neg.	27 (67.5)
Pos.	13 (32.5)
Requiring nonnarcotic medication	5
Requiring narcotic medication	8

ECOG = Eastern Cooperative Oncology Group; PSA = prostate-specific antigen; HRPC = hormone-refractory prostate cancer.

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Table 2 – Prior therapy

	No. (%)
Hormone therapy (maximum androgen blockade)	40 (100)
LH-RH analogue	31 (77.5)
Surgical castration	9 (22.5)
Diethylstilbesterol	2 (5.0)
Chemotherapy	8 (20.0)
Single estramustine phosphate	6 (15.0)
Single 5-fluorouracil	1 (2.5)
Estramustine phosphate, 5-fluorouracil	1 (2.5)
Corticosteroid	11 (27.5)
Surgery (prostatectomy)	1 (2.5)
Radiation	3 (7.5)
Prostate/pelvis	3 (7.5)

LH-RH = luteinizing hormone-releasing hormone.

present in 17 (42.5%) patients, and 36 (90.0%) demonstrated bone metastasis. Of 39 (97.5%) patients with primary lesions, 32 (80.0%) demonstrated positive biopsies of the prostate. Cancer-induced pain was present in 13 (32.5%) patients. A median of six consecutive cycles of therapy was administered per patient (range: 2–47). The prior therapies of these patients are summarized in Table 2. Two patients had previously been treated with diethylstilbestrol (DES) as second- or third-line hormone therapy. Eight patients had received prior cytotoxic chemotherapy. Prior corticosteroid therapy had been performed in 11 (27.5%) patients. A total of three (7.5%) patients had received prior radiation therapy, and one (2.5%) had undergone prior radical prostatectomy.

3.2. *Clinical outcomes*

Clinical outcomes are listed in Table 3. PSA levels decreased by >50% in 95.0% of the patients as a

Table 3 – Clinical outcome

	Effective no. pts/ total no. pts (%)
PSA decrease (%)	
50 or greater	38/40 (95.0)
75 or greater	37/40 (92.5)
90 or greater	27/40 (67.5)
Measurable extraosseous disease	14/21 (66.7)
Lymph nodes	9/13 (69.2)
Liver	3/5 (60.0)
Lung	2/3 (66.7)
Osseous disease	3/36 (8.3)
Prostatic biopsy	27/32 (84.4)
Cancer pain	11/13 (84.6)
Overall response	31/40 (77.5)

PSA = prostate-specific antigen.

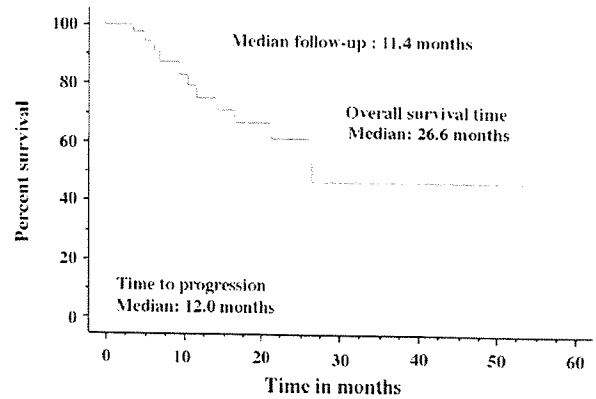

Fig. 1 – Progression-free survival (broken line) and overall survival (solid line) of hormone-refractory prostate cancer patients who underwent combination chemotherapy with DTX/EMP/CBDCA, as determined by the Kaplan-Meier method.

maximum response. The median PSA decrease was 96.3% (range: 55.9–99.9). PSA normalized to <4.0 ng/mL in 45% of the patients. Of 13 assessable patients with lymphadenopathy, 9 (69.2%) attained PR, and of 8 patients with measurable extraosseous disease except for lymphadenopathy, 5 attained PR. The final response rate for all measurable lesions was 66.7%. Of 36 patients with positive bone metastasis, bone scans revealed improvement in only 3. Of the remaining 33 patients, 32 were stable and only 1 was PD. Tumor volume reduction and/or antitumor therapeutic effects were exhibited in 27 (84.4%) of 32 patients with positive biopsies. Thirteen patients complained of cancer-induced pain on bone metastasis. In 11 patients (84.6%), NSAIDS and/or narcotic drugs dosage were reduced because of a decrease in cancer-induced pain during treatment. In addition, cancer-induced pain was completely relieved in eight patients without NSAID and/or narcotic drugs. As for overall combined response, CR was demonstrated in no patients, PR in 31 (77.5%), and PD in 1 (2.5%) with lymphadenopathy and bone metastasis. The disease was considered stable in the remaining 8 patients (20.0%). The median follow-up time was 11.4 mo, the median TTP was 12.0 mo (95% confidence interval [CI], 8.0–20.0 mo) (Fig. 1: broken line), and the median OST was 26.6 mo (95%CI, 16.9–54.0 mo) (Fig. 1: solid line). There was no statistical differentiation in TTP and OST between both groups (positive and negative) divided on the basis of several factors including measurable response.

3.3. *Adverse effects*

All of the patients were assessable for toxicity (Table 4). Most adverse events were moderate

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Table 4 – Percentage of major toxicities

Toxicity ($n = 40$)	Grades 1–2	Grades 3–4	Total
	No. (%)		
Hematologic			
Anemia	18 (45.0)	13 (32.5)	31 (77.5)
Leukopenia	22 (55.0)	8 (20.0)	30 (75.0)
Thrombocytopenia	16 (40.0)	7 (17.5)	23 (57.5)
Cardiac/thromboembolic	1 (2.5)	0 (0.0)	1 (2.5)
Thrombosis/embolism	0 (0.0)	0 (0.0)	0 (0.0)
Neurosensory toxicity	1 (2.5)	1 (2.5)	2 (5.0)
Transaminase	3 (7.5)	–	3 (7.5)
Anorexia	3 (7.5)	1 (2.5)	4 (10.0)
Malaise/fatigue/asthenia	3 (7.5)	2 (5.0)	5 (12.5)
Alopecia	15 (37.5)	–	15 (37.5)
Erythema	1 (2.5)	1 (2.5)	2 (5.0)
Nail changes	5 (12.5)	0 (0.0)	5 (12.5)
Pleural effusion	3 (7.5)	3 (7.5)	6 (15.0)
Pneumonitis/pulmonary infiltrates	0 (0.0)	1 (2.5)	1 (2.5)

Therapy-related deaths: none.

in intensity and were managed medically. Major toxicities consisted of grade 3–4 anemia in 32.5% of the patients, leukopenia in 20.0%, thrombocytopenia in 17.5%, and pleural effusion in 7.5%. G-CSF was utilized in seven patients (17.5%), and eight patients (20.0%) underwent blood transfusion. Severe neuropathy and gastrointestinal toxicity were uncommon. Abnormalities in liver function were slight and reversible. Venous thromboembolic events were not observed. Although DTX and CBDCA dose reductions were required because of toxic effects in 17 patients (42.5%), most patients demonstrated no severe hematologic side effects after dose reduction, and temporary cessation of DTX and CBDCA. The mean received dose intensities of DTX and EMP in this study were 24.7 mg/m^2/wk and 7.8 mg/kg/d (82.3% and 78% of the planned dose intensity), respectively. The subsequent dose levels of CBDCA were AUC; 6 (23 patients), AUC; 5 (15 patients), and AUC; 4 (2 patients). One patient demonstrated a hypersensitivity reaction to CBDCA. Furthermore, there were no treatment-related deaths.

4. Discussion

In this phase 2 trial, weekly DTX, daily EMP, and monthly CBDCA showed significant activity and an acceptable toxicity profile. Post-therapy reductions of serum PSA and measurable responses were higher, and the median TTP (12.0 mo) and OST (26.6 mo) were longer than those previously reported in most phase 2 trials evaluating combination chemotherapy regimens for HRPC; It is impossible, however, to simply compare our results with other studies, because the backgrounds of the patients enrolled in each trial are different [21]. Recently, several groups have reported pretreatment nomograms for predicting the survival of patients with HRPC [22–24]. Depending on the most recent nomogram [24], the median probability of HRPC-specific survival was 0.45 at 24 mo after HRPC diagnosis of our patients enrolled in this study. Therefore, our median OST results were considered better than nomogram-estimated survival. These findings support the compelling rationale that improving response proportions by using active, synergistic, or additive combinations may subsequently lead to an improvement in survival [25].

Because it is desirable for heavily pretreated elderly patients with HRPC to be treated by chemotherapy with less toxicity, we choose a weekly DTX regimen associated with less myelotoxicity than an every 3-wk regimen [26]. We believe the lower hematologic toxicity in this study must be caused by the weekly DTX schedule, except for the myeloprotective effect of EMP on CBDCA-induced myelosuppression, as reported previously [17]. These toxicities were temporary and reversible with dose reduction and withholding of chemotherapeutic agents in the majority of patients. Although the incidence of thromboembolic and/or cardiovascular side effects in trials utilizing EMP has been reported to be approximately 7–15% [16,17], no such events were observed in our previous trial without routine anticoagulants [18,19]. Therefore, we suggest that anticoagulation should only be administered in cases in which patients have a past history of thromboembolism or risk factors for cardiovascular toxicity, or when the administration of EMP must be continued long term. Actually, no grade 3–4 thromboembolic and/or cardiovascular toxicity was observed in this study, although only three patients with the risk factors were pretreated with anticoagulants. These results may represent a race-specific variation, because the incidence of cardiovascular toxicity in Japanese patients is generally lower than that in Western patients. In this way, this combination regimen seems to have an acceptable safety profile in spite of such accumulative toxicity as fatigue, hyperlacrimation, nail toxicity, and alopecia. The Cancer and Leukemia Group B (CALGB) also reported that the combination of EMP, DTX, and CBDCA with G-CSF support was found to have significant clinical activity with an acceptable toxicity profile in patients with progressive HRPC [16]. Our and others' results seem to encourage going on to large randomized phase 3 trials. On the other hand, two recent phase 3 studies (SWOG 9916,

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TAX327) have questioned both the need for EMP as well as the relative efficacy of weekly chemotherapy for HRPC [1,2]. On the basis of these findings, the administration every 3 weeks of DTX-based chemotherapy without EMP is now considered the first-line standard option for metastatic HRPC [27]. However, in the present state, we were unfortunately unable to find which regimen was best. Nevertheless, we believe our regimen should be tested in selected patients, because HRPC is not untreatable, although it is an incurable condition that consists of a spectrum of disease [28].

Now there are also increasing options for second-line therapies in the palliative treatment of HRPC [29]. We suggest several possible clinical scenarios in which our regimen could be useful. The first opportunity may exist in high-risk elderly patients since weekly DTX chemotherapy is associated with less myelotoxicity than DTX administered every 3 wk [26]. Second, patients with a short response to androgen-deprivation therapy and severe cancer-induced pain resulting from measurable soft tissue disease may represent an opportunity to test our triplet regimen, because the measurable response rates in SWOG 9916 and TAX327 trials were not significantly higher than those with mitoxantrone plus prednisone. Other opportunities may exist in patients who have a lower likelihood of responding to standard chemotherapy or whose levels of neuronendocrine differentiation markers are high, because the transition of prostate cancer from a hormone-sensitive to a hormone-refractory state is associated with increased neuroendocrine differentiation, and CBDCA has activity against cancers with neuronendocrine differentiation [30]. Moreover, our regimen should be examined as second-line chemotherapy in patients who have progressed after initial DTX chemotherapy without EMP and/or CBDCA. Although future studies will be required to characterize patients for whom our regimen may provide clinical benefit beyond the effects of standard DTX-chemotherapy, we believe that our data indicate an important clue to clarify the role of CBDCA for HRPC under the present situation in which the role of CBDCA has never been fully evaluated in a comparative study.

5. Conclusions

Combination chemotherapy with DTX/EMP/CBDCA was found to have significant clinical activity with an acceptable toxicity profile in HRPC patients. Although further studies are necessary to characterize patients for whom this regimen may provide clinical benefit beyond the effects of standard DTX-chemotherapy, our report contains an important clue to develop more suitable HRPC clinical trials in the future.

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Suicide Gene Therapy With Adenoviral Delivery of HSV-tK Gene for Patients With Local Recurrence of Prostate Cancer After Hormonal Therapy

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We conducted a Phase I study of *in situ* herpes simplex virus thymidine kinase (HSV-tk) plus ganciclovir (GCV) gene therapy, which was approved by the Japanese government as the first prostate cancer gene therapy trial. Major inclusion criteria were local recurrence of prostate cancer after hormonal therapy and no metastasis. Adv.HSV-tk was injected directly into the prostate in escalating doses from 10^9 to 10^{10} infection units, followed by intravenous administration of GCV for 14 days. Eight patients received nine courses of this gene therapy. The detection of vector DNA in blood/urine was only transient and no remarkable adverse events were observed in any patient. With regard to clinical response, significant prolongation of the median serum prostate-specific antigen (PSA) doubling time from 2.9 to 6.2 months ($P=0.041$) was detected. In five patients (six injections), a clear decrease of PSA values was observed. One patient showed repeated clinical response after repeated injections. Serum cytokine analysis showed no notable changes after treatment. Fluorescence-activated cell sorting analysis also showed no influence on phenotypic distribution in peripheral blood samples, except for an increasing trend of $CD8^+$/HLA-DR^+ after therapy. This study confirmed the safety profile and possibility of clinical response at the surrogate marker level in a clinical trial of HSV-tk gene therapy for hormone-refractory prostate cancer.

Received 6 September 2006; accepted 30 November 2006; advance online publication 27 February 2007. doi:10.1038/sj.mt.6300096

INTRODUCTION

Although early-stage diagnosis of prostate cancer has become more prevalent by widespread screening for prostate-specific antigen (PSA), there are still a large number of patients with locally advanced disease whose treatment is controversial. Hormonal manipulation (orchiectomy or luteinizing hormone-releasing hormone agonist) is the main treatment option for patients who are not candidates for or who are not willing to undergo radical prostatectomy or radiation therapy. However, a large number of patients undergoing hormonal treatment alone show disease progression with the development of hormonal-refractory status. In some cases, micrometastases are present but undetectable at the time of initiation of the treatment. Therefore, a new therapeutic modality for local lesions and systemic disease is needed. Gene therapy is a promising modality because it affords an opportunity to affect both localized and systemic disease.

Gene therapy refers to the transfer of genetic material into cells and the expression of this material for therapeutic purposes. One experimental cancer gene-therapy approach is the transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene, followed by the administration of ganciclovir (GCV). This antiherpetic pro-drug is a poor substrate for mammalian thymidine kinases, but can be phosphorylated into an effective cytotoxic drug by HSV-tk.[1] The phosphorylated drug is a nucleotide analog that is incorporated into DNA during cell division, leading to termination of DNA replication and cell death.[2] We selected it as a cytotoxic gene-therapy approach for our experimental therapy for hormonal-refractory local advanced disease, primarily because of the following advantages: (1) the "bystander" effect, a well-documented phenomenon whereby many more cells are killed than are transduced with the therapeutic gene, and (2) evidence for the initiation of a local as well as a systemic immune response.[3,4] The clinical trials using this approach in the United States on patients who have local recurrence after definitive radiation therapy have documented that this therapy is safe in clinical use[5] and that it results in a decline in the serum PSA level in some patients.[6] These trials have also shown that this therapy produces a characteristic, recognizable pattern of cytopathic changes in prostate cancer tissue. Thereafter, neoadjuvant approaches of this therapy for patients with radical surgery for clinically localized cancers were reported.[7–9] However, there has been no clinical trial, except for a

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study using a different adenoviral vector,[10] on patients with hormone-refractory status.

The primary end point of this study was toxicity, with anticancer response evaluated by serum PSA serving as a secondary end point. We confirmed the safety of *in situ* Adv.HSV-tk plus GCV gene therapy for patients with hormone-refractory status. The data also suggest an anticancer effect, as judged by the reduction of serum PSA levels in these patients with this therapy.

RESULTS

Patients

We enrolled eight patients in the study: three patients in the low-dose (10^9 infection units (IU)) group and six in the high-dose (10^{10} IU) group, including one who was also treated with the low dose (patient no. 7 as the second treatment of patient no. 3). The median age was 67.5 years (61–80 years). Pre-treatment PSA levels ranged from 4.0 to 29.9 μg/ml (median 11.2 ng/ml) (**Table 1**). The median follow-up period from the time of vector injection to the time of treatment change was 7.1 months (range 1.6–13.0 months). Two patients died of prostate cancer at 60 and 50 months after treatment. Six patients were alive at 9–52 months (median 25 months).

Safety considerations and adverse events

No adenoviral DNA was detected in the blood of low-dose patients by real-time polymerase chain reaction (PCR). In the high-dose group, adenoviral DNA was observed in all blood samples obtained 30 min after viral injection. DNA was detected only on day 0 and not on day 1 or thereafter in all patients. The highest quantity of DNA determined by real-time PCR was 4.1×10^3 copies/μg in one patient 60 min after viral injection. Adenoviral DNA in urine was detected on the day of injection in all cases. In one of three patients receiving low-dose injections and four of six patients receiving the high dose, viral DNA was detected by PCR on day 1. Viral DNA in the urine of one patient remained positive on day 2. Thereafter, no DNA was detected in urine (**Table 2**).

No patient in the study group experienced grade 3 or 4 toxicity. As for symptomatic adverse events, one patient showed grade 1 complications with urinary outflow obstruction and frequency on day 6. Grade 1 headache in two patients and micturition pain, hot flushes, nausea, and fever in each patient were observed on day 0 or 1. Grade 1 lumbago was observed in three patients on day 0, and grade 1 hematuria was observed in one low-dose patient on day 3 and in two high-dose patients on day 1. Temporary eczema was observed in one patient on day 4. As for laboratory data abnormalities, four of six patients in the high-dose vector group had temporary elevation of grade 1 C-reactive protein values. The details of adverse events are shown in **Table 3**. All adverse events were transient and patients spontaneously returned to normal within 2 weeks without discontinuation of treatment.

PSA response

A reduction in serum PSA was observed immediately after six of the nine treatments (vector injection and GCV therapy) in eight patients (**Table 1**). PSA reduction (PSAR), which is defined as a percentage of pre-treatment value to nadir value after treatment, ranged from 6.7 to 43.9% in these six responders with a median PSAR of 24.1%. No patient showed more than a 50% decrease in PSA, which is the minimum criterion for an objective response. Based on multiple measurements of serum PSA during follow-up, we calculated the linear regression line for the change in the log of serum PSA after the nadir of the PSA response and estimated the time for the serum PSA to return to the pre-treatment value. The time of return to the initial PSA (TR-PSA) ranged from 2 months to at least 13 months with a median of 5.0 months (mean 5.8 months) (**Table 1**).

Table 1 Patient characteristics and PSA response (PSAR, TR-PSA, and PSADT)

Patient no.	Age (years)	Pre-treatment PSA	Nadir PSA[a]	PSAR	TR-PSA (months)	Pre-treatment PSADT	Post-treatment PSADT (months)
Low dose							
1	65	23.2	(34.9)	None	None	0.8	1.6
2	61	4.1	(5.68)	None	None	1.4	1.9
3 (3-1)	78	4.0	(3.58)	10.1	7.0	9.4	28.9
High dose							
4	64	26.0	24.4	6.7	6.0	3.1	16.1
5	69	21.8	13.3	38.7	2.0	5.5	1.7
6	70	29.9	20.7	30.6	4.0	8.1	19.3
3 (3-2)	80	7.0	3.9	43.9	>13.0	2.9	12.2
7	80	8.8	7.3	17.6	3.0	1.8	6.2
8	66	14.6	(26.2)	None	None	0.9	1.1

PSA, prostate-specific antigen. PSAR: PSA reduction—the percent reduction in the serum PSA value determined before vector injection to the nadir value after injection. TR-PSA: the time of return to initial PSA—estimation by plotting the log PSA values versus time and performing logistic regression analysis after the PSA nadir. The time from vector injection to the time when the linear regression line of log PSA intercepted with the value of PSA before vector injection was used as the TR-PSA. PSADT: PSA doubling time—the linear regression of the log PSA was also used to calculate PSADT according to the following formula: PSADT 5 log(2)/slope of the linear regression fit of log PSA versus time. Pre-treatment PSADT was calculated from PSA nadir to the next point (at least 4 weeks apart) before the vector injection, and post gene therapy PSADT was calculated from PSA nadir (or time of the vector injection) to 4 weeks after. [a]Four weeks after the vector injection.

Table 2 Quantitative kinetic analysis of adenoviral DNA in blood and urine by real-time PCR

Patient no.	30 min after injection	60 min after injection	90 min after injection	Day 1	Day 2
Adenoviral DNA quantity in blood (copies/ug)					
1	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND
4	1.2×10^2	1.2×10^2	ND	ND	ND
5	1.1×10^2	ND	ND	ND	ND
6	1.2×10^3	3.0×10^2	ND	ND	ND
7	1.3×10^2	ND	ND	ND	ND
8	1.5×10^3	4.1×10^3	6.6×10^2	ND	ND
9	1.3×10^3	ND	ND	ND	ND
Adenoviral DNA quantity in urine (copies/μg)					
1	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND
4	9.1×10^3	2.9×10^3	6.3×10^2	ND	ND
5	1.5×10^2	ND	ND	ND	ND
6	1.0×10^2	3.2×10^2	4.4×10^2	2.5×10^2	ND
7	1.5×10^3	1.8×10^2	ND	1.7×10^2	ND
8	4.0×10^3	1.8×10^2	2.2×10^3	ND	ND
9	9.3×10^2	1.1×10^2	1.3×10^2	ND	ND
Detection of adenoviral DNA in urine					
1	+	+	+	+	–
2	+	+	+	+	–
3	+	+	+	–	–
4	+	+	+	–	–
5	+	–	–	–	–
6	+	+	+	+	–
7	+	+	+	+	–
8	+	+	+	+	–
9	+	+	+	+	+

ND, not detectable; PCR, polymerase chain reaction. Detection of adenoviral DNA in urine by PCR. In patient no. 9, no adenoviral DNA in urine was confirmed on day 3.

From the linear regression of the log of serum PSA, we also calculated PSA doubling time (PSADT). Median pre-treatment PSADT was 2.9 months (range 0.3–9.1 months; mean 3.8 months) and median post gene therapy PSADT was prolonged significantly by more than twofold to 6.2 months (range 1.1–28.9 months; mean 9.9 months) ($P = 0.041$) (**Table 1**) (**Figure 1**). A repeated cycle of gene therapy for one patient showed PSADT of 9.4–28.9 months following the first injection and 2.9–12.2 months following the second, and resulted in increased PSAR from 10.1 to 43.9% and TR-PSA from 7 weeks to over 13.0 months.

Detection of *HSV-tK* gene in biopsy specimens

To confirm the transduction of the *HSV-tK* gene into target lesions, the *HSV-tK* gene from the biopsy samples was amplified by PCR and visualized in ethidium bromide-stained gels. The *HSV-tK* gene was detected after viral injection (**Figure 2**).

Anti-adenovirus neutralizing antibody response

The antibody response was evaluated by analyzing adenovirus-specific neutralization. In the low vector dose group, all three patients demonstrated a clear boosting of adenovirus-specific neutralizing antibody responses. In the high vector dose group, a neutralizing antibody response was evidenced in three of six patients. No dose dependencies were observed. The plateaus of neutralizing antibody response were documented on day 14 in seven of nine treatments, with diminishment confirmed later in the remaining two patients.

Systematic immunoresponses

Cytokine analysis revealed no response to the intraprostatic injection of Adv.HSV-tk (**Table 4**). Peripheral blood was obtained for phenotypic analysis by fluorescence-activated cell sorting from eight of nine patients before vector injection and at selected intervals after the course of this therapy. The percentage of peripheral blood lymphocytes expressing CD56 and CD16 markers characteristic of natural killer cells showed no consistent pattern (**Table 5**). There was also no consistent alteration in the percentage of either $CD4^+$ or $CD8^+$ T cells (CD31). The human leukocyte antigen dimensional reconstruction (HLA-DR) marker of activation was used to double label $CD4^+$ or $CD8^+$ lymphocytes as a relative measure of activated T cells detectable by fluorescence-activated cell sorting in all patients after vector injection. No consistent changes were observed in $CD4^+$/HLA-DR^+ cells after vector injection. However, a trend of increasing frequencies of $CD8^+$ T cells positive for the HLA-DR marker of activation was observed post-treatment.

DISCUSSION

In previous studies, our collaborative group at BCM demonstrated the efficacy and safety of adenovirus-mediated herpes simplex virus thymidine kinase gene transduction and ganciclovir therapy (Adv.HSV-tk + GCV) in orthotopic mouse models for prostate cancer. These preclinical studies resulted in the initiation of a clinical Phase I/II study analyzing escalating doses of intraprostatic Adv.HSV-tk in patients with locally recurrent prostate carcinoma after radiotherapy.[6] This study was completed without significant side effects from intraprostatic injection of a replication-deficient adenovirus and biological responses including increases in PSADT, PSAR, and TR-PSA and some systemic immune reactions indicating T-cell activation. In collaboration with BCM, we tested the safety and efficacy of this strategy for patients with locally recurrent prostate carcinoma after hormonal therapy as the first prostate cancer gene therapy in Japan.

In situ HSV-tk plus GCV gene therapy for local recurrence of prostate cancer after hormonal therapy showed a safety profile and clinical response comparable to those of the clinical trial performed for local recurrence of prostate cancer after definitive radiation therapy at BCM. Although Kubo *et al.*[10] reported a histological effect in their clinical trial for hormone-refractory prostate cancer with osteocalcin promoter-driven HSV-tk gene

Table 3 Adverse events

Patient no.	Increased CRP	Hematuria	Headache	Dermoreaction	Leukocytopenia	Thrombocytopenia	Fever	Others (possibly related)
Low dose								
1		Grade 1						
2								Voiding disturbance, pollakisuria, increased LD
3			Grade 1					Nausea
High dose								
4	Grade 1	Grade 1		Grade 2	Grade 1		Grade 1	Eczema
5	Grade 1		Grade 1					Increased direct bilirubin
6	Grade 1							Micturition pain
7		Grade 1						
8	Grade 1							
9								

CRP, C-reactive protein; LD, lactate dehydrogenase. All adverse events were spontaneously cured or returned to normal.

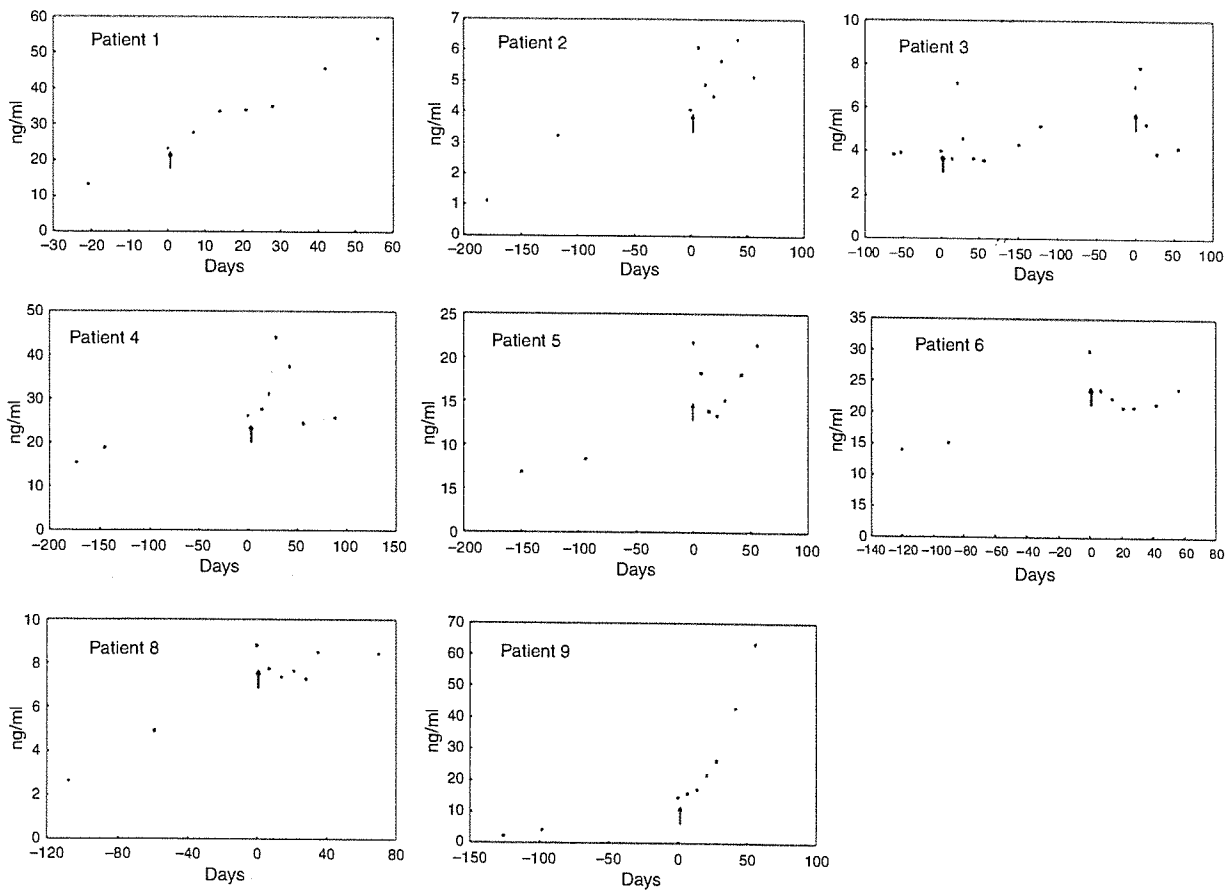

Figure 1 PSA values of treated patients. Vector was injected on day 0 (arrow). Pre-treatment PSADT was calculated from PSA nadir to the next point (shown in the previous two points), and post gene therapy PSADT was calculated from PSA nadir to 4 weeks after. In the case of no PSA decline, PSADT was calculated from the time of vector injection.

therapy, our study is the first clinical trial of HSV-tk gene therapy for prostate cancer that showed clinical response for hormone-refractory prostate cancer. Unfortunately, we have not analyzed a histological response, such as HSV-tk, coxsackie and adenovirus receptor, and apoptotic cells in the injected sites, which might predict clinical response, or the local distribution of T cells/macrophages, which might demonstrate local immune response.[7,10] A sufficient volume of tissue specimen for those

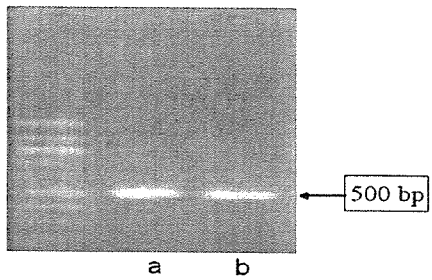

Figure 2 Detection of *HSV-tK* gene in biopsy specimens. The biopsy samples were obtained from both lobes of the prostate in patients 1. The *HSV-tK* gene was amplified by PCR and visualized in ethidium bromide-stained gels. The *HSV-tK* gene was detected in both lesions. (**a**) Right lobe and (**b**) left lobe.

Table 4 Cytokine analysis in the serum samples

	Pre-treatment	Day 7	Day 14	Day 28
Interferon-γ (IU/ml)	0.78±0.85	1.05±1.25	1.17±1.47	1.08±1.40
TNF-α (pg/ml)	0.5>	0.5>	0.5>	0.5>
IL-6 (pg/ml)	2.60±3.27	1.59±0.56	2.10±2.28	1.55±0.81
IL-8 (pg/ml)	18.06±39.7	6.31±5.94	10.31±9.95	8.10±6.95
IL-10 (pg/ml)	2>	2>	2.75±2.12	2>

IL, interleukin; IU, infection unit; TNF, tumor necrosis factor. No serum cytokines showed any response to the intraprostatic injection of Adv.HSV-tk. Days: after the vector injection; ±SD.

Table 5 FACS analysis in peripheral white blood cells

	Pre-treatment	Day 7	Day 14	Day 28
CD4	1	0.95	0.93	0.92
CD8	1	1.05	1.07	1.02
CD4/CD8	1	0.88	0.84	0.84
CD56	1	1.00	0.99	0.94
CD11b	1	1.08	1.03	1.05
CD16	1	0.99	1.02	0.97

FACS, fluorescence-activated cell sorting. Each value is mean ratio to pre-treatment values.

analyses was not obtained for several reasons: (i) the ethical consideration of the possibility of complications due to the large biopsy amount; (ii) not enough biopsy specimen to analyze T cells/macrophages distribution; (iii) no reliable antibody for HSV-tk and coxsackie and adenovirus receptor.

Considering safety with minimal toxicity in this study, self-resolving adverse symptoms were modest, as in other clinical trials, and never peaked higher than grade 2.[6,11] Temporary elevation in serum C-reactive protein (maximum 0.6–3.0 mg/dl in each patient) on early days, which was the most frequent adverse event, was related to local inflammation from the needle-stick procedure and not to active systemic infection. No severe cytolytic effects of viral infections, as observed after intra-arterial gene therapy,[12] were seen. No viral DNA in blood was detected after the day of injection. This result supports the fact that no vector was intra-vascularly disseminated, which could cause severe systemic adverse events.[13] Although viral DNA in urine was detected on the day of injection, it diminished by day 2, indicating no continuous viral infection in the urinary tract (**Table 2**).

Immune responses to viral infection are predominantly antibody mediated.[14] In our study, the overall antibody response was comparable among low and high vector dose patients, as in other clinical studies.[6]

Systemic adverse reactions in adenoviral vector dissemination may be detected or monitored by systemic cytokine reaction and the distribution of peripheral leukocytes.[15–17] Therefore, we monitored serum cytokine kinetics of interferon-γ, tumor necrosis factor-α, interleukin (IL)-6, IL-8, and IL-10 in this study, and no active cytokine reaction to intraprostatic injection of Adv.HSV-tk was observed. Fluorescence-activated cell sorting analysis also showed no obvious influence on the distributions. These results indicate that the *in situ* Adv.HSV-tk plus GCV gene therapy did not cause severe systemic biological adverse events. Only the increasing frequencies of $CD8^+$ T cells positive for the HLA-DR marker of activation were observed, which is comparable to a prior report.[6] Although we could not overlook nonspecific therapy effects and/or the presence of the virus proper, the results might indicate that the anticancer effect of this therapy could induce a systemic anticancer cellular immune response with cancer cell apoptosis. However, our results were obtained from only a small number of patients, as the analysis for activated T lymphocytes was started in the last four patients.

We measured PSAR that could potentially reflect acute-cell cytotoxic responses to this experimental therapy. PSAR in response to *in situ* Adv.HSV-tk plus GCV gene therapy was observed in the study carried out for local recurrence of prostate cancer after definitive radiation therapy at BCM.[6] Seventy-five percent of their patients showed a response to the initial vector injection. In our study, six of nine patients (67%) responded even in hormonal-refractory cancer. A higher response rate (83%, 5/6) was observed in patients treated with high-dose vector (1×10^{10}). These results suggest that *in situ* Adv.HSV-tk plus GCV gene therapy could have a cytotoxic effect on hormonal-refractory prostate cancer. We also measured TR-PSA, which could reflect a clinically significant response. In our study, TR-PSA showed a relatively shorter time (mean 5.8 months) than that in Miles *et al.*[6] One possible reason is the difference in pre-treatment cancer conditions between our study and theirs. We calculated PSADT, generally accepted as a reasonable surrogate end point to document therapeutic response or failure for many protocols over time, which could significantly prolong ($P=0.041$) PSADT in eight of nine patients. PSADT before treatment in our series (mean 3.8 months) was shorter than in Miles *et al.* (14.3 months).[6] Historically, the mean PSADT in patients with hormone-refractory cancer has been reported to be 3.9 months,[18] but in patients with local failure after irradiation therapy it was reported to be 12.6 months[19] to 13.2 months.[20] Hormone-refractory cancer can be more biologically active than irradiation-resistant cancer. This difference in background might explain why our post-treatment PSADT was shorter than theirs (9.8 versus 42.5 months). On the other hand, the criticism of our

short interval used to calculate the PSADT, based on the study design, still remains, and longer follow-up and more data points are needed in the next study. Our results, however, indicate that a single cycle of *in situ* Adv.HSV-tk plus GCV gene therapy might result in anticancer effects even in active cancer.

Further studies are needed to develop intravenous forms of the vector to treat prostate cancer with metastases, and therapy combined with existing therapeutic modalities or with other gene therapies should be planned.

Intraprostatic injections of Adv.HSV-tk followed by GCV for local recurrence of prostate cancer after definitive hormonal therapy show a safety profile comparable to that of the clinical trial carried out for local recurrence of prostate cancer after definitive radiation therapy. This gene therapy for hormone-refractory prostate cancer is feasible and safe in intraprostatic vector doses up to 1×10^{10}, with minimal toxicity, and no infectious vector spread was observed.

MATERIALS AND METHODS

Treatment protocol. The protocol was approved by the Okayama University Institutional Review Board and the Japanese Government. This protocol was a dose escalation study without the inclusion of control subjects. The study was an open-label trial and not randomized. Patients reviewed the informed consent document and received individual counseling with a thorough discussion as to alternative treatments, including non-participation. Initially, we planned to use three escalating doses of 10^9–10^{11} IU, but an optimum dose for Adv.HSV-tk was confirmed during the study;[6] therefore, we omitted the highest dosage of 10^{11} IU.

Inclusion criteria. The major inclusion criteria of the study were local recurrence of histologically proven adenocarcinoma of prostate cancer after hormonal therapy and no evidence of metastasis. Patients receiving hormonal ablation treatment including antiandrogen therapy underwent at least a 4-week antiandrogen withdrawal period before enrollment to exclude the possible effects of antiandrogen withdrawal syndrome. Luteinizing hormone-releasing hormone agonist was continued throughout the study, and serum testosterone had to be confirmed at the castration level. Serum PSA levels had to be elevated at three 2-week intervals to become finally greater than 4.0 ng/ml. Patients were required to have completed any other treatment that may have influenced this study. Normal hematopoietic function (platelet count > 100,000/ml, neutrophil count > 2,000/ml), a normal coagulation profile, and normal kidney and liver functions (serum creatinine < 1.5 mg/dl, bilirubin < 1.5 mg/dl) were required.

Vector. The vector used was an adenovirus of serotype Ad5 that contained the herpesvirus thymidine kinase gene and a Rous sarcoma virus long terminal repeat promoter in the region of the excised E1/E2 wild-type adenoviral genes. It was produced in the Baylor College of Medicine Center of Cell and Gene Therapy gene vector laboratory, in accordance with good manufacturing practices (21 CFR210 and 211). The vector was characterized for purity and potency and approved for clinical use. The vector and dilution buffer were provided by the BCM. The viral particle/IU ratio was 20:1. Each viral particle contained 100 μl of vector at 2×10^{11} IU/ml (00496ATKA). Dilution buffer contained 10 mM Tris, 4% sucrose, and 2 mM $MgCl_2$. The virus was stored at − 80°C and before use diluted to the dose specified for each patient cohort. After the vector was delivered, *in vitro* cytotoxicity assay was performed using PC-3 human prostate cancer cells based on the assay protocol of the Baylor College of Medicine Center of Cell and Gene Therapy gene vector laboratory, and 50.6% cytotoxicity was recognized at 50 MOI of HSV-tk adenoviral vector plus GCV compared with 50 MOI of adenoviral vector plus phosphate-buffered saline. Thereafter, patients received a 1-h intravenous infusion of 5 mg/kg of GCV (Tanabe) every 12 h for a total of 2 weeks.

Course of treatment. Adv.HSV-tk was injected directly into the prostate in two escalating doses from 10^9 IU (three patients) to 10^{10} IU (six patients including one who was treated with low dosage). After the patient was anesthetized, the vectors (10^9 or 10^{10} IU) were thawed and immediately diluted with 1–2 ml of the buffer dependent on tumor volume. HSV-tk was injected into the prostate using a transrectal approach in the target lesion mapped by saturation biopsy (**Figure 3**). The procedure was carried out by an injection machine that can control injection speed and solution volume under ultrasound guidance, which showed the distribution of liquid medium throughout the target lesion. No significant extraprostatic extravasation was noted. Patients were given 2 weeks of intravenous 5 mg/kg GCV following the intraprostatic injection of HSV-tk. Each patient was carefully monitored for adverse events throughout the therapy. Toxicity was graded according to the Cancer Therapy Evaluation Program Common Toxicity Criteria (version 2.0) published by the National Cancer Institute.

Sample collection. Blood samples were collected before viral injection; 30, 60, and 90 min after viral injection; on days 1, 2, 3, 5, 7, and 14; and thereafter every 2 weeks. Urine samples were collected before viral injection; 30, 60, and 90 min after viral injection; and on days 1, 2, 3, 4, 5, 6, and 7 for PCR analysis of the distribution of Adv.HSV-tk sequence and gene expression. Biopsies of the target lesion for the detection of the *HSV-tK* gene were taken on day 28 from three patients, whose consent was obtained. However, we did not plan to obtain an additional biopsy core for immunohistological analysis for ethical reasons and the influence it may have on PSA values.

Monitoring viral DNA detection and neutralizing antibody. Adenoviral DNA in blood was determined by real-time PCR before viral injection; 30, 60, and 90 min after viral injection; and on days 1, 2, and 3. Gene expression in urine was measured PCR/real-time PCR as in the above schedule. PCR and real-time PCR were analyzed in a commercial-based laboratory (SRL, Tokyo, Japan). Neutralizing antibody titers were determined by neutralization tests in a commercial-based laboratory (SRL, Tokyo, Japan) on days 1, 14, and 28.

PSA monitoring and patient follow-up. PSA levels were monitored on days 7, 14, and 28, and every month thereafter. Blood count, serum hepatic enzymes, and creatinine measurements were performed on days 1, 2, 3, 5, 7, and 14 and at each hospital visit thereafter. Adverse events were monitored and recorded at the times of every visit.

Detection of* HSV-TK gene *in biopsy specimens. Whole cellular DNA of biopsy specimens from patients was extracted with a QIAamp tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. One microgram of cellular DNA from each sample was used for PCR analysis to determine the *HSV-tK* gene copy number. Deoxyoligonucleotide primers were as follows: sense CCCAGATAACAATAGGCATG and antisense GGTAAATAACGTGTCCCCGA. Each PCR cocktail preparation consisted of 10× PCR buffer (5 μl), deoxynucleoside triphosphates (10 mM each, 1 μl), 20 mM hsv-*TK* primer set (0.5 μl each), 25 mM $MgCl_2$ (4 μl), 20 mM *Taq* polymerase (0.5 μl), and H_2O (5.2–36.5 μl, depending on each sample). The final PCR volume was 50 μl including each cellular DNA. PCR conditions were as follows: 95°C for 2 min for one cycle; 95°C for 30 s, 56°C for 1 min, and 72°C for 2 min for 35 cycles. The product (10 μl) was loaded onto a 1.8% agarose gel, and a predicted 500 base pair product could be seen.

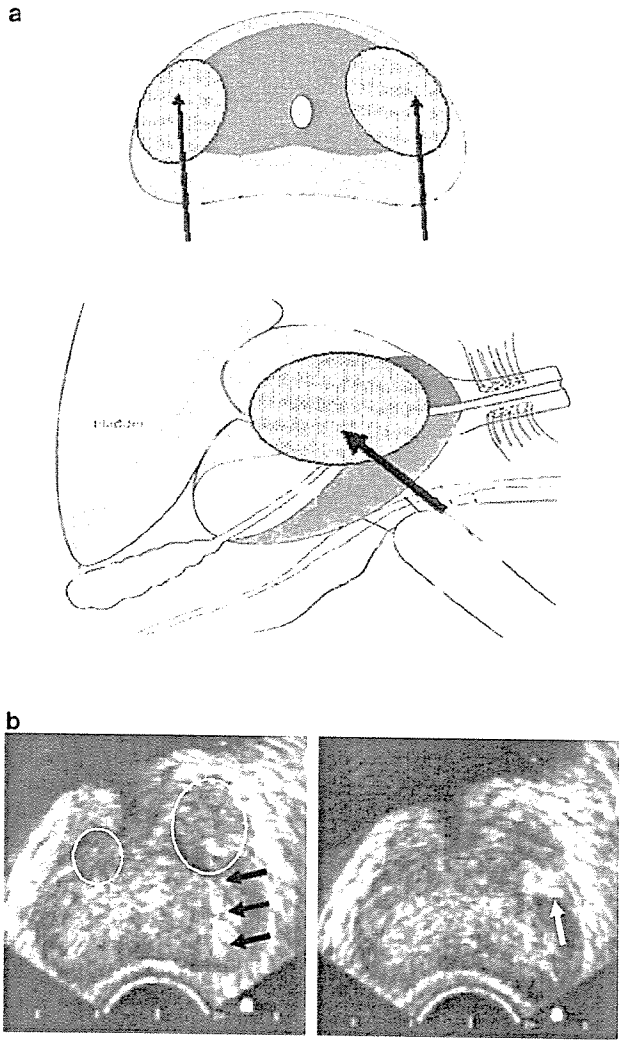

Figure 3 Vector injection. (**a**) Schema for cancer lesion and vector injection. Cancer lesion was confirmed by needle biopsy under transrectal ultrasound. Arrows show the site of vector injection. (**b**) Transverse view of prostate being injected with vector. White circles indicate cancer lesions. Black arrows show the injection needle. The white arrow shows the filtration of injection fluid into the cancer lesion.

Cytokine analysis. Serum levels of interferon-γ were measured using an established, commercially available enzyme immunoassay kit (SRL, Japan). Tumor necrosis factor-α, IL-6, IL-8, and IL-10 were measured using commercially available enzyme-linked immunosorbent assay kits (SRL, Japan). These cytokines were measured before viral injection and on days 7, 14, and 28.

Fluorescence-activated cell sorting analysis. Blood samples obtained before viral injection and on days 7, 14, and 28 were incubated with the following antibodies: CD4, CD8, CD11b, CD56, CD16, CD8/HLA-DR, and CD4/HLA-DR. After incubation at room temperature for 30 min, red blood cells were lysed with formic acid and the samples were fixed by paraformaldehyde. The immunofluorescence analysis was performed in a commercial-based laboratory (SRL, Japan). Analyses for CD4, CD8, CD11b, CD56, and CD16 were carried out in eight of nine treatments, and analyses for CD8/HLA-DR and CD4/HLA-DR were carried out in four of nine treatments.

Natural killer cell activity. The activity of natural killer cells obtained before viral injection and on days 7, 14, and 28 was determined by standard Cr^{51} releasing assay.

Statistical analyses. Statistical analyses were performed with Statview 4.0 (SAS Institute, Cary, NC). The *t*-test was used to evaluate differences in the PSADT.

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