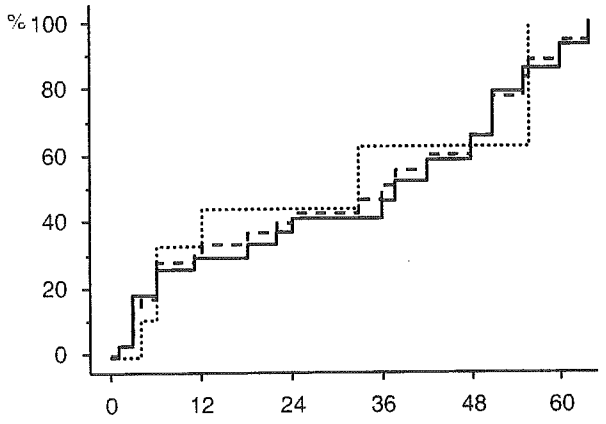
Table 1 Patients' characteristics

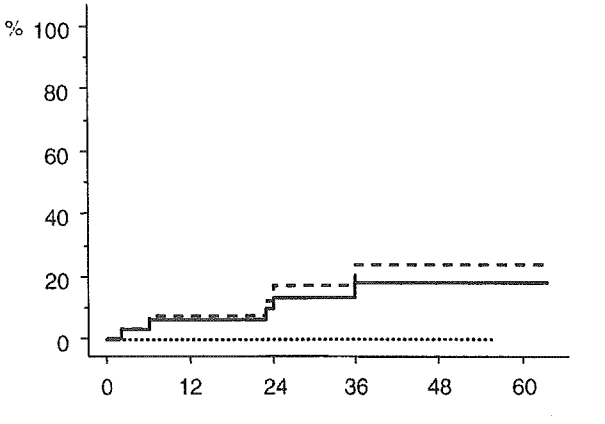
Preoperative erectile function	Overall	Normal	ED	*P*-value
Number of patients	48	36	12	
Median age (years, range)	66 (54–73)	65 (54–73)	68 (62–73)	0.016†
Median follow-up (months, range)	32 (3–66)	36 (8–62)	27 (3–66)	0.058†
Median PSA (ng/mL, range)	7.2 (1.4–37.0)	7.2 (2.1–37.0)	5.2 (1.4–15.1)	0.053†
Clinical stage				0.264‡
T1b (%)	3 (6.3)	3 (8.3)	0 (0)	
T1c (%)	25 (52.1)	20 (55.6)	5 (41.7)	
T2a (%)	14 (29.2)	8 (22.2)	6 (50.0)	
T2b (%)	6 (12.5)	5 (13.9)	1 (8.3)	
Median NPT (mm)	25 (0–50)	25 (15–50)	15 (0–45)	0.008†

†Mann–Whitney *U*-test; ‡Fisher's exact test. ED, erectile dysfunction; NPT, nocturnal penile tumescence (determined with nocturnal penile circumferential change by the erectometer); PSA, prostate-specific antigen.



Patients at risk	12 months	36 months	60 months
Overall: 36 patients	24 (33.3%)	12 (50.6%)	1 (94.3%)
Bilateral NS: 27 patients	19 (29.6%)	10 (46.7%)	1 (93.1%)
Unilateral NS: 9 patients	5 (44.4%)	2 (63.0%)	0 (100%)

Fig. 1 Recovery of any degree of erection following nerve-sparing-radical retropubic prostatectomy. The recovery rate of any degree of erection with time and patients at risk determined by the Kaplan-Meier Method. There was no statistically significant difference between bilateral and unilateral nerve-sparing groups (log-rank test $P = 0.761$).



Patients at risk	12 months	36 months	60 months
Overall: 36 patients	32 (5.6%)	16 (17.7%)	3 (17.7%)
Bilateral NS: 27 patients	23 (7.4%)	12 (23.1%)	3 (23.1%)
Unilateral NS: 9 patients	9 (0.0%)	4 (0.0%)	0 (0.0%)

Fig. 2 Recovery of sufficient erection to penetrate following nerve-sparing-radical retropubic prostatectomy. The recovery rate of sufficient erection to penetrate with time and patients at risk determined by the Kaplan-Meier Method. Recovery was seen only in the bilateral nerve-sparing group.

We assessed the recovery rate of erectile function following nerve-sparing RRP according to its quality by the Kaplan-Meier method. The overall estimated recovery rates of any degree of erection were 50.6% at 36 months and 94.3% at 60 months (Fig. 1). The rate of sufficient erection was 17.7% at 36 months without further improvement (Fig. 2). Sufficient erection to penetrate appeared only in the bilateral nerve-sparing group; however, as for any degree of erection, there was no statistically significant difference in recovery between bilateral and unilateral nerve-sparing.

The efficacy of sildenafil was determined in 13 patients with ED following nerve-sparing RRP (Table 2). Efficacy was found in nine patients (62.9%). Of the nine patients with normal erectile function before surgery, sildenafil was effective for six (66.6%): four were bilateral and two were unilateral nerve-sparing patients. In the four patients with bilateral nerve-sparing, sildenafil was effective at a dosage of 100 mg for two and 50 mg for two. For the two patients with the

Table 2 The efficacy of sildenafil for postoperative ED

Preoperative erectile function	Overall	Normal	ED
Number of patients	13	9	4
Mean age: years (range)	66.4 (57–73)	66.8 (62–71)	65.5 (57–73)
NS (bilateral/unilateral)	8/5	6/3	2/2
Efficacy of sildenafil (%)			
25 mg	1/2 (50.0)	0/1 (0.0)	1/1 (100)
50 mg	4/6 (66.7)	3/4 (75.0)	1/2 (50.0)
75 mg	1/1 (100.0)	1/1 (100.0)	–/– (00.0)
100 mg	3/4 (75.0)	2/3 (66.7)	1/1 (100)
Total	9/13 (69.2)	6/9 (66.7)	3/4 (75.0)

ED, erectile dysfunction; NS-RRP, nerve-sparing retropubic radical prostatectomy; NS, nerve-sparing.

unilateral procedure, sildenafil was similarly effective at a dosage of 100 mg and 50 mg. Of three men who showed no response to sildenafil, one patient with bilateral sparing spontaneously achieved erection sufficient to penetrate 24 months after RRP. Another patient with the bilateral procedure who did not respond to 100 mg sildenafil achieved sufficient erection with intracavernous injection of 20 μg of alprostadil. The other one with unilateral sparing did not request further treatment after failure with 25 mg of sildenafil.

For four patients with ED before surgery, sildenafil was effective in three (75%). For two patients with bilateral nerve-sparing, sildenafil was effective at the dosages of 25 mg and 50 mg, respectively. For one patient with the unilateral procedure, sildenafil was effective at a dosage of 100 mg. However, the drug was not effective at a dosage of 50 mg for the one remaining patient with unilateral nerve-sparing.

Discussion

We set out to examine functional erectile recovery following nerve-sparing RRP, the efficacy of sildenafil for ED following nerve-sparing RRP and the rationale of the surgery for preoperative ED patients.

Introduction of nerve-sparing has been reported to enable the preservation of erectile function. However, the outcomes reported previously varied widely (13.3–86%) for the maintenance rate of erectile function.[3,12,17] Reasons for the variations might be the reliability of cavernous nerve preservation, the difference in assessment procedures and period of ED following the surgery, as well as the definition of erection before and after surgery.

Walsh *et al.* reported that the recovery rate for erectile function was high, being 86% within 18 months following surgery.[17] Although it was also reported that the recovery rate at more than 6 months following surgery was 87.6% with any degree of erection, most of the erections were not sufficient in quality and the recovery rate of erection sufficient to penetrate was only 9.8%.[14] In the current study, although most patients noticed their erection, the rate of sufficient erection without sildenafil was low, being 17.7% at 36 months. That suggested the possibility of a lower recovery rate in terms of being 'sufficient to penetrate' without any aid, even following the nerve-sparing procedure.

A previous study suggested that the recovery rates for sufficient erectile function were 31.2% and 13.3% in patients with bilateral and unilateral nerve-sparing, respectively.[15] It seems that bilateral nerve-sparing provides a more favorable outcome than the unilateral type. In the current study, recovery of sufficient erection to penetrate was seen only in the bilateral nerve-sparing group. Indeed, this result suggested that the amount of preserved nerve fiber might be responsible for the more favorable recovery of the function. A recent basic experiment revealed that nerve injury induced apoptosis of smooth muscle, which led to volume loss of cavernous tissue and finally caused ED due to venous leakage.[18] The maintenance of the cavernous nerve is important for both nNOS-NO-cGMP pathway preservation and the amount of cavernous tissue. Thus, bilateral, rather than unilateral, nerve-sparing might be essential for preservation of sufficient erection for sexual intercourse without any aid. There are some reports on the efficacy of sildenafil for patients with ED after RRP.[5,19–21] The rates of efficacy of sildenafil were reported to be 71.7%, 50% and 15.4% with patients who underwent bilateral, unilateral and non-nerve-sparing RRP, respectively.[5] Although the assessment of the efficacy of sildenafil in the current study was preliminary due to the limited number of patients, sildenafil was effective for both nerve-sparing groups. These results suggest that at least unilateral nerve-sparing is crucial for the efficacy of

sildenafil. Again, maintenance of the nNOS-NO-cGMP pathway may be essential for both spontaneous functional recovery and the efficacy of sildenafil.

In the current study, sildenafil was effective postoperatively in three of four preoperative ED patients. Thus, a postoperative favorable effect of sildenafil might be expected for patients with preoperative ED if they have mild dysfunction and a good response to sildenafil before surgery. If sildenafil is effective in ED patients prior to RRP, nerve-sparing is also able to maintain the effectiveness postoperatively even in a unilateral nerve-sparing group. In the present study, we chose nerve-sparing RRP for preoperative ED patients when they strongly hoped for nerve-sparing during the detailed informed consent process before surgery. In the future, preoperative assessment for the response to sildenafil may be useful to select candidates for nerve-sparing surgery. In the PDE5 inhibitor era, this highly effective oral agent and nerve-sparing procedure play key roles in the QOL of RRP patients.

The current study has some limitations. The major limitation is the small number of patients. Additionally, the study is retrospective and the time of recovery of erectile function was not clearly determined. Furthermore, the questionnaire has not been completely validated. The reason we developed a new questionnaire was for the purpose of assessing the quality of erectile function regardless of sexual activity and the time when they recovered from ED. However, as far as we know, there is no report on the efficacy of sildenafil after nerve-sparing RRP for patients having ED preoperatively. The current results are encouraging for urologists and patients with ED before surgery. Additionally, we should confirm the efficacy of sildenafil for those who have ED and hope for nerve-sparing RRP in advance. A prospective large-scale trial with a validated questionnaire is crucial to reach a definite conclusion.

Conclusion

Sufficient erection without any aid was achieved only with bilateral nerve-sparing RRP in the current study. On the other hand, unilateral nerve-sparing did not lead to sufficient erection without sildenafil. However, sildenafil was effective for both nerve-sparing groups regardless of the preoperative erectile function.

References

1 Bacon CG, Giovannucci E, Testa M, Glass TA, Kawachi I. The association of treatment-related symptoms with quality-of-life outcomes for localized prostate carcinoma patients. *Cancer* 2002; **94**: 862–71.

2 Arai Y, Okubo K, Aoki Y *et al.* Patient-reported quality of life after radical prostatectomy for prostate cancer. *Int. J. Urol.* 1999; **6**: 78–86.

3 Miyao N, Adachi H, Sato Y *et al.* Recovery of sexual function after nerve-sparing radical prostatectomy or cystectomy. *Int. J. Urol.* 2001; **8**: 158–64.

4 Walsh PC, Mostwin JL. Radical prostatectomy and cystoprostatectomy with preservation of potency. Results using a new nerve-sparing technique. *Br. J. Urol.* 1984; **56**: 694–7.

5 Zippe CD, Jhaveri FM, Klein EA *et al.* Role of Viagra after radical prostatectomy. *Urology* 2000; **55**: 241–5.

6 Ogura K, Ichioka K, Terada N, Yoshimura K, Terai A, Arai Y. Role of sildenafil citrate in treatment of erectile dysfunction after radical retropubic prostatectomy. *Int. J. Urol.* 2004; **11**: 159–63.

7 NIH Consensus Conference. Impotence. NIH Consensus Development Panel on Impotence. *JAMA* 1993; **270**: 83–90.

8 Kato R, Sato Y, Horita H *et al.* [Validity analysis of Sapporo Medical University-sexual function questionnaire]. *Nippon Hinyokika Gakkai Zasshi* 1999; **90**: 872–7. (In Japanese.)

9 Suzuki K, Sato Y, Horita H *et al.* The correlation between penile tumescence measured by the erectometer and penile rigidity by the RigiScan. *Int. J. Urol.* 2001; **8**: 594–8.

10 Sobin LH, Fleming ID. TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer* 1997; 80: 1803–804.

11 Lange PH, Ercole CJ, Lightner DJ, Fraley EE, Vessella R. The value of serum prostate specific antigen determinations before and after radical prostatectomy. *J. Urol.* 1989; **141**: 873–9.

12 Rabbani F, Stapleton AM, Kattan MW, Wheeler TM, Scardino PT. Factors predicting recovery of erections after radical prostatectomy. *J. Urol.* 2000; **164**: 1929–34.

13 Quinlan DM, Epstein JI, Carter BS, Walsh PC. Sexual function following radical prostatectomy: influence of preservation of neurovascular bundles. *J. Urol.* 1991; **145**: 998–1002.

14 Kao TC, Cruess DF, Garner D *et al.* Multicenter patient self-reporting questionnaire on impotence, incontinence and stricture after radical prostatectomy. *J. Urol.* 2000; **163**: 858–64.

15 Geary ES, Dendinger TE, Freiha FS, Stamey TA. Nerve sparing radical prostatectomy: a different view. *J. Urol.* 1995; **154**: 145–9.

16 Gralnek D, Wessells H, Cui H, Dalkin BL. Differences in sexual function and quality of life after nerve sparing and nonnerve sparing radical retropubic prostatectomy. *J. Urol.* 2000; **163**: 1166–9; discussion 1169–70.

17 Walsh PC, Marschke P, Ricker D, Burnett AL. Patient-reported urinary continence and sexual function after anatomic radical prostatectomy. *Urology* 2000; **55**: 58–61.

18 User HM, Hairston JH, Zelner DJ, McKenna KE, McVary KT. Penile weight and cell subtype specific changes in a post-radical prostatectomy model of erectile dysfunction. *J. Urol.* 2003; **169**: 1175–9.

19 Feng MI, Huang S, Kaptein J, Kaswick J, Aboseif S. Effect of sildenafil citrate on post-radical prostatectomy erectile dysfunction. *J. Urol.* 2000; **164**: 1935–8.
20 Zagaja GP, Mhoon DA, Aikens JE, Brendler CB. Sildenafil in the treatment of erectile dysfunction after radical prostatectomy. *Urology* 2000; **56**: 631–4.
21 Lowentritt BH, Scardino PT, Miles BJ *et al.* Sildenafil citrate after radical retropubic prostatectomy. *J. Urol.* 1999; **162**: 1614–17.

Appendix

Questions for erectile function

1) Did you have sexual intercourse before surgery?
   1) never
   2) less than once a month
   3) once or twice a month
   4) once or twice a week
   5) more than three times a week
2) Did you notice erection regardless of sexual activity before surgery?
   1) never
   2) less than once a month
   3) once or twice a month
   4) once or twice a week
   5) more than three times a week
3) How firm was your erection? (except for the persons who answered 1 in question 2)
   1) Swollen but not firm at all
   2) Insufficient to penetrate
   3) Sufficient to penetrate but not satisfactory
   4) Very sufficient to penetrate
4) When did you notice any degree of erection after surgery?
   _____months after surgery
5) When was your erection sufficient to penetrate after surgery?
   _____months after surgery

Jpn J Clin Oncol 2005;35(3)158–161
doi:10.1093/jjco/hyi040

Cancer Genetics Report

Association of a Genetic Polymorphism of the *E-cadherin* Gene with Prostate Cancer in a Japanese Population

Toshiyuki Kamoto[1], Yoshiaki Isogawa[1], Yousuke Shimizu[1], Sachiko Minamiguchi[2], Hidefumi Kinoshita[1], Yoshiyuki Kakehi[3], Kenji Mitsumori[4], Shingo Yamamoto[1], Tomonori Habuchi[1], Tetsuro Kato[4] and Osamu Ogawa[1]

[1]Department of Urology and [2]Department of Clinical Pathology, Kyoto University Graduate School of Medicine, Kyoto, [3]Department of Urology, Kagawa University, Takamatsu and [4]Department of Urology, Akita Medical University, Akita, Japan

Received August 26, 2004; accepted January 8, 2005

The *E-cadherin* gene has been identified as having a physiological role in cellular attachment, and is hypothesized to participate in carcinogenesis. A polymorphism (an A to C substitution) in the 5′-untranslated region has a direct effect on *E-cadherin* gene transcriptional regulation. We explored the association between *E-cadherin* gene polymorphism and the risk of prostate cancer in a Japanese population. The subjects consisted of 236 patients with prostate cancer, 209 benign prostatic hyperplasia (BPH) patients and 139 male controls. A marginally significant difference was found between prostate cancer patients and male controls ($P = 0.053$). No significant difference was observed between prostate cancer and BPH patients. When patients with prostate cancer were divided into two groups, stage A+B and stage C+D, a significant difference was observed between progressive cancer patients (stage C+D) and male controls (odds ratio = 1.93, $P = 0.016$). It is possible that the presence of one A allele resulted in an increased risk of cancer progression.

Key words: E-cadherin – prostate cancer – single nucleotide polymorphism

INTRODUCTION

Prostate cancer is the most common malignancy in North American and European men, and represents a major public health challenge. Traditionally considered a disease of elderly men, a considerable proportion of cases now occur in men in their pre-retirement years. New means of identifying individuals at risk and strategies for early detection and preventive care are urgently needed.

There are some risk factors that have thus far been clearly established for prostate cancer, e.g. familial aggregation and ethnic origin (1,2). Many epidemiological studies have been conducted to evaluate the effects of environmental factors on the risk of prostate cancer in an attempt to explain the large ethnic variations in risk. However, no single environmental or lifestyle factor has consistently been associated with the risk of prostate cancer. Genetic polymorphisms of genes encoding for the androgen receptor, vitamin D receptor and cytochrome P450c17alpha enzyme have been associated with different degrees of risk for prostate cancer (3–5).

E-cadherin is a 120 000 MW glycoprotein that plays a critical role in many aspects of cell adhesion, epithelial development and the establishment and maintenance of epithelial polarity (6). Loss of E-cadherin expression and the subsequent loss of homotypic cellular adhesiveness may be a critical step allowing epithelial tumor cells to invade and metastasize. E-cadherin expression is decreased or absent in poorly differentiated carcinomas of the breast, prostate and many other tumors (7,8). In prostate cancer, it is possible that the loss of E-cadherin is related to tumor aggressiveness (8).

The association between E-cadherin abnormality and tumorigenesis has been previously reported. Guilford et al. (9) reported germline mutations in the *E-cadherin* gene in three familial gastric cancer kindreds of New Zealand Maori. Germline mutation of the *E-cadherin* gene has also been implicated in the pathogenesis of sporadic colorectal cancers (10,11).

There is a polymorphism (an A to C substitution) at −160 from the transcriptional start site of the *E-cadherin* gene promoter that might influence transcriptional efficacy (12). The present study was conducted to explore the association of this polymorphism of the *E-cadherin* gene with prostate cancer risk

For reprints and all correspondence: Osamu Ogawa, Department of Urology, Kyoto University Graduate School of Medicine, Shogoin Kawahara-cho 54, Sakyo-ku, Kyoto City, Kyoto 606-8507, Japan. E-mail: ogawao@kuhp.kyoto-u.ac.jp

in Japanese men. In addition, we set up a group of men with apparent benign prostatic hyperplasia (BPH), and another group of older men without any evidence of prostate cancer or BPH.

SUBJECTS AND METHODS

SUBJECTS

All subjects included in the study were unrelated Japanese. A total of 584 subjects with appropriate informed consent were studied. The subjects consisted of 236 prostate cancer patients who were diagnosed from 1995 to 2001, 209 BPH patients and 139 male controls who were recruited at Kyoto University Hospital and Akita University Medical Center. All of the prostate cancer patients were diagnosed histologically with specimens obtained from transrectal needle biopsy or transurethral resection of the prostate for voiding symptoms. All of the BPH patients had various degrees of lower urinary tract symptoms and an apparent prostatic enlargement by digital rectal examination. Serum PSA levels were measured in all BPH patients, as well as in men with elevated PSA levels (≥4.0 ng/ml by the Tandem-R assay; Hybritech Inc., San Diego, CA) who were shown not to have prostate cancer by transrectal sextant biopsies. The male control group comprised 139 volunteers over 65 years old without any voiding symptoms. They were mainly selected from patients admitted to several community hospitals because of various non-urological diseases. They all had normal serum PSA levels (<4.0 ng/ml by the Tandem-R assay), showed no signs of prostate cancer and had no prostatic enlargement by digital rectal examination. Serum PSA levels were measured using the Tandem-R assay in most cases. When serum PSA levels were measured by kits other than the Tandem-R, the measured PSA level was adjusted to that of the Tandem-R assay using a formula published by Kuriyama et al. (13). The mean ages (±SD) of prostate cancer patients, BPH patients and male controls were 72.2 ± 7.9, 69.8 ± 8.8 and 74.7 ± 6.9 years, respectively. There was no statistically significant difference in the mean age between these groups by unpaired two-tailed *t* test.

HISTOPATHOLOGICAL GRADING

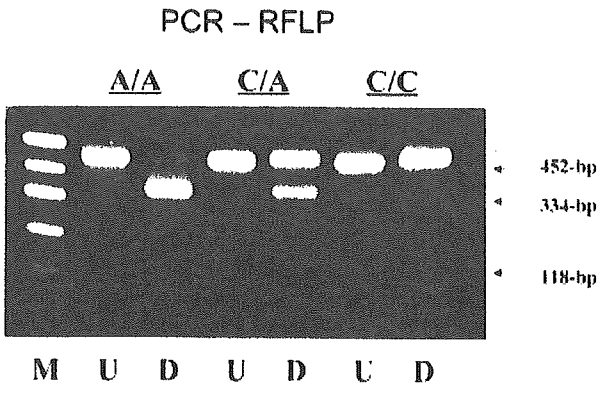
Pathological grading of the prostate cancers was determined according to the General Rule for Clinical and Pathological Studies of Prostate Cancer by the Japanese Urological Association and the Japanese Society of Pathology, which is based on the WHO criteria and the Gleason pattern (14). A pathologist (S.M.) reviewed prostate cancer specimens in order to determine Gleason Grade and WHO criteria. The clinical stage was determined by review of the medical records and was classified using the Whitmore–Jewett system. In localized cancer (stages A and B), the allele distributions of A/A, C/A and C/C in cases from Akita University were 1, 7 and 34 cases, respectively, and those from Kyoto was 4, 12 and 31 cases, respectively. In invasive cancer (stages C and D), the distributions were 1, 16 and 40, and 4, 29 and 39 cases from Akita and Kyoto, respectively. There was not enough information on 18 patients with prostate cancer who did not have their clinical stage determined and thus they were excluded from the analysis.

GENOTYPING OF E-CADHERIN GENE PROMOTER POLYMORPHISMS

DNA was extracted from blood samples collected from each subject using a QIAamp Blood Kit (QIAGEN, Germany) or by the standard method with proteinase K digestion followed by phenol/chloroform extraction. There is a polymorphism (an A to C substitution) at −160 from the transcriptional start site of the *E-cadherin* gene promoter. The A allele created an *Afl*III site and the C allele created a *Hph*I site. The 452 bp fragment encompassing either the *Afl*III or *Hph*I polymorphic site in the promoter region of the *E-cadherin* gene was amplified using the primers 5′-TTCTGATCCCAGGTCTTAGTGAGC-3′ and 5′-GGTACCTGCAGCAGCAGCAGCAG-3′. The 30 µl PCR mixture contained 1 µl of genomic DNA as a template, 10× PCR buffer (3 µl), 2.5 mM $MgCl_2$ (1.8 µl), 200 µM each dNTPs, 15 pmol of each primer and 0.2 U of AmpliTaq polymerase (Roche Molecular Systems). Thirty-three cycles of PCR were performed, with each cycle consisting of 94°C for 50 s, 65°C for 40 s and 72°C for 40 s. The 452 bp fragment was divided into 334 and 118 bp with *Afl*III-digestion, or into PCR products digested for at least 2 h with either *Hph*I or *Afl*III. The digestion reactions were fractioned on a 2% agarose gel. The genotypes were designated as 'A' when the *Afl*III restriction site was present and the *Hph*I site was absent, and as 'C' when the *Afl*III site was absent and the *Hph*I site was present (Fig. 1).

STATISTICAL METHODS

Associations between disease and genotype were assessed by calculating odds ratios (ORs) and 95% confidence intervals



U:undigested PCR product; D: digested with *Afl*III. M:size marker

A/A : 334bp+118bp C/A : 452bp+334bp+118bp C/C : 452bp

Figure 1. Representative PCR–RFLP pattern digested with *Afl*III. U, undigested PCR product; D, digested with *Afl*III; M, size marker.

Table 1. Frequencies [*n* (%)] of *E-cadherin* gene polymorphisms in patients with prostate cancer, patients with BPH and male controls

	E-cadherin gene genotype				Age-adjusted OR (95% CI)			
	A/A	A/C	C/C	A allele	Compared with BPH	*P* value	Compared with male control	*P* value
Prostate cancer	11 (4.7)	71 (30)	154 (65.3)	34.7%	1.22 (0.82–1.84)	0.329	1.61 (0.99–2.59)	0.053
Stage A,B*	5 (5.6)	19 (21.4)	65 (73.0)	27.0%	0.85 (0.49–1.50)	0.581	1.10 (0.60–2.04)	0.755
Stage C,D*	5 (3.9)	45 (34.9)	79 (61.2)	38.8%	1.47 (0.92–2.34)	0.107	1.93 (1.13–3.29)	0.016
BPH	6 (2.9)	56 (26.8)	147 (70.3)	29.7%				
Male control	5 (3.6)	29 (20.9)	105 (75.5)	24.5%				

*Eighteen patients were not informative and were therefore excluded.
OR, odds ratio; CI, confidence interval.

(CIs). *E-cadherin* genotype distribution in prostate cancer was compared with BPH and male controls. Multivariate logistic regression analysis was performed for each polymorphism alone in order to adjust for age. A probability of <0.05 was required for statistical significance.

RESULTS

Frequencies of the *E-cadherin* promoter genotype and allele types in the three groups (prostate cancer, BPH and male control) are shown in Table 1. The *E-cadherin* gene allelic distribution in each group was found by the Hardy–Weinberg equilibrium ($P > 0.05$).

The *E-cadherin* gene polymorphism was further analyzed to clarify the influence of the A allele by dividing it into two groups, i.e. the C/C genotype or the C/A+A/A genotypes. Because there was an extremely small frequency of A/A, we divided these groups according to the presence of the A allele. To adjust for age, multivariate logistic regression analysis was performed for each polymorphism alone. A marginally significant difference was found in prostate cancer patients and male controls ($P = 0.053$). No significant differences were observed between the prostate cancer and BPH patients. When we divided patients with prostate cancer into two groups, stage A+B and stage C+D, a significant difference was observed between progressive cancer (stage C+D) and male controls ($P = 0.016$). It is possible that having one A allele resulted in an increased risk of cancer progression (Table 1). We examined the distribution of *E-cadherin* promoter genotypes in prostate cancer patients by Gleason score (GS). The frequency of the genotype C/C was 75% in those with a GS <6, 56.6% with a GS = 7 and 59.1% with a GS >7. There was no statistically significant difference among these GS groupings. Indeed, in the prostate cancer patients, the frequency of at least one A allele being present was higher in a GS > 9 compared with a GS <6 (48.9% versus 25%; $P = 0.038$).

DISCUSSION

The *E-cadherin* gene has been identified as having a physiological role in cellular attachment, and several investigators have hypothesized that this gene participates in carcinogenesis (9,10,15). Abnormalities in the expression and cellular localization of E-cadherin are frequently associated with high tumor grading, infiltrative growth, and lymph node metastasis in a variety of human malignancies (7,8,16,17). For patients with prostate cancer, it is now well documented that decreased expression of E-cadherin is associated with a poor prognosis (8). The biological functional relationship between the loss of E-cadherin expression and the acquisition of invasive behavior has been described in several reports. Moreover, the restoration of an epithelial phenotype and a concomitant reduction in invasiveness after DNA-mediated transfection of E-cadherin has been reported by several investigators (18,19). These results suggest that *E-cadherin* gene abnormalities participate in several human malignancies, including carcinoma of the prostate. At present, it is not clear that the single nucleotide polymorphism (SNP) of this gene determines susceptibility to developing carcinomas as a common human disease.

Recently, the association of genetic polymorphism of the *E-cadherin* gene with prostate cancer has been reported (20–22). Verhage et al. (20) reported that the A-allele frequencies among 82 low grade prostate cancer cases consisted of 52 sporadic cases, and 25 familial cases and 188 controls were 39.0% and 24.7%, respectively. Thus, in their study population, A-allele carriers had an increased risk of prostate cancer, which was statistically significant (OR = 3.6; 95% CI 2.0–6.4). In our present study, the frequencies of the A allele were 34.7% in prostate cancer, 29.7% in BPH and 24.5% in male controls. Although our age-adjusted data did not support an association of the A allele with prostate cancer, we cannot exclude the possibility of a relationship between prostate cancer and this polymorphism.

On the other hand, however, we did find a significant association between the *E-cadherin* genotype and disease status in prostate cancer patients. Considering the function of E-cadherin intracellular attachment, it seems natural that the SNP of the *E-cadherin* gene promoter correlates with tumor invasiveness rather than tumorigenesis. In this study, there was a statistically significant difference between high stage prostate cancer (stage C+D) and male controls. It is possible that the presence of one A allele resulted in an increased risk of prostate cancer progression. Tsukino et al. (22) have recently reported that this E-cadherin polymorphism is not related to the incidence and progression of prostate cancer in the Japanese population. These previous results differ from our study. One of the reasons for this discrepancy might be a

difference in the setting of the control group. Tsukino et al. set the control as the age-matched general population. This may have included a number of people who had latent prostate cancer. On the other hand, in our study we set up two control groups. One group was a normal population without BPH with a serum PSA value of <4 ng/ml, while the other was those with BPH. In addition, the male volunteer controls were examined by both PSA test and digital rectal examination to confirm the absence of detectable prostate cancer.

Factors that affect susceptibility to prostate cancer include genetic factors, diet and environmental factors. There are many reports about the differences in prevalence of prostate cancer. Because there is so much variance in the prevalence of prostate cancer between races, especially Asians including Japanese and Caucasians, further studies on different racial groups with different prevalences of clinical prostate cancer are required to establish the association of *E-cadherin* gene polymorphism.

This genetic polymorphism seems to be correlated with a risk of developing prostate cancer. The polymorphism of the *E-cadherin* gene can then not only identify those at high risk for prostate cancer, but may become a marker to determine the clinical significance of the disease.

References

1. Carter BS, Beaty TH, Steinberg GD, Child B, Walsh PC. Mendelian inheritance of familial prostate cancer. *Proc Natl Acad Sci USA* 1992;89:3367–71.
2. Parkin DM, Pisani P, Frelay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999;80:827–41.
3. Giovannucci E, Stampfer MJ, Krithivas K, et al. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc Natl Acad Sci USA* 1997;94:3320–23.
4. Tomonori H, Suzuki T, Sasaki R, et al. Association of vitamin D receptor gene polymorphism with prostate cancer and benign prostatic hyperplasia in a Japanese population. *Cancer Res* 2000;60:305–8.
5. Tomonori H, Liqing Z, Suzuki T, et al. Increased risk of prostate cancer and benign prostatic hyperplasia associated with a CYP17 gene polymorphism with a gene dosage effect. *Cancer Res* 2000;60: 5710–13.
6. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991;251:1451–5.
7. Oka H, Shiozaki H, Kobayashi K, et al. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res* 1993;53:1696–701.
8. Umbas R, Schalken JA, Aalders TW, et al. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. *Cancer Res* 1992;52:5104–9.
9. Guilford P, Hopkins J, Harraway J, et al. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998;392:402–5.
10. Richards FM, McKee SA, Rajpar MH, et al. Germline E-cadherin gene (CDH1) mutations predispose to familial gastric cancer and colorectal cancer. *Hum Mol Genet* 1999;8:607–10.
11. Kim HG, Wheeler JMD, Kim JC, et al. The E-cadherin gene (CDH1) variants T340A and L599V in gastric and colorectal cancer patients in Korea. *Gut* 2000;47:262–7.
12. Li L, Chui RM, Sasaki M, et al. A single nucleotide polymorphism in the E-cadherin gene promoter alters transcriptional activities. *Cancer Res* 2000;60:873–6.
13. Kuriyama M, Akimoto S, Akaza H, et al. Comparison of various assay systems for prostate-specific antigen standardization. *Jpn J Clin Oncol* 1992;22:393–9.
14. Gleason DF. Classification of prostatic carcinomas. *Cancer Chemother Rep* 1966;50:125–8.
15. Berx G, Cleton-Jansen AM, Nollet F, et al. E-cadherin is a tumor/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J* 1995;14:6107–15.
16. Mayer B, Johnson JP, Leitl F, et al. E-cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res* 1993;53:1690–5.
17. Kinsella AR, Lepts GC, Hill CL, Jones M. Reduced E-cadherin expression correlates with increased invasiveness in colorectal carcinoma cell lines. *Clin Exp Metastasis* 1994;12:335–42.
18. Vleminkx K, Vakeat L, Mareel M, Fiers W, Van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 1991;66:107–19.
19. Frixen UH, Behrens J, Sachs M, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 1991;113:173–85.
20. Verhage BAJ, Houweligen K, Ruijter TE, Kiemeney LA, Schalken JA. Single-nucleotide polymorphism in the E-cadherin gene promoter modifies the risk of prostate cancer. *Int J Cancer* 2002;100:683–5.
21. Jonsson BJ, Adami HO, Hagglund M, et al. –160C/A polymorphism in the E-cadherin gene promoter and risk of hereditary, familial and sporadic prostate cancer. *Int J Cancer* 2004;109:348–52.
22. Tsukino H, Kuroda Y, Imai H, et al. Lack of evidence for the association of E-cadherin gene polymorphism with increased risk or progression of prostate cancer. *Urol Int* 2004;72:203–7.

ELSEVIER

ANDROGEN RECEPTOR, Ki67, AND p53 EXPRESSION IN RADICAL PROSTATECTOMY SPECIMENS PREDICT TREATMENT FAILURE IN JAPANESE POPULATION

TAKAHIRO INOUE, TAKEHIKO SEGAWA, TAIZOU SHIRAISHI, TORU YOSHIDA, YOSHINOBU TODA, TOMOMI YAMADA, NAOKO KINUKAWA, HIDEFUMI KINOSHITA, TOSHIYUKI KAMOTO, AND OSAMU OGAWA

ABSTRACT

Objectives. To evaluate multiple known prognostic markers in localized prostate cancer using tissue microarrays in Japanese patients. Molecular studies have suggested that ethnicity influences prostate tumor biology.

Methods. Specimens were studied from 52 patients who underwent radical surgery at our institution between 1997 and 2001 without neoadjuvant hormonal therapy and with three or more available and complete cancer spots. Ki67, p53, and androgen receptor antigen expression were examined. Immunohistochemical scores were compared with outcomes of chemical relapse as monitored using prostate-specific antigen.

Results. Pathologic tumor classification ($P = 0.047$), World Health Organization score ($P = 0.026$), World Health Organization histologic grade ($P = 0.026$), and surgical margin status ($P = 0.018$) were significant conventional clinicopathologic variables for predicting biochemical failure. The tissue microarray Gleason sum ($P = 0.038$), tissue microarray primary Gleason grade ($P = 0.013$), Ki67 labeling index ($P < 0.0001$), p53 ($P = 0.0097$), and androgen receptor ($P = 0.0113$) antigen expression also were significant. Moreover, surgical margin status and Ki67 labeling index were independently associated with treatment failure.

Conclusions. Especially together, the Ki67 labeling index and p53 and androgen receptor expression in localized prostate cancer often predicted postoperative progression in Japanese patients. UROLOGY **66:** 332–337, 2005. © 2005 Elsevier Inc.

Substantial differences exist in the incidence of clinical prostate cancer (PCa) among ethnic groups, with black Americans having a 10-fold and American whites a 5-fold greater incidence than Japanese men.[1,2] Despite the striking racial variation in the incidence of clinical PCa, the prevalence of latent carcinoma seems to be similar across populations, suggesting that promotion of microscopic carcinoma to clinically significant cancer differs substantially among these ethnic groups. Moreover, PCa risk increases for Japanese migrants to Hawaii and Japanese migrants to Los Angeles.[1,2] This epidemiologic evidence emphasizes that the incidence and progression of PCa is genetically and environmentally influenced. In fact, latent tumors in men from Japan showed more ras mutations than those from U.S. whites and blacks.[3] Additionally, a difference was noted between Japanese and Americans in the p53 mutational spectrum.[4] All these facts allow us to assess the risk of disease progression more accurately in Japanese patients with PCa to determine the appropriate treatment options better. We constructed a tissue microarray (TMA) of prostate carcinoma from Japanese patients with comprehensive clinical data and evaluated the significance of known

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

From the Department of Urology, Kyoto University Graduate School of Medicine, Kyoto, Japan; Department of Second Pathology, Mie University School of Medicine, Tsu, Japan; Anatomical Center for Kyoto University Graduate School of Medicine, Kyoto, Japan; Division of Medical Informatics, Kyusyu University Hospital, Kyusyu, Japan; Department of Urology, Kansai Medical University, Moriguchi, Japan

Reprint requests: Osamu Ogawa, M.D., Ph.D., Department of Urology, Kyoto University Graduate School of Medicine, 54 Kawaharacho, Shogoin, Sakyoku, Kyoto 606-8507, Japan. E-mail: ogawao@kuhp.kyoto-u.ac.jp

Submitted: November 2, 2004, accepted (with revisions): February 24, 2005

0090-4295/05/$30.00
doi:10.1016/j.urology.2005.02.028

prognostic markers such as Ki67,[5,6] p53,[7–9] and androgen receptor (AR).[10]

MATERIAL AND METHODS

Specimens and Clinicopathologic Features

The entry criteria for this retrospective cohort study to construct a PCa tissue array included clinically localized PCa resected between June 1997 and August 2001, whole mount tissue specimens processed by standard histologic methods and available at our institution, and PCa of sufficient size (greater than 5 mm) present in the specimens to be cored for TMAs. A total of 80 patients with PCa fulfilled these criteria, and their specimens were cored to produce TMAs. Among these 80 patients, 52 were enrolled in this study and had met the following criteria: no preoperative treatment, and at least three available cancer spots for evaluation of all molecular markers. All prostate specimens were processed by standard histologic methods.[11] All the specimens were graded by two of us (Taizou S. and Takahiro I.) independently according to the Gleason method and staged pathologically according to the 2002 American Joint Committee on Cancer TNM classification and the Japanese General Rules for Clinical and Pathological Studies on Prostate cancer (third edition). All clinical and pathologic data were obtained from the medical records. The clinicopathologic data are summarized in Table I. The date of failure was considered the time of the initial postoperative blood sampling yielding detectable prostate-specific antigen (PSA; 0.1 mg/mL or greater). When the serum PSA level after surgery did not decline to less than 0.1 ng/mL, the date of failure was defined as the time of surgery. The most recent date of follow-up was December 31, 2003. All the patients involved in this study provided informed consent before participating in this investigation.

TMA Construction

All samples were arrayed, as previously described, with some modifications.[12–15] Tissue cylinders 1.0 mm in diameter were punched from the selected areas of each block by a precision instrument (Beecher Instruments, Silver Spring, Md) and were embedded into paraffin blocks in a systematic fashion. For each case, three cores with the most undifferentiated or the main tumor area were selected and arrayed.

Immunohistochemistry

Standard indirect immunoperoxidase procedures using monoclonal antibodies were applied to detect Ki67 (1:100, MIB-1, DAKO, Kyoto, Japan), p53 (1:400, Do7, Novocastra, New Castle, UK), and AR (1:100, 2F12 Novocastra). Tumors with known positivity were used as positive controls for all antibodies. As negative controls, the primary antibodies were omitted. All slides were evaluated by two of us (Takehiko S. and Takahiro I.) independently. Nuclear staining was considered representative for Ki67 and p53, and both cytoplasmic and nuclear staining of epithelium was considered positive for AR. Therefore, we defined the Ki67 labeling index and p53 expression as the percentage of nuclear area stained with these antibodies. One to three nonoverlapping measurements, including the most intense staining area, were made at high magnification (×200) in each cancer spot. Each measurement included at least 100 epithelial cell nuclei, which resulted in counts including 300 to 1200 nuclei for each case. AR immunopositivity was graded semiquantitatively as weak (no staining or light immunostaining involving less than 10% of the epithelium), moderate (light-to-moderate immunostaining involving 10% to 50%), and strong (moderate-to-strong immunostaining involving more than 50%). Thereafter, we determined a score as follows: strong AR staining was scored as 3; moderate AR staining as 2; and weak AR staining as 1. To evaluate the prognostic value of each marker subjected to immunostaining, the mean Ki67 labeling index (sum of the labeling index of each cancer spot in each case divided by the total number of evaluated spots), maximal percentage of p53 expression (greatest percentage of expression among all cancer spots in each case), and mean AR staining score (sum of the AR score of each cancer spot in each case divided by the total number of evaluated spots) were determined and recorded for each case.

TABLE I. *Demographic and clinicopathologic features of 52 patients*

Feature	
Age (yr)	50–76 (68; 64, 72)
Preoperative PSA (ng/mL)	3.8–120 (9.95; 7.75, 17.15)
Follow-up (days)	727–2343 (1274; 1039, 1759)
Time to PSA failure (mo)	0–648 (157; 0, 415)
Pathologic T stage (n)	
pT2	14
pT3	38
Whole mount tissue Gleason sum (n)	
5	2
6	2
7	45
8	0
9	3
10	0
Primary Gleason score	
3	40
4	11
5	1
WHO score	
2	1
3	19
4	27
5	2
6	2
7	1
WHO histologic grade	
0	1
1	19
2	27
3	3
4	2

Key: PSA = prostate-specific antigen; WHO = World Health Organization.
Data presented as ranges, with median and 25th and 75th percentiles in parentheses, unless otherwise noted.

Statistical Analysis

The survival time from the date of prostatectomy to treatment failure or last follow-up was estimated using the Kaplan-Meier method. A log-rank test was used to examine the relationship between each molecular marker, histologic and clinical data, and PSA relapse-free survival. In multivariate analysis with a Cox proportional hazards model, a stepwise method was used to determine the parameters with the greatest influence on the risk of progression. Spearman's rank order correlation analysis was used to analyze the statistical signifi-

TABLE II. *Univariate analysis of time to PSA-defined treatment failure*

Factor Investigated	No. of Cases (No. of PSA Failure Cases)	*P* Value
Age (<70 vs. ≥70 yr)	34 (12) vs. 18 (8)	0.665
Preoperative PSA (<10 vs. ≥10 ng/mL)	26 (8) vs. 26 (12)	0.286
pT stage (<T3 vs. ≥T3)	14 (2) vs. 38 (18)	0.047
Gleason sum (<7 vs. ≥7)	4 (1) vs. 48 (19)	0.500
Primary Gleason score (<4 vs. ≥4)	40 (13) vs. 12 (7)	0.061
WHO score (<4 vs. ≥4)	20 (4) vs. 32 (16)	0.026
WHO histologic grade (<2 vs. ≥2)	20 (4) vs. 32 (16)	0.026
Surgical margin (positive vs. negative)	36 (18) vs. 16 (2)	0.018
Perineural invasion (positive vs. negative)	29 (13) vs. 23 (7)	0.196
TMA Gleason sum (<7 vs. ≥7)	17 (3) vs. 35 (17)	0.038
TMA primary Gleason grade (<4 vs. ≥4)	25 (4) vs. 27 (16)	0.001
p53 expression (positive vs. negative)	23 (13) vs. 29 (7)	0.0097
Ki67 LI (positive vs. negative)	24 (16) vs. 28 (4)	<0.0001
AR (high vs. low)	26 (14) vs. 26 (6)	0.0113

KEY: PSA = prostate-specific antigen; WHO = World Health Organization; TMA = tissue microarray; LI = labeling index; AR = androgen receptor.

cance of the correlations among the Ki67 labeling index, AR expression, and p53 expression in cancer spots. Statistical analysis was performed using StatView, version 5.0 (SAS Institute, Cary, NC). All *P* values were two-tailed, and $P < 0.05$ was considered to indicate significance.

RESULTS

Clinical and Histopathologic Parameters

The known clinical and pathologic variables of whole mount prostatic tissues were dichotomized. Table II summarizes the results of univariate analysis for disease-free survival.

Histopathologic Parameters and Molecular Markers in TMA

Both the TMA Gleason sum and TMA Gleason grade significantly influenced PSA-free survival ($P = 0.038$ and $P = 0.0013$, respectively).

In the 52 cases analyzed in this study, the range of the Ki67 labeling index was 0 to 34.3 (median 4.475, 25th percentile 2.38, 75th percentile 7.77). The percentage of p53 positive expression was 0% to 90% (median 11%, 25th percentile 2.165%, 75th percentile 23.67%). The range of the AR expression score was 1 to 3 (median 1.5, 25th percentile 1.0, 75th percentile 1.835). The labeling indexes for Ki67 and p53 expression were dichotomized according to a cutoff of 5 and 15, respectively, as previously reported.[3-4] AR immunostaining was dichotomized as high (mean AR score 1.6 or greater) vs. low (mean AR score less than 1.6). By univariate dichotomized log-rank analysis, the Ki67 labeling index ($P < 0.0001$), p53 ($P = 0.0097$), and AR ($P = 0.0113$) were significantly associated with PSA-defined disease-free survival (Table II and Fig. 1).

Multivariate Analysis of All Variables

In stepwise multivariate analysis with a Cox proportional hazards model, the surgical margin status ($P = 0.0263$, hazards ratio 5.299) and Ki67 labeling index ($P = 0.0004$, hazards ratio 7.517) were independent, significant determinants of PSA-defined disease-free survival.

COMMENT

This report is of an initial study of the utility of PCa TMAs in a Japanese population. Our study included a small number of patients. Nevertheless, according to the assumption that at a significance level of less than 5% and a power of 80%, we can detect a statistically significant difference between a good prognostic group with a 3-year PSA-free survival rate of 80% and a poor prognostic group with a 3-year PSA-free survival rate of 40% using 54 patients (27 patients for each group), we had a sufficient number of patients in our study at 52 patients. Hence, our sample number permitted adequate discrimination of the statistical significance between our subsets of analysis.

According to univariate analysis, the Gleason sum was not related to PSA failure, inconsistent with the findings of many other reports.[16,17] This might have been because the histologic results of most of our patients were Gleason sum 7 or less, resulting in an imbalance of numbers in each dichotomized category. Considering that the primary Gleason score was marginally related to PSA failure, if more patients were analyzed in future studies, the same results as in previous reports in terms of the relationship between the Gleason sum and PSA failure might be seen.

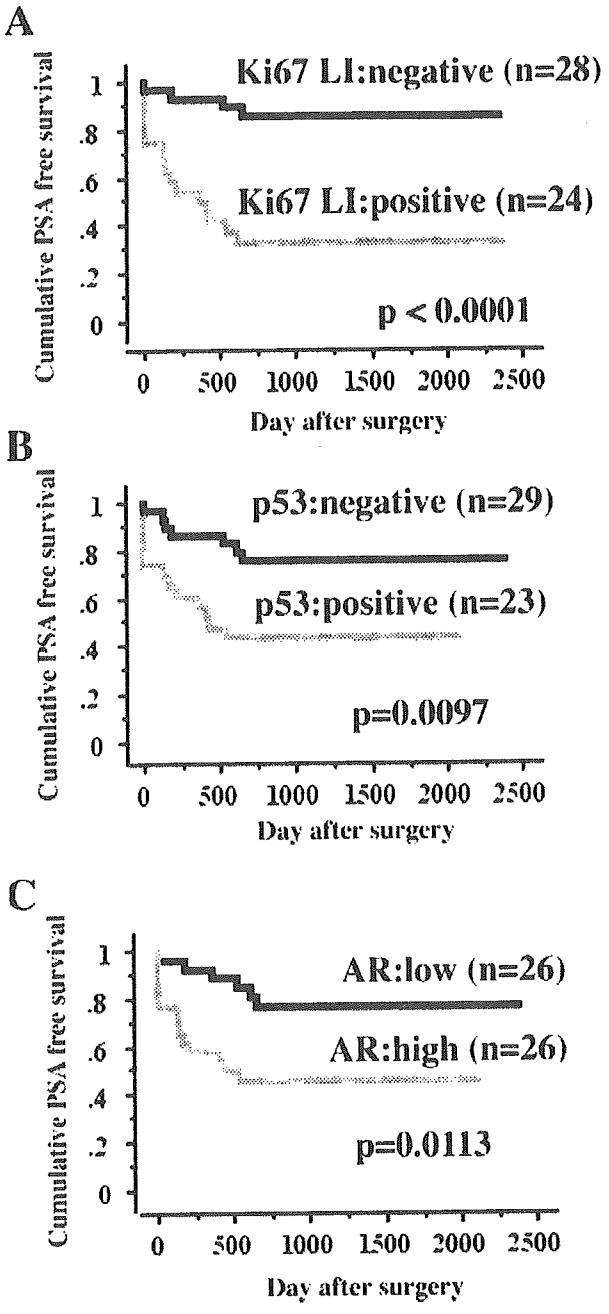


FIGURE 1. *Kaplan-Meier PSA-free survival curve according to (A) Ki67 labeling index (LI) (P <0.0001), (B) p53 expression (P = 0.0097), and (C) AR expression (P = 0.0113).*

On multivariate analysis, our data showed that surgical margin status and Ki67 labeling index were independent predictors of PSA failure after radical prostatectomy. Several other reports have also revealed that positive surgical margins are significantly associated with the risk of biochemical progression after radical prostatectomy.[18,19] Recently, Shuford *et al.*[20] reported that patients with capsular incision after radical prostatectomy were more likely to have biochemical recurrence than patients with pT2 and pT3 and negative surgical margins, even after adjusting for the effect of Gleason score, preoperative PSA level, and tumor volume. This finding shows that residual cancer at the prostatic bed is an adverse prognostic indicator. Therefore, there is no doubt that among the various conventional clinicopathologic factors, only surgical margin status remains as an independent factor after multivariate analysis. Positivity included both incision into the capsule by the surgeon and tumors extending to the edge of the specimen without adequate periprostatic tissue to provide a histologic diagnosis of extraprostatic extension.

The usefulness of the Ki67 labeling index as an independent prognostic marker has been confirmed in previous reports,[9,10,21] making this protein clinically applicable to PCa in Japanese, as well as other populations.

Our results showed p53 to be an important prognostic marker for patients with localized PCa treated by radical prostatectomy, in agreement with recent studies finding p53 protein immunohistochemical overexpression to have prognostic significance in PCa.[7–9] We used spots with a maximal percentage of p53 staining as representative of p53 staining for each case, because p53 overexpression is an aggressive feature of cancer.[7–9] Considering the mean percentage of p53 as representative staining, a relationship was found between the prognosis and the mean value, although this relationship was not statistically significant ($P = 0.11$, data not shown). In the present study, p53 nuclear staining was demonstrated in only 3 patients (21.4%) with less than Stage pT3 disease (Stage pT2 or less). Moreover, none of these 3 patients had PSA failure. Conversely, 20 patients (52.6%) demonstrated a positive p53 status with a tumor of Stage pT3 or greater. Among them, 13 patients (65%) had PSA failure. Therefore, p53 accumulation is associated with locally high advanced stage cancer. Moreover, considering that a reduction of wild-type p53 expression in an androgen-dependent cell line, LNCaP, induced androgen-independent proliferation, some PCa cells with nuclear p53 accumulation might pose androgen independence.[22] Although conflicting data have been reported regarding whether p53 status is prognostic in PCa, especially in the United States,[23] and some conflicts exist concerning analyzing p53 expression using TMAs,[24] we demonstrated the prognostic value of p53 staining in our TMAs of a Japanese population.

High AR protein expression predicted shorter disease-free survival in our investigation. Moreover, Ayala *et al.*[25] recently reported high AR expression in PCa to be associated with aggressive disease. Using the highest AR expression score as the representative of each case, we considered the

statistical analysis to be under-power for the imbalance of numbers of each category dichotomized. Therefore, in this study, we used the mean AR expression score as representative of each case, although a significant correlation was also found between the highest AR score and the prognosis ($P = 0.0464$, data not shown). Although the mechanisms underlying poor prognosis in AR overexpression are not fully understood, considering that the androgen/AR complex may regulate cell proliferation and survival of the prostate epithelium,[26] it is conceivable that high AR expression correlates with aggressive disease. Recently, Chen *et al.*[27] reported that AR overexpression induced cell proliferation at low androgen concentrations in androgen-dependent cell lines, which suggests that PCa cells with AR overexpression might be highly adaptable to a low androgen environment due to aging.[28] Moreover, this aggressiveness was confirmed by a significant positive relationship between AR immunostaining and p53 expression or the Ki67 labeling index in each of our TMA cancer spots (data not shown).

In this study, 1 (10%) of 10 patients developed recurrence with a negative Ki67 labeling index and positive p53 expression. Moreover, no patient developed recurrence with a negative Ki67 labeling index and positive AR expression. This might give some explanation of why only the Ki67 labeling index of the three molecular markers was an independent factor on multivariate analysis. Our multivariate analysis showed that Ki67 labeling index positivity was a consequence of earlier events involving other protein expression abnormalities such as p53 or AR overexpression. Nevertheless, examination of the other molecular markers together with the Ki67 labeling index might be desirable to delineate an intermediate risk group with longer follow-up.

CONCLUSIONS

We have demonstrated that our PCa TMA is useful for evaluating survival-related tissue markers. Furthermore, Ki67, p53, and AR expression in localized PCa is useful for predicting recurrence after radical prostatectomy. Admittedly the present study included fewer patients than many papers from Western countries, indicating a need for additional information with more markers in a large Japanese study population.

REFERENCES

1. Taylor JD, Holmes TM, and Swanson GM: Descriptive epidemiology of prostate cancer in metropolitan Detroit. Cancer 73: 1704–1707, 1994.

2. Oishi K, Yoshida O, and Schroeder FH: The geography of prostate cancer and its treatment in Japan. Cancer Surv 23: 267–280, 1995.

3. Shiraishi T, Muneyuki T, Fukutome K, *et al:* Mutations of ras genes are relatively frequent in Japanese prostate cancers: pointing to genetic differences between populations. Anticancer Res 18: 2789–2792, 1998.

4. Watanabe M, Ushijima T, Kakiuchi H, *et al:* p53 gene mutations in human prostate cancers in Japan: different mutation spectra between Japan and Western countries. Jpn J Cancer Res 85: 904–910, 1994.

5. Bubendorf L, Sauter G, Moch H, *et al:* Ki67 labeling index: an independent predictor of progression in prostate cancer treated by radical prostatectomy. J Pathol 178: 437–441, 1996.

6. Bubendorf L, Tapia C, Gasser TC, *et al:* Ki67 labeling index in core needle biopsies independently predicts tumor-specific survival in prostate cancer. Hum Pathol 29: 949–954, 1998.

7. Bauer JJ, Sesterhenn IA, Mostofi FK, *et al:* Elevated levels of apoptosis regulator proteins p53 and bcl-2 are independent prognostic biomarkers in surgically treated clinically localized prostate cancer. J Urol 156: 1511–1516, 1996.

8. Leibovich BC, Cheng L, Weaver AL, *et al:* Outcome prediction with p53 immunostaining after radical prostatectomy in patients with locally advanced prostate cancer. J Urol 163: 1756–1760, 2000.

9. Kuczyk MA, Serth J, Bokemeyer C, *et al:* The prognostic value of p53 for long-term and recurrence-free survival following radical prostatectomy. Eur J Cancer 34: 679–686, 1998.

10. Henshall SM, Quinn DI, Lee CS, *et al:* Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer. Cancer Res 61: 423–427, 2001.

11. Inoue T, Hioki T, Hayashi N, *et al:* Preoperative predictors of cancerous involvement of the neurovascular bundles in patients with localized prostate cancer. Int J Urol 9: 47–53, 2002.

12. Kononen J, Bubendorf L, Kallioniemi A, *et al:* Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4: 844–847, 1998.

13. Bubendorf L, Nocito A, Moch H, *et al:* Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol 195: 72–79, 2001.

14. Torhorst J, Bucher C, Kononen J, *et al:* Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 159: 2249–2256, 2001.

15. Rubin MA, Dunn R, Strawderman M, *et al:* Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol 26: 312–319, 2002.

16. Roehl AK, Han M, Ramos GC, *et al:* Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3478 consecutive patients: long-term results. J Urol 172: 910–914, 2004.

17. Han M, Partin WA, Zahurak M, *et al:* Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. J Urol 169: 517–523, 2003.

18. van den Ouden D, Bentvelsen FM, Boeve ER, *et al:* Positive margins after radical prostatectomy: correlation with local recurrence and distant progression. Br J Urol 72: 489–494, 1993.

19. Paulson DF, Moul JW, Walther JP, *et al:* Radical prostatectomy for clinical stage T1-2N0M0 prostatic adenocarcinoma: long-term results. J Urol 144: 1180–1184, 1990.

20. Shuford DM, Cookson SM, Chang SS, *et al:* Adverse prognostic significance of capsular incision with radical retropubic prostatectomy. J Urol 172: 119–123, 2004.

21. Keshgegian AA, Johnston E, and Cnaan A: Bcl-2 oncoprotein positivity and high MIB-1 (Ki-67) proliferation rate are independent predictive markers for recurrence in prostate carcinoma. Am J Clin Pathol 110: 443–449, 1998.

22. Burchardt M, Burchardt T, Shabsigh A, *et al:* Reduction of wild-type p53 function confers a hormone resistant phenotype on LNCaP prostate cancer cell. Prostate 48: 225–230, 2001.

23. Brooks DJ, Bova SG, Ewing MC, *et al:* An uncertain role for p53 gene alterations in human prostate cancers. J Urol 56: 3814–3822, 1996.

24. Merseburger AS, Kuczyk MA, Serth J, *et al:* Limitations of tissue microarrays in the evaluation of focal alterations of bcl-2 and p53 in whole mount derived prostate tissues. Oncol Rep 10: 223–228, 2003.

25. Ayala G, Li R, Weigel N, *et al:* High levels of androgen receptor in PCA is associated with aggressive disease and is an independent predictor of survival. Am J Surg Pathol 28: 928–934, 2004.

26. Isaacs JT: Antagonistic effect of androgen on prostatic cell death. Prostate 5: 545–557, 1984.

27. Chen DC, Welsbie SD, Tran C, *et al:* Molecular determinants of resistance to antiandrogen therapy. Nat Med 10: 33–39, 2003.

28. Iwamoto T, Yanase T, Koh E, *et al:* Reference ranges of serum total and free testosterone in Japanese male adults. Jpn J Urol 95: 751–760, 2004.

Prevention of Cancer Cachexia by a Novel Nuclear Factor κB Inhibitor in Prostate Cancer

Kenji Kuroda,[1] Yutaka Horiguchi,[1] Jun Nakashima,[1] Eiji Kikuchi,[1] Kent Kanao,[1] Akira Miyajima,[1] Takashi Ohigashi,[1] Kazuo Umezawa,[2] and Masaru Murai[1]

Abstract

Purpose: To investigate the association between serum interleukin-6 (IL-6) and cachexia in patients with prostate cancer and the inhibitory effect of a new nuclear factor κB (NF-κB) inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), on IL-6 production and cachexia in an animal model of hormone-refractory prostate cancer.

Experimental Design: The association between serum IL-6 levels and variables of cachexia was evaluated in 98 patients with prostate cancer. The inhibitory effects of DHMEQ on IL-6 secretion and cachexia were investigated in *in vitro* and *in vivo* studies using JCA-1 cells derived from human prostate cancer.

Results: Serum IL-6 levels were significantly elevated and cachexia developed in JCA-1 tumor-bearing mice as well as in prostate cancer patients with progressive disease. IL-6 secretion was significantly inhibited in JCA-1 cells exposed to DHMEQ. Intraperitoneal administration of DHMEQ (8 mg/kg) to tumor-bearing mice produced a significant amelioration of the reduction in body weight, epididymal fat weight, gastrocnemius muscle weight, hematocrit, and serum levels of triglyceride and albumin when compared with administration of DMSO or no treatment. DHMEQ caused a significant decrease of serum IL-6 level in JCA-1 tumor-bearing mice (all $P < 0.05$).

Conclusions: These results suggested an association between serum IL-6 and cachexia in patients with prostate cancer and in JCA-1 tumor-bearing mice and that a new NF-κB inhibitor, DHMEQ, could prevent the development of cachexia in JCA-1 tumor-bearing mice presumably through the inhibition of IL-6 secretion. DHMEQ seems to show promise as a novel and unique anticachectic agent in hormone-refractory prostate cancer.

Cancer cachexia, which features the loss of muscle and fatty tissue as well as anorexia, asthenia, and anemia (1, 2), makes therapeutic intervention difficult (3) and is an important cause of death in cancer patients (4). Although little is known about the detailed mechanisms of cachexia, recent studies have revealed that inappropriate production and release of cytokines such as interleukin-6 (IL-6) is involved in the induction of cachexia (5–8). Progressive prostate cancer is often associated with anorexia, weight loss, and accelerated malnutrition that lead to cachexia, even if metastases are confined to the bones. It has been reported that human prostate cancer cells produce IL-6 and that the serum level of IL-6 is elevated in patients with prostate cancer (9, 10). However, few studies have investigated the association between the serum IL-6 level and cachexia in patients with prostate cancer. Production of IL-6 is regulated by several transcription factors, among which nuclear factor κB (NF-κB) is one of the pivotal regulators of cytokine-inducible gene expression (11). Schwarz et al. (12) have suggested that suppression of NF-κB may result in the amelioration of cachexia in a mouse tumor model. It is also well known that NF-κB shows often constitutive activation in hormone-refractory prostate cancer cells (13, 14). As far as we know, no investigators have explored a treatment strategy for cachexia based on the regulation of NF-κB by administration of a compound synthesized from a natural product. Recently, we have investigated the effectiveness of a new NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), which is a 5-dehydroxymethyl derivative of epoxyquinomicin C that shows anti-NF-κB activity in cultured human leukemia Jurkat cells and inhibits type II collagen–induced rheumatoid arthritis in mice (15). The present study was undertaken to evaluate the association between IL-6 and cachexia in patients with prostate cancer, as well as the inhibitory effect of DHMEQ on IL-6 production and cachexia in an animal model of hormone-refractory prostate cancer.

Materials and Methods

Patients. The association between serum IL-6 and cachexia in patients with prostate cancer was evaluated in this retrospective study. Ethics approval was obtained from our institutional ethics committee. Ninety-eight archival serum samples from patients with histologically

Authors' Affiliations: [1]Department of Urology, Keio University, School of Medicine, Tokyo, Japan and [2]Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan

Received 12/11/04; revised 4/23/05; accepted 5/9/05.

Grant support: Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan; Sato Memorial Foundation for Cancer Research; and Japanese Foundation for Multidisciplinary Treatment of Cancer.

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Requests for reprints: Jun Nakashima, Department of Urology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: 81-3-5363-3825; Fax: 81-3-3225-1985; E-mail: njun@sc.itc.keio.ac.jp.

confirmed prostate cancer were examined. There were 55 patients with untreated disease, 23 patients in remission after endocrine therapy, and 20 patients with relapse. The definitions of remission and tumor progression were previously reported (8). Remission included any of the following: (*a*) reduction or disappearance of tumor masses; (*b*) a decrease in the number, size, or intensity of lesions on successive bone scans; or (*c*) a significant decrease of serum prostate-specific antigen. In addition, there had to be no new lesions and no deterioration of symptoms or performance status. Any of the following events was considered evidence of tumor progression: (*a*) the appearance of any new metastasis; (*b*) an increase in the number, size, or intensity of lesions on successive bone scans; or (*c*) significant cancer-related deterioration of symptoms or performance status. Classification of the patients with prostate cancer was done in accordance with Modified Jewett Staging System (16). Serum levels of IL-6 were measured using the Quantikine enzyme-linked immunoadsorbent assay kit (R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. Laboratory tests included analysis of serum albumin and hematocrit. Performance status was assessed in accordance with the Eastern Cooperative Oncology Group scale, in which 0 indicated that the patient had no symptoms; 1, the patient had symptoms but was ambulatory; 2, the patient was bedridden less than half the day; 3, the patient was bedridden half the day or longer; and 4, the patient was chronically bedridden and required assistance with activities of daily living. Body mass index (BMI) was calculated by the following formula: weight (kg) / height^2 (m^2).

Cell line. JCA-1 cells derived from human prostate cancer (17) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin (Life Technologies, Inc., Grand Island, NY), and 100 IU/mL penicillin (Life Technologies).

Chemicals. DHMEQ was synthesized in our laboratory (15). We also referred to the article by Suzuki et al. (18) in which the molecular structure was shown. It was dissolved in DMSO to prepare a 10 mg/mL solution and was subsequently diluted in culture medium to a final DMSO concentration of <0.1%.

In vitro *interleukin-6 assay.* JCA-1 cells (1×10^5) were seeded in a total volume of 1 mL of medium in each well of 24-well tissue culture plates and allowed to grow overnight. Then cells were treated with 1.0 or 1.5 μg/mL of DHMEQ, whereas other cells treated with the same concentrations of DMSO served as controls. After 48 hours of incubation, the supernatant of each well was collected and stored at −80°C until assay, and the number of viable cells was determined by trypan blue dye exclusion. The IL-6 concentration was measured using an enzyme immunoassay specific for human IL-6 (R&D Systems QuantiGlo Human IL-6 Immunoassay kit) according to the instructions of the manufacturer.

Animal model. All procedures involving animals and their care in this study were approved by the animal care committee of our institution in accordance with institutional and Japanese government guidelines for animal experiments. Male Balb/C-nu/nu mice were obtained from Sankyo Lab Service Corp. (Tokyo, Japan). The mice were housed at a constant temperature and humidity and received a standard diet and water. JCA-1 cells (1×10^7) were inoculated s.c. into the right flank of each mouse. When the tumors reached ~ 10 mm in diameter, mice were randomly assigned to three groups. DHMEQ (8 mg/kg) was administered i.p. in a volume of 0.2 mL once daily for 25 days to group 2 ($n = 12$). This group was labeled Tumor (+), DHMEQ. To clarify the effect of the vehicle, the same dose of DMSO given to group 2 was injected in another group of mice (group 3, $n = 16$), and this group was labeled Tumor (+), DMSO, and another group of tumor-bearing mice was observed without any treatment [group 4, $n = 11$; this group was labeled Tumor (+), No drug]. As a healthy control, age-matched mice were observed without any treatment [group 1, $n = 14$; this group was labeled Tumor (−), No drug]. During the treatment period, mice were carefully monitored and body weight was measured every other day. At the time of sacrifice, the tumor, gastrocnemius muscle, and epididymal fat were dissected and weighed. Blood samples were collected into nonheparinized tubes, and serum was separated within 1 hour of sacrifice. The serum samples were stored at −80°C and thawed just before testing. Serum IL-6 activity was determined using an enzyme immunoassay specific for human IL-6 (R&D Systems QuantiGlo Human IL-6 Immunoassay kit) according to the instructions of the manufacturer. At the same time, the hematocrit and the serum levels of triglycerides and albumin were also measured in each mouse.

Statistical analysis. All values are expressed as the mean ± SE. Variables for different groups were compared using Student's *t* test or ANOVA; $P < 0.05$ was considered statistically significant.

Results

The serum IL-6 level of 20 patients with relapse was significantly higher than that of 55 untreated patients or that of 23 patients in remission (Table 1). Serum levels of prostate-specific antigen were significantly higher in patients with relapse than in untreated patients or patients with remission. Serum albumin levels, hematocrit, and BMI were significantly lower in patients with relapse than in untreated patients or patients in remission (Table 1). The performance status was also significantly worse in patients with relapse when compared with untreated patients or patients in remission (Table 1). The serum albumin level and hematocrit were significantly lower (all $P < 0.05$) in patients with serum IL-6 level ≥ 7 pg/mL than in patients with serum IL-6 level < 7 pg/mL (data not shown). BMI was significantly lower in patients with serum IL-6 level ≥ 7 pg/mL (19.38 ± 0.41 kg/m^2) than in patients with serum IL-6 level < 7 pg/mL (22.94 ± 0.28 kg/m^2; $P < 0.0001$; Fig. 1).

The IL-6 level in the culture medium of JCA-1 cells treated with DHMEQ at 1.0 or 1.5 μg/mL for 48 hours was 4.03 ± 0.35 and 2.98 ± 0.17 pg/mL/10^5 cells, respectively, being significantly lower than when cells were treated with DMSO alone (10.54 ± 3.73 pg/mL/10^5 cells; $P = 0.031$ and $P = 0.015$, respectively; Fig. 2).

When mice had developed tumors ~ 10 mm in diameter after inoculation of JCA-1 cells, treatment was initiated and the day when treatment was started was designated as day 0. At the end of experiments, the mean weight of tumors of DHMEQ-treated mice was 3.02 ± 0.54 g, which was smaller than that of DMSO-treated mice (4.16 ± 0.81 g) and untreated mice (4.15 ± 1.10 g), but the differences were not statistically significant. The body weight, epididymal fat weight, gastrocnemius muscle weight, hematocrit, and serum levels of triglyceride and albumin were significantly lower in untreated JCA-1 tumor-bearing mice (group 4) than in healthy control mice without tumors (group 1), and serum IL-6 levels were significantly elevated in group 4 mice at the time of sacrifice (Figs. 3 and 4; Table 2). Although the body weight of untreated tumor-bearing mice and tumor-bearing mice treated with DMSO decreased in a time-dependent manner, the weight of JCA-1 tumor-bearing mice treated with DHMEQ did not decline significantly (Fig. 3). On day 26, body weight (28.24 ± 1.44 g), epididymal fat weight (197.11 ± 31.67 mg), and gastrocnemius muscle weight (499.27 ± 30.26 mg) were significantly greater in DHMEQ-treated mice (group 2) than in mice treated with DMSO alone (group 3; 24.09 ± 1.30 g, 117.12 ± 19.10 mg, and 306.28 ± 24.46 mg; $P = 0.018$, $P = 0.044$, and $P < 0.001$, respectively) or untreated mice (group 4; 21.46 ± 1.08 g, 43.48 ± 2.97 mg, and 261.13 ± 14.54 mg; $P = 0.002$, $P = 0.001$, and $P < 0.001$, respectively; Table 2).

In addition, the hematocrit (41.89 ± 1.54%) and the serum triglyceride level (60.63 ± 7.36 mg/dL) were significantly higher in group 2 mice than in group 3 mice (36.71 ± 1.81% and

Table 1. Disease status and variables

	No. patients	Serum levels of IL-6 (pg/mL)		Rate of serum IL-6 levels of ≥7 pg/mL (%)	Prostate-specific antigen (ng/mL)	Albumin (g/dL)	Hematocrit (%)	BMI (kg/m^2)	Performance status
		Range	Mean ± SE		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Untreated patients									
Total	55	0.69-111.06	5.21 ± 2.06	10.9 (6/55)	171.09 ± 58.73	3.58 ± 0.06	36.26 ± 0.70	22.74 ± 0.31	0.75 ± 0.14
Stage A	3	0.86-1.6	1.20 ± 0.22	0 (0/3)	2.27 ± 0.71	3.57 ± 0.27	41.73 ± 3.35	20.97 ± 1.54	0
Stage B	15	0.69-5.65	2.45 ± 0.40	0 (0/15)	42.52 ± 19.18	3.57 ± 0.09	38.27 ± 1.26	22.21 ± 0.46	0.33 ± 0.13
Stage C	11	1.07-7.45	2.16 ± 0.45	9.1 (1/11)	50.94 ± 14.90	3.76 ± 0.06	38.16 ± 1.07	23.18 ± 0.65	0.09 ± 0.09
Stage D	26	1.08-111.06	8.55 ± 4.30	19.2 (5/26)	315.57 ± 118.37	3.52 ± 0.11	33.67 ± 0.94	23.05 ± 0.50	1.35 ± 0.22
Patients with remission as a result of endocrine therapy									
	23	0.26-4.77	2.45 ± 0.26	0 (0/23)	79.34 ± 53.48	3.78 ± 0.09	36.60 ± 1.03	23.12 ± 0.59	0.48 ± 0.16
Patients with relapsed bone metastatic disease after endocrine therapy									
	20	4.46-135.53	41.58 ± 8.07	85.0 (17/20)	14.35 ± 1210.34	2.78 ± 0.08	31.00 ± 1.37	18.99 ± 0.32	2.83 ± 0.19

NOTE: The untreated patients were separated into subgroups of stage A to D in accordance with Modified Jewett Staging System. Performance status was assessed in accordance with the Eastern Cooperative Oncology Group scale.

36.50 ± 6.16 mg/dL; $P = 0.018$ and $P = 0.020$, respectively) or group 4 mice (34.70 ± 1.80% and 32.00 ± 2.91 mg/dL; $P = 0.005$ and $P = 0.007$, respectively). The serum level of albumin was significantly higher in group 2 (2.04 ± 0.07 g/dL) than in group 4 (1.77 ± 0.08 g/dL; $P = 0.019$; Table 2). The serum IL-6 level of group 2 (238.83 ± 72.59 pg/mL) was significantly lower than that of group 3 (1,009.51 ± 316.35 pg/mL; $P = 0.030$) or group 4 (1,312.09 ± 368.66 pg/mL; $P = 0.006$; Fig. 4).

Discussion

It has been reported that elevation of the serum levels of IL-6 is strongly associated with cachexia in patients with various types of advanced cancer. Serum cytokine levels are increased in prostate cancer patients who have weight loss when compared with those who show no weight loss (8, 19). In the present study, prostate cancer patients with relapse showed more severe cachexia and had higher serum IL-6 levels than untreated patients or patients in remission. In addition, the prostate cancer patients with higher serum IL-6 levels were more cachectic than those with lower IL-6 levels. Wallenius et al. investigated the effect of IL-6 on the physique in mice lacking the gene encoding IL-6 ($Il6^{-/-}$ mice) and found that they developed mature-onset obesity that was partly reversed by IL-6 replacement. Taken together, these results suggest a strong relationship between the serum level of IL-6 and weight loss (20). In the present study, DHMEQ produced a significant decrease in the IL-6 level and a significant improvement in the body weight of tumor-bearing mice.

Alexandrakis et al. (21) showed that IL-6 was significantly higher and hemoglobin was significantly lower in patients with multiple myeloma than in the controls, and a significant decrease in hemoglobin concentration and hematocrit was also found in patients with higher serum IL-6 levels. Ishiko et al.

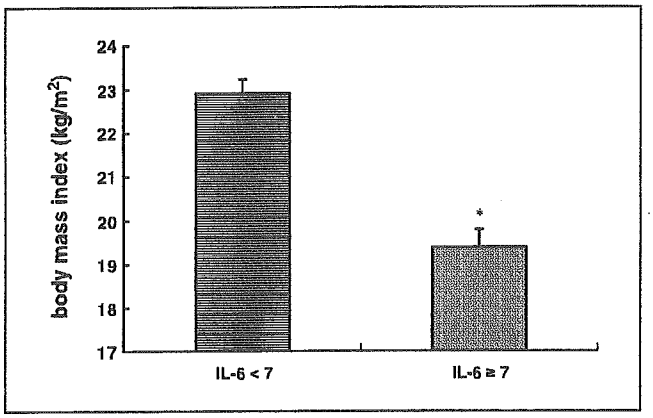

Fig. 1. Relationship between IL-6 and BMI. BMI was significantly lower in patients with serum IL-6 level ≥ 7 pg/mL (19.38 ± 0.41 kg/m^2) than in patients with serum IL-6 level < 7 pg/mL (22.94 ± 0.28 kg/m^2; $P < 0.0001$). *, significantly different from the mean value of patients with serum IL-6 level < 7 pg/mL.

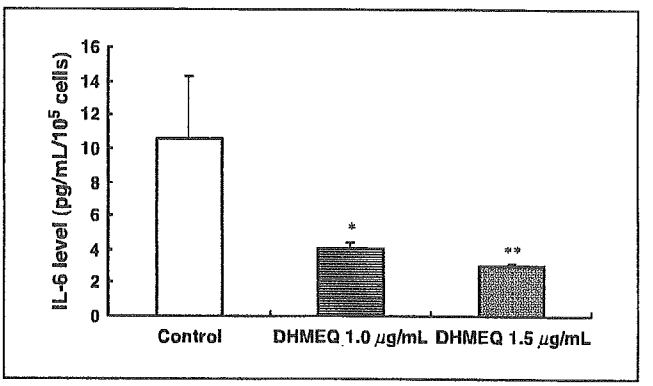

Fig. 2. Effect of DHMEQ on IL-6 secretion by JCA-1 cells. At concentrations of 1.0 and 1.5 μg/mL DHMEQ significantly inhibited IL-6 secretion by JCA-1 cells. *Columns*, mean value of samples; *bars*, SE. *, significantly different from control (DMSO alone; $P = 0.031$). **, significantly different from control (DMSO alone; $P = 0.015$).

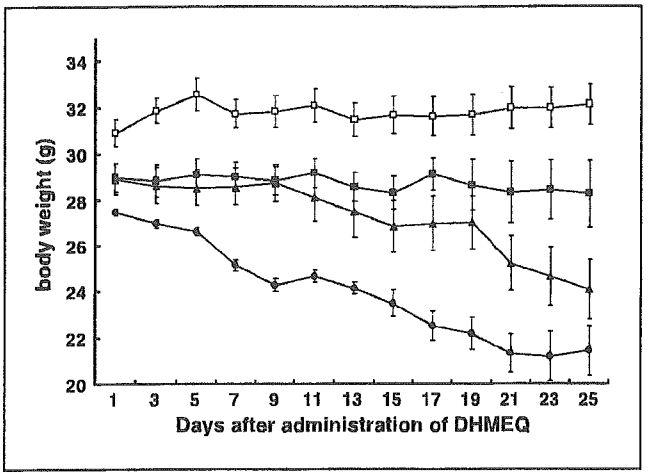

Fig. 3. Effect of DHMEQ on body weight. JCA-1 cells (1×10^7) were inoculated s.c. into the right flank of each mouse. When palpable tumors had developed (largest diameter >10 mm), the mice were randomly assigned to three groups. Then DHMEQ (8 mg/kg) was administered i.p. once daily for 25 days to one group of mice [■, Tumor (+), DHMEQ; $n = 12$], DMSO (the vehicle for DHMEQ) was given to another group [▲, Tumor (+), DMSO; $n = 16$], and no drug was given to another group [●, Tumor (+), No drug; $n = 11$]. The group Tumor (−), No drug were healthy control mice with no tumors and no treatment (□; $n = 14$). *Points,* mean body weight of mice per group; *bars,* SE. Body weight of group Tumor (+), DMSO was statistically significantly lower at day 23 ($P = 0.032$) and day 25 ($P = 0.018$) than that of group Tumor (+), DHMEQ, and body weight of group Tumor (+), No drug was statistically significantly lower at each day than that of group Tumor (+), DHMEQ (all $P < 0.05$).

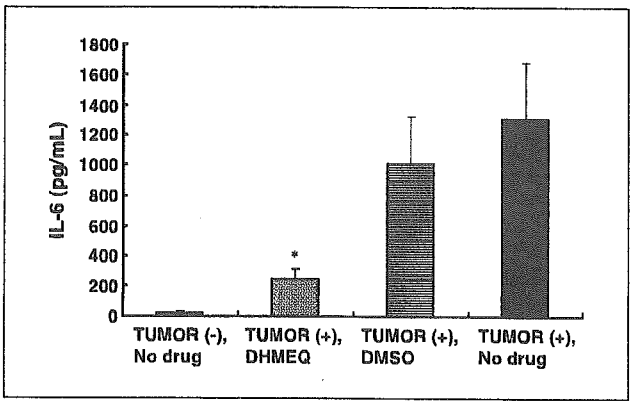

Fig. 4. Effect of DHMEQ on serum IL-6 level. Serum IL-6 levels were significantly lower in Tumor (+), DHMEQ mice than in Tumor (+), DMSO mice or Tumor (+), No drug mice. *Columns,* mean IL-6 levels of samples; *bars,* SE. *, significantly different from Tumor (+), DMSO ($P = 0.030$) and Tumor (+), No drug ($P = 0.006$).

(22) showed that there is severe anemia in cancer-bearing rabbits, whereas the mean hemoglobin value of normal rabbits was much higher. In our study, the serum IL-6 level was significantly higher and hematocrit was significantly lower in JCA-1 tumor-bearing mice than in healthy control mice, and DHMEQ significantly prevented the development of anemia and the increase of serum IL-6.

Soda et al. (23) transplanted mice with clone 20 (a subclone of murine colon adenocarcinoma), causing profound weight loss by 15 days after inoculation, and showed that body fat was lost preferentially along with a decrease in the plasma triglyceride level. Fat constitutes 90% of the fuel reserve in adults and depletion of fat is commonly seen in cancer patients with cachexia, whereas Strassmann and Kambayashi (24) reported that IL-6 may decrease carcass lipids. Path et al. investigated the effects of IL-6 on adipocyte functions. They found that chronic incubation of adipocytes with 1 nmol/L IL-6 during differentiation reduced glycero-3-phosphate dehydrogenase activity, a marker of adipocyte differentiation, and triglyceride synthesis to 67 ± 9% of the basal level ($P < 0.05$; ref. 25). Naoe et al. (26) showed that a significant decrease in the circulating levels of triglyceride was found in the saline-treated tumor-bearing mice compared with the saline-treated normal mice. In the present study, DHMEQ significantly inhibited the decrease of epididymal fat weight and serum triglycerides, presumably through the suppression of IL-6 production.

Oka et al. reported that serum albumin levels were significantly lower in patients with esophageal squamous cell carcinoma who had high serum levels of IL-6 when compared with patients who had lower IL-6 levels. They suggested that IL-6, which is produced by tumor cells, may be related to various disease variables in patients with esophageal squamous cell carcinoma, as well to the nutritional status (27). Tisdale (28) also reported that IL-6 may play a role in muscle wasting in certain animal tumors, possibly via both lysosomal (cathepsin) and nonlysosomal (proteasome) pathways. We showed that JCA-1 tumor-bearing mice showed a significant decrease in gastrocnemius muscle weight and serum albumin, and changes were significantly prevented by DHMEQ.

The regulation of many cytokine genes, including IL-6, is relatively simple, and the transcriptional factor NF-κB has been reported to up-regulate various cytokines (29–31). Several approaches to inhibit the activation of NF-κB have been investigated (32). A recombinant adenovirus vector expressing

Table 2. Effects of DHMEQ on epididymal fat weight, gastrocnemius muscle weight, hematocrit, and serum levels of triglyceride and albumin

	Tumor (−), No drug	Tumor (+), DHMEQ	Tumor (+), DMSO	Tumor (+), No drug
Epididymal fat weight (mg)	314.40 ± 40.23	197.11 ± 31.67*	117.12 ± 19.10	43.48 ± 2.97
Gastrocnemius muscle weight (mg)	558.66 ± 19.62	499.27 ± 30.26*	306.28 ± 24.46	261.13 ± 14.54
Hematocrit (%)	42.14 ± 1.07	41.89 ± 1.54*	36.71 ± 1.81	34.70 ± 1.80
Triglyceride (mg/dL)	80.82 ± 13.77	60.63 ± 7.36*	36.50 ± 6.16	32.00 ± 2.91
Albumin (g/dL)	2.45 ± 0.09	2.04 ± 0.07†	1.95 ± 0.08	1.77 ± 0.08

*Significantly different from the group of Tumor (+), DMSO and Tumor (+), No drug.
†Significantly different from the group of Tumor (+), No drug (all $P < 0.05$).

the stable form (IκBα) induces apoptosis of cancer cells showing constitutive NF-κB activity *in vitro* (33). Kawamura et al. developed synthetic double-stranded oligodeoxynucleotides for use as "decoy" *cis* elements that block the binding of nuclear factors to the promoter regions of target genes. They injected decoy oligodeoxynucleotide targeting NF-κB directly into adenocarcinoma colon 26 tumors in mice to examine whether or not cachexia was alleviated by inhibiting the action of cytokines, and their results suggested that cytokines regulated by NF-κB may play a pivotal role in the induction of cachexia in the colon 26 model (34). However, the clinical feasibility of gene therapy for inhibiting NF-κB is limited by the need for intratumoral delivery of a vector that expresses the NF-κB inhibitor, and few studies have assessed the usefulness of this strategy with *in vivo* models. In contrast, we assessed a novel agent for inhibiting the activity of NF-κB. There have been no previous reports about the therapeutic effect of an agent synthesized from a natural product on cachexia mediated through the regulation of cytokines by NF-κB.

We have previously reported that DHMEQ produces a significant decrease in NF-κB activity in JCA-1 cells with constitutive NF-κB activation (35). Because of these encouraging *in vitro* findings, we investigated the effect of DHMEQ on cachexia induced by JCA-1 tumor secreting IL-6. We found that DHMEQ significantly inhibited IL-6 production and significantly prevented the development of cachexia in a JCA-1 tumor model.

In conclusion, we showed a significant association between IL-6 and cachexia in patients with progressive prostate cancer, as well as in JCA-1 tumor-bearing mice, and we showed that DHMEQ inhibits NF-κB and thus prevents the development of cachexia induced by prostate cancer in an animal model. Prevention of the complex syndrome of cachexia will improve the quality of life for cancer patients. The new NF-κB inhibitor, DHMEQ, seems to be a promising novel anticachectic agent for the treatment of hormone-resistant prostate cancer.

Acknowledgments

We thank Azusa Yamanouchi and Hiroshi Nakazawa for their excellent technical assistance in cell culture.

References

1. Norton JA, Peacock JL, Morrison SD. Cancer cachexia. Crit Rev Oncol Hematol 1987;7:289–327.
2. Langstein HN, Norton JA. Mechanisms of cancer cachexia. Hematol Oncol Clin North Am 1991;5:103–23.
3. van Eys J. Effect of nutritional status on responses to therapy. Cancer Res 1982;42:747–53S.
4. Tisdale MJ. Cancer cachexia. Br J Cancer 1991;63:337–42.
5. Strassmann G, Fong M, Kenney JS, Jacob CO. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. J Clin Invest 1992;89:1681–4.
6. Soda K, Kawakami M, Kashii A, Miyata M. Manifestations of cancer cachexia induced by colon 26 adenocarcinoma are not fully ascribable to interleukin-6. Int J Cancer 1995;62:332–6.
7. Costelli P, Carbo N, Tessitore L, et al. Tumor necrosis factor-α mediates changes in tissue protein turnover in a rat cancer cachexia model. J Clin Invest 1993;92:2783–9.
8. Nakashima J, Tachibana M, Ueno M, Miyajima A, Baba S, Murai M. Association between tumor necrosis factor in serum and cachexia in patients with prostate cancer. Clin Cancer Res 1998;4:1743–8.
9. Twillie DA, Eisenberger MA, Carducci MA, Hseih WS, Kim WY, Simons JW. Interleukin-6: a candidate mediator of human prostate cancer morbidity. Urology 1995;45:542–9.
10. Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, Thompson TC. Elevated levels of circulating interleukin-6 and transforming growth factor-β1 in patients with metastatic prostatic carcinoma. J Urol 1999;161:182–7.
11. Baeuerle PA, Baltimore D. NF-κB: ten years after. Cell 1996;87:13–20.
12. Schwartz SA, Hernandez A, Mark Evers B. The role of NF-κB/IκB proteins in cancer: implications for novel treatment strategies. Surg Oncol 1999;8:143–53.
13. Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IκB kinase α and NF-κB in prostate cancer cells is inhibited by ibuprofen. Oncogene 1999;18:7389–94.
14. Sumitomo M, Tachibana M, Nakashima J, et al. An essential role for nuclear factor κB in preventing TNF-α-induced cell death in prostate cancer cells. J Urol 1999;161:674–9.
15. Matsumoto N, Ariga A, To-e S, et al. Synthesis of NF-κB activation inhibitors derived from epoxyquinomicin C. Bioorg Med Chem Lett 2000;10:865–9.
16. Jewett HJ. The present status of radical prostatectomy for stages A and B prostatic cancer. Urol Clin North Am 1975;2:105–24.
17. Muraki J, Addonizio JC, Choudhury MS, et al. Establishment of new human prostatic cancer cell line (JCA-1). Urology 1990;36:79–84.
18. Suzuki Y, Sugiyama C, Ohno O, Umezawa K, et al. Preparation and biological activities of optically active dehydroxymethylepoxyquinomicin, a novel NF-κB inhibitor. Tetrahedron 2004;60:7061–6.
19. Pfitzenmaier J, Vessella R, Higano CS, Noteboom JL, Wallace D Jr, Corey E. Elevation of cytokine levels in cachectic patients with prostate carcinoma. Cancer 2003;97:1211–6.
20. Wallenius V, Wallenius K, Ahren B, et al. Interleukin-6-deficient mice develop mature-onset obesity. Nat Med 2002;8:75–9.
21. Alexandrakis MG, Passam FH, Ganotakis ES, et al. The clinical and prognostic significance of erythrocyte sedimentation rate (ESR), serum interleukin-6 (IL-6) and acute phase protein levels in multiple myeloma. Clin Lab Haematol 2003;25:41–6.
22. Ishiko O, Hirai K, Nishimura S, et al. Elimination of anemia-inducing substance by cyclic plasma perfusion of tumor-bearing rabbits. Clin Cancer Res 1999;5:2660–5.
23. Soda K, Kawakami M, Kashii A, Miyata M. Characterization of mice bearing subclones of colon 26 adenocarcinoma disqualifies interleukin-6 as the sole inducer of cachexia. Jpn J Cancer Res 1994;85:1124–30.
24. Strassmann G, Kambayashi T. Inhibition of experimental cancer cachexia by anti-cytokine and anti-cytokine-receptor therapy. Cytokines Mol Ther 1995;1:107–13.
25. Path G, Bornstein SR, Gurniak M, Chrousos GP, Scherbaum WA, Hauner H. Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by β-adrenergic activation and effects of IL-6 on adipocyte function. J Clin Endocrinol Metab 2001;86:2281–8.
26. Naoe Y, Kawamura I, Inami M, et al. Anti-cachectic effect of FK317, a novel anti-cancer agent, in colon26 and LX-1 models in mice. Jpn J Cancer Res 1998;89:1318–25.
27. Oka M, Yamamoto K, Takahashi M, et al. Relationship between serum levels of interleukin 6, various disease parameters and malnutrition in patients with esophageal squamous cell carcinoma. Cancer Res 1996;56:2776–80.
28. Tisdale MJ. Loss of skeletal muscle in cancer: biochemical mechanisms. Frontiers in Bioscience 2001;6:D164–74.
29. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-κB. Annu Rev Cell Biol 1994;10:405–55.
30. Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141–79.
31. Brennan DC, Jevnikar AM, Takei F, Reubin-Kelley VE. Mesangial cell accessory functions: mediation by intercellular adhesion molecule-1. Kidney Int 1990;38:1039–46.
32. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science 1995;267:1485–8.
33. Oya M, Ohtsubo M, Takayanagi A, Tachibana M, Shimizu N, Murai M. Constitutive activation of nuclear factor-κB prevents TRAIL-induced apoptosis in renal cancer cells. Oncogene 2001;20:3888–96.
34. Kawamura I, Morishita R, Tomita N, et al. Intratumoral injection of oligonucleotides to the NF-κB binding site inhibits cachexia in a mouse tumor model. Gene Ther 1999;6:91–7.
35. Kikuchi E, Horiguchi Y, Nakashima J, et al. Suppression of hormone-refractory prostate cancer by a novel nuclear factor κB inhibitor in nude mice. Cancer Res 2003;63:107–10.

):-5347/05/1735-1541/0
JOURNAL OF UROLOGY®

Vol. 173, 1541–1545, May 2005
Printed in U.S.A.
DOI: 10.1097/01.ju.0000154636.24375.76

PROSTATE SPECIFIC ANTIGEN ADJUSTED FOR TRANSITION ZONE EPITHELIAL VOLUME: THE POWERFUL PREDICTOR FOR THE DETECTION OF PROSTATE CANCER ON REPEAT BIOPSY

TAKASHI OHIGASHI*, KENT KANAO, EIJI KIKUCHI, KEN NAKAGAWA, JUN NAKASHIMA, KEN MARUMO AND MASARU MURAI

From the Department of Urology, School of Medicine, Keio University, Tokyo, Japan

ABSTRACT

Purpose: The indications for repeat prostate biopsy for persistently increased prostate specific antigen (PSA) in men with prostate cancer never detected on previous biopsy are not clear. In this study we determined that PSA adjusted for transition zone (TZ) epithelial volume is the most powerful predictor for detecting prostate cancer on repeat biopsy.

Materials and Methods: Repeat prostate biopsies including additional TZ cores were performed in 75 men with PSA between 4.0 and 10.0 ng/ml. TZ epithelial volume was calculated by multiplying TZ volume by the percent of epithelium, which was measured by morphometric analysis using image analysis computer software.

Results: Prostate cancer was detected on repeat biopsy in 19 of the 75 patients. Patients with prostate cancer had a significant smaller percent area of epithelium or glandular lumen than those without cancer. In patients without prostate cancer TZ epithelial volume significantly correlated with total PSA. According to ROC analysis PSA adjusted for TZ epithelial volume had the greatest AUC for cancer detection (0.879). This parameter was able to avoid more than 90% of unnecessary repeat biopsies with 90% sensitivity. Multiple logistic regression analysis showed that PSA complex adjusted for TZ epithelial volume was the significant independent predictor of cancer.

Conclusions: PSA adjusted for TZ epithelial volume is the most powerful predictor of cancer in men who have undergone previous negative prostate biopsies and in whom PSA remains between 4.0 and 10.0 ng/ml.

KEY WORDS: prostate, prostatic neoplasms, biopsy, prostate-specific antigen

Controversy still persists regarding how men with previous negative prostate biopsies and prolonged abnormal prostate specific antigen (PSA) should be followed. There have been several attempts to predict positive prostate cancer at repeat biopsy, such as by calculating PSA velocity or PSA adjusted for prostate volume (PSAD). Our previous study has demonstrated that PSA adjusted for transition zone (TZ) volume (PSATZD) provides the most predictive information for prostate cancer in men who have previously undergone negative prostate biopsies.[1] According to that study PSATZD can avoid about 50% of unnecessary biopsies with 90% sensitivity in patients with PSA between 4.0 and 10.0 ng/ml. This observation means that patients with larger transition volume are at lower risk for positive cancer detection at repeat biopsy, especially if total PSA increases moderately. Prostatic tissue can be separated into 3 components, namely the epithelium, fibromuscular stroma and glandular stroma. Of them only epithelial cells can produce PSA. It was reported that serum PSA closely correlates with TZ epithelial volume.[2] Therefore, we speculated that PSA adjusted for TZ epithelial volume (PSATZepiD) can predict cancer in repeat biopsy more accurately than other PSA based parameters. To our knowledge no studies have compared PSA adjusted for the volume of special histological component, volume, to other parameters in the prediction of prostate cancer. The current study was designed to determine the usefulness of PSATZepiD for detecting prostate cancer in repeat biopsy in patients with an initial negative biopsy and with PSA consistently between 4.0 and 10.0 ng/ml.

Submitted for publication July 27, 2004.
* Correspondence: Department of Urology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan (telephone: +81–3-5363–3825; FAX: +81–3-3225–1985; e-mail: ohigashi@sc.itc.keio.ac.jp).

MATERIALS AND METHODS

Between October 1997 and January 2000, 565 consecutive patients underwent systemic biopsies under transrectal ultrasonography guidance. Of these patients 144 with initially negative systematic biopsies underwent repeat biopsies. The indications for repeat biopsy were serum PSA greater than 4.0 ng/ml or suspicious digital rectal examination findings. A total of 75 patients with PSA between 4.0 and 10.0 ng/ml at repeat biopsy were enrolled in the study. Patients with prostatitis and those who underwent urethral catheterization were excluded from study. Patient age was 49 to 83 years (mean 67.6).

Determination of PSA based parameters. Serum specimens for determining total and free PSA were obtained before digital rectal examination or transrectal ultrasound when repeat biopsy was scheduled. Total PSA was measured using an AIA-PACK PA enzyme immunoassay (Tosoh, Foster City, California). Serum-free PSA was determined using a γ-SM-MP kit (Mitsui Pharmaceutical Co., Tokyo, Japan). The detection limits of total and free PSA were 0.1 and 0.06 ng/ml, respectively. Serum α1-antichymotrypsin-PSA complex (PSA-ACT) was measured using a PSA-ACT Kit (Chugai Pharmaceutical Co., Tokyo, Japan).

Biopsy under transrectal ultrasonographic guidance. The first systemic transrectal biopsies comprised 6 cores from the peripheral zone (PZ) (bilateral base, middle and apex) using