Persistence of Prostatic Intraepithelial Neoplasia After Effective Chemoprevention of Microscopic Prostate Cancer With Antiandrogen in a Rat Model

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Purpose: We determined the chemopreventive effect of the antiandrogen bicalutamide (Zeneca Co., Ltd., Osaka, Japan) on Fisher 344 rat prostate carcinogenesis induced by DMAB (3,2'-dimethyl-4-aminobiphenyl) (Nard Co., Ltd., Osaka, Japan). We have previously reported that rat prostate microscopic carcinogenesis in this model was paradoxically enhanced when continuous treatment with bicalutamide was begun 20 weeks after the initiation of DMAB. In the current study we determined whether antiandrogen would promote or suppress the prostate carcinogenesis when administration was begun at a later period of carcinogenesis.

Materials and Methods: DMAB at a dose of 50 mg/kg was injected subcutaneously into all animals 10 times at 2-week intervals. To clarify the target lesions of bicalutamide we used 2 control groups (groups 1 and 2). Animals in groups 1 and 2 were autopsied at 60 and 74 weeks, respectively, after the initiation of DMAB. Treatment with bicalutamide began in the 60th week in group 3 rats and continued for 14 weeks. They were sacrificed in the 74th week.

Results: Microscopic cancer was revealed in 27% of group 1 rats and the incidence was increased to 42% in group 2 (statistically not significant). Delayed bicalutamide treatment significantly suppressed the cancer lesion. No cancerous lesion was detected in the ventral or other lobes of the prostate of the rats in group 3. In contrast, bicalutamide did not affect the incidence of PIN. The difference in the incidence of PIN in groups 2 and 3 (84% and 78%, respectively) was not significant.

Conclusions: The current investigation indicates that, if bicalutamide is started in the later period, it can efficiently eradicate existing microscopic cancer. Despite this suppressive effect on microscopic cancer bicalutamide permits the persistence of PIN. The latter finding suggests that the sensitivity of PIN to antiandrogen might be more complicated than previously recognized.

Key Words: prostate, prostatic neoplasms, androgen antagonists, chemoprevention, prostatic intraepithelial neoplasia

Prostate cancer is the most rapidly increasing cancer in Asia. Because there is growing evidence to suggest that a substantial risk of prostate cancer can be prevented by changes in diet and life-style, and/or by using drugs, many investigators consider that primary prevention would be the ideal strategy for decreasing mortality from this disease. In our previous studies the preventive effect of 5α-reductase inhibitor and pure antiandrogen given during the stage of progression from microscopic to macroscopic cancer has been verified in a rat carcinogenesis model using DMAB and high dose exogenous testosterone.[1,2] However, in this model androgen dependency in carcinogenesis and the development process was not proved, especially in the point of similarity with clinical prostate cancer. On the other hand, Iwasaki et al reported that, when the observation period was prolonged to more than 40 weeks, DMAB alone was able to initiate the induction of androgen dependent cancer in the ventral lobe.[3]

Although this carcinogenesis model needs a long-term experimental period (beyond 60 weeks), it represents a relevant and reliable model for testing chemopreventive agents. Therefore, we have previously tested the chemopreventive activity of bicalutamide using this model. However, the results contradicted those of the prior study using DMAB with high dose exogenous testosterone.[4] Rat prostate microscopic carcinogenesis was markedly enhanced when continuous treatment with pure antiandrogen (eg bicalutamide) was begun 20 weeks after the initiation of DMAB. In addition, even when it was continued for 40 weeks, bicalutamide treatment did not eradicate PIN of the ventral lobe.

Although this observation was paradoxical, we considered that it would provide an important clue to the complicated actions of androgen in prostate carcinogenesis. Moreover, it is clinically important to evaluate the effect of antiandrogen on prostate carcinogenesis because antiandrogen[5] and 5α-reductase inhibitor[6] are the most promising

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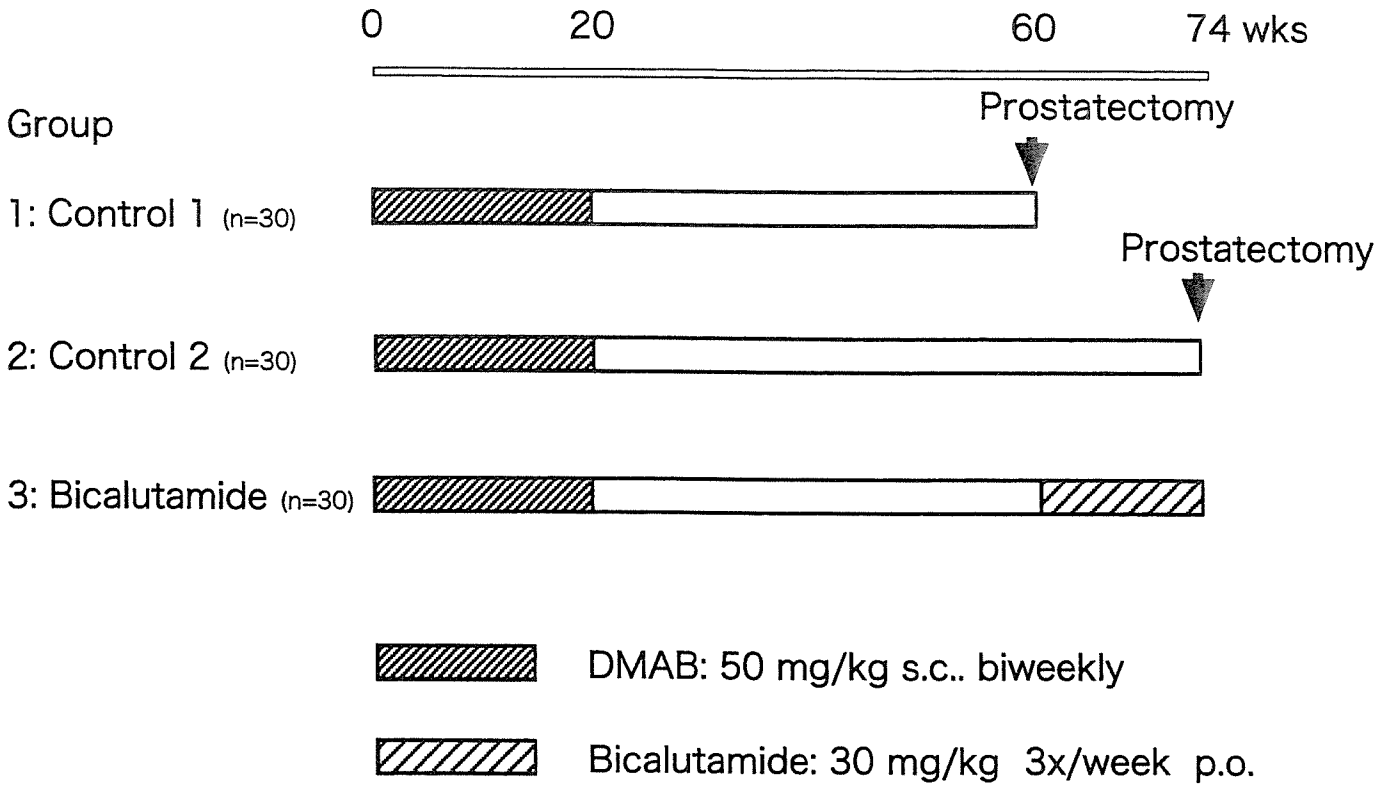

FIG. 1. Experimental design. DMAB at 50 mg/kg body weight was injected subcutaneously once every 2 weeks for total of 10 doses. Six-week-old Fisher 344 rats were divided into 3 groups of 30 each. Groups 1 and 2 were administered DMAB only. In addition, group 3 was administered bicalutamide at 30 mg/kg by oral gavage (*p.o.*) 3 times weekly beginning from 61st week of experiment until study completion.

agents due to their potential activity and limited side effects. Actually the Prostate Cancer Prevention Trial revealed that the a 5α-reductase inhibitor finasteride is effective for preventing prostate cancer. In addition, a double-blind, randomized, phase 2 trial of the pure antiandrogen flutamide vs placebo is currently under way in the United States.[5] Based on this background we performed the current study with an altered duration and initiation of bicalutamide treatment after DMAB initiation.

MATERIALS AND METHODS

Figure 1 shows the experimental design of the current study. A total of 90 Fisher 344 male rats (Charles River Japan, Inc., Kanagawa, Japan) at age 6 weeks were divided randomly into 3 groups. The rats were housed on hardwood chips in plastic cages in an air conditioned room with a 12/12-hour light-dark cycle and given free access to food (Oriental MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water.

DMAB at a dose of 50 mg/kg body weight was injected subcutaneously into all animals 10 times at 2-week intervals according to the method of Shirai et al.[7] Groups 1 and 2 of 30 rats each were administered only DMAB and autopsied at 60 and 74 weeks, respectively. The 30 rats in group 3 were administered bicalutamide orally by gavage at a dose of 30 mg/kg 3 times weekly starting at the 60th week of the experiment and continuing for 14 consecutive weeks. The experimental design was determined based on our previous experiment, which showed that approximately 30% of animals had microscopic prostate cancer 60 weeks after DMAB initiation.[4] All rats in groups 2 and 3 that survived to the end of the 74-week experimental period were autopsied. At autopsy serum samples for measuring the concentration of testosterone and LH were collected from 5 rats randomly selected from groups 2 and 3. All accessory sex organs were examined for gross abnormalities and fixed in 10% neutral buffered formalin. They were then separated into the ventral, dorsolateral and anterior lobes of the prostate gland and seminal vesicle, and their weights were recorded. The ventral lobe of the prostate was prepared as 2 sections, the dorsolateral lobe was prepared as 1 coronal section including the urethra, and the anterior lobe and seminal vesicle were prepared as 1 left and 1 right longitudinal section with the vertical axis as the cut surface. Sections were embedded in paraffin, cut at 4 μm and stained with hematoxylin and eosin for histopathological examination. The incidence of carcinoma and PIN that occurred in the ventral prostate lobe in the treatment group was examined. All histological examination was performed under the supervision of one of us (TS). Because the experimental period was long, almost consuming the lifespan of the animals, many of them in the control and experimental groups died during the experiment. Thus, all animals that died before the end of experiment were not included in the effective number.

As statistical analyses, data on the final body weight, accessory sex organ weight, and incidence of microscopic carcinoma and PIN were compared by ANOVA using Fisher's PLSD method (StatView®). Student's t test was used to compare serum testosterone and LH. All animal experi-

TABLE 1. *Final body and organ weight, and incidence of microscopic adenocarcinoma and PIN*

	Group 1	Group 2	Group 3
No. rats	18	19	14
Body wt (gm)	415	369	369
% Relative sex + accessory sex organ wt:			
Ventral*	0.095	0.062	0.025
Dorsolat*	0.125	0.093	0.049
Anterior*	0.047	0.026	0.012
Seminal vesicle*	0.194	0.112	0.033
Testis	0.762	0.809	0.692†
No. ventral (%):			
PIN	13 (72)	16 (84)	11 (78)
Ca	5 (27)	8 (42)	0†
No. seminal vesicle PIN (%)	17 (94)	18 (95)	12 (85)

No rats had dorsolateral or anterior PIN or cancer, or seminal vesicle cancer.
* Groups 1 vs 2 and 2 vs 3 $p < 0.0001$.
† Vs group 2 $p < 0.05$.

ments were performed under protocols approved by the Institutional Animal Care Guidelines of Yamanouchi Research Laboratory.

RESULTS

Table 1 shows the mean final body weight in rats in each treatment group and a comparison of the weight of each portion of the accessory sex organs as a percent of body weight. There was no significant difference in total body weight among the 3 groups. The weight of accessory sex organs was significantly suppressed in group 2 compared with group 1 ($p < 0.0001$). The weight of accessory sex organs in group 3 was further suppressed. There was a significant difference in the weight of accessory sex organs between groups 2 and 3 ($p < 0.0001$).

Table 1 also shows the incidence of prostate carcinoma and PIN. All cancer was microscopic adenocarcinoma detected only in the ventral lobe and no visible nodule was observed in any specimen. These findings completely agreed with our previous study. In group 1, which was administered only DMAB and autopsied at the 60th week, 27% of the rats had cancer. The incidence of cancer in group 2 rats, which were autopsied at the 74th week, was 42%. However, the difference in the cancer incidence was not statistically significant. On the other hand, no cancer was detected in the ventral or other lobes of the prostate in group 3. The difference in the cancer incidence was statistically significant compared to group 2 ($p < 0.05$).

In contrast to cancer, PIN was observed not only in the ventral lobe, but also in the seminal vesicle in each group. In control groups 1 and 2 the incidence of PIN in the ventral lobe was 72% and 84%, and the incidence of PIN in the seminal vesicle was 94% and 95%, respectively. Interestingly there was no tendency for the incidence of PIN to decrease in group 3. The incidence of PIN of this group was 78% in the ventral lobe and 85% in the seminal vesicle. There was no statistically significant difference in the incidence of PIN among the 3 groups. Figure 2 shows typical microscopic findings of microscopic prostate cancer and PIN.

Table 2 lists the average serum testosterone and LH in 5 rats each in groups 2 and 3. Serum testosterone in group 3 was somewhat higher than that in group 2 but there was no statistically significant difference. Also, there was no diff ence in LH between groups 2 and 3.

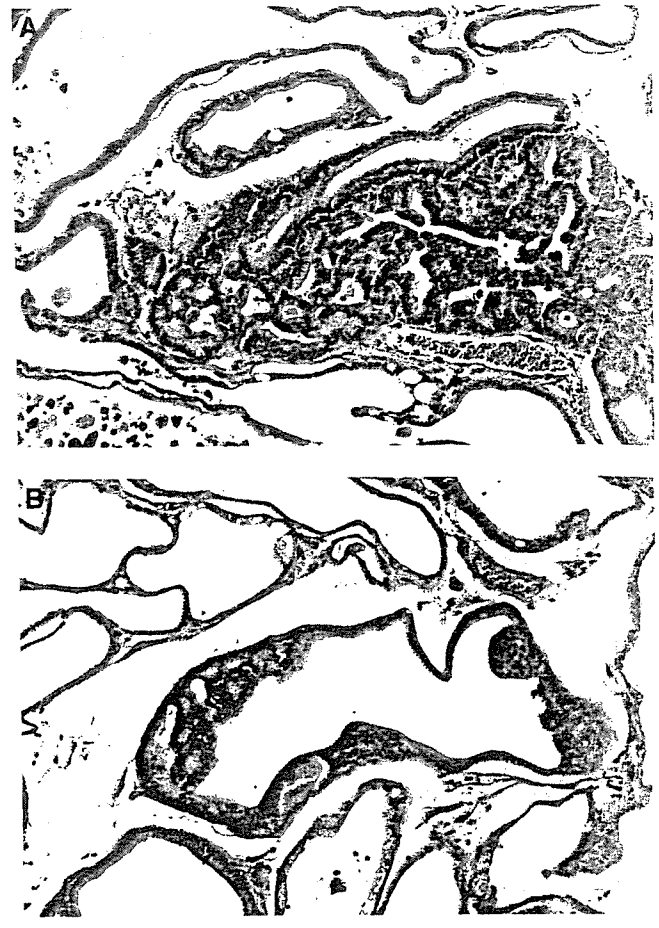

FIG. 2. Ventral prostate. *A*, microscopic carcinoma induced DMAB administration. *B*, PIN. H & E, reduced from ×200.

DISCUSSION

We have previously reported that rat prostate microsco carcinogenesis is markedly enhanced when continu treatment with pure antiandrogen, eg bicalutamide, v begun 20 weeks after the initiation of DMAB. In additi treatment with bicalutamide, even when continued for weeks, did not eradicate PIN of the ventral lobe. considered certain possible mechanisms accounting the paradoxical enhancement of carcinogenesis. 1) cause bicalutamide was begun 20 weeks after DMAB ministration, initiated prostate cells that existed in t early phase might have reacted contradictorily to anti drogen. 2) The increased serum testosterone induced bicalutamide might have enhanced microscopic carci genesis. The latter hypothesis was also supported by a chemoprevention model using a high dose 5α-reduct inhibitor.[8]

Because this paradoxical effect of bicalutamide on pr tate carcinogenesis may provide a clue to the complica effects of androgen in prostate carcinogenesis, we p formed the current study to determine whether anti drogen promotes prostate carcinogenesis even when i begun in the later period of this long-term carcinogen

TABLE 2. *Serum testosterone and LH at experimental week 74*

Group	Av Testosterone (ng/ml)	Av LH (ng/ml)
2	0.69	1.3
3	0.96	1.4

model. To clarify the target lesions of bicalutamide we used 2 control groups. Animals in group 1 were autopsied at 60 weeks after the DMAB initiation. At this time treatment with bicalutamide was begun in group 3 rats and continued for 14 weeks. At the 74th week of the experiment all surviving rats in group 2 (another untreated control group) and group 3 were autopsied. The current investigation yielded several significant findings when we varied the duration and onset of bicalutamide treatment after DMAB initiation.

1. Microscopic cancer was revealed in 27% of rats in group 1 and the incidence was increased to 42% in group 2. Although there is a possibility that some small volume tumors existing at the 60th week were missed, finding suggests that the process of malignant conversion from PIN to microscopic cancer might have occurred between the 60th and 74th weeks of the current experiment. Since PIN is known to be the most important target of clinical chemoprevention trials for prostate cancer,[5,9] this model seems to be a useful tool for testing the activity of chemopreventive agents.
2. Delayed bicalutamide treatment significantly suppressed the cancer lesion. No cancerous lesion was detected in the ventral or other lobes of the prostate in rats in group 3. The observation suggests to us that delayed bicalutamide actively eradicated the existing microscopic prostate cancer. Preclinical studies using Dunning R3327H transplantable rat prostate tumor demonstrated that bicalutamide at a daily oral dose of 25 mg/kg showed a significant reduction in tumor growth equivalent to surgical or medical castration.[10] Therefore, we considered that the direct antitumor effect of antiandrogen had a significant role in prostate cancer regression in the current study. Also, there is a possibility that bicalutamide might have an inhibitory effect on malignant conversion of PIN to microscopic cancer. In the current study serum testosterone was relatively higher in the bicalutamide treated group than in the control group (statistically not significant), a phenomenon that was also observed in our previous experiment. Thus, the increased serum testosterone induced by bicalutamide did not have a promoting effect on microscopic prostate cancer in this later phase of carcinogenesis.
3. To our interest delayed bicalutamide did not affect the incidence of PIN. There was no difference in the incidence of PIN between groups 2 and 3. This is in striking contrast to the apparent suppressive effect of bicalutamide on microscopic cancer. Persistent PIN in the ventral lobe was also observed in our previous study using bicalutamide begun 20 weeks after DMAB initiation. The results of the 2 studies suggest the possibility that the sensitivity of PIN to antiandrogen might be more complicated than previously recognized. Conversely in clinical investigation most investigators have reported that androgen deprivation decreases the prevalence of PIN in prostatectomy specimens. However, it should be noted that those patients were treated with CAD by medical castration and antiandrogen, and also that PIN was not completely eradicated in most studies despite CAD treatment.[11–13] Thus, currently limited data are available about the clinical effects on PIN of antiandrogen as a single agent. Since CAD is not a realistic method for chemoprevention due to its side effects,[9] it is important to determine the single agent activity of antiandrogen against PIN. Also, it is noteworthy that 5α-reductase as a single agent has little or no effect on PIN in clinical treatment.[14] Theoretically persistent PIN makes it likely that the cessation of antiandrogen would allow PIN again to transform into microscopic and even macroscopic cancer. van der Kwast et al reported persistent MIB-1 positive dysplastic cells in the majority of PINs after 6 months of CAD.[15] They pointed out the possibility of PIN reactivation after therapy cessation. They also observed a slow decrease in the frequency of PIN after androgen deprivation, which suggests that eradicating PIN would require long androgen deprivation treatment. In the current study the rats in group 3 were treated with bicalutamide until the termination of the experiment. Therefore, the reactivation of PIN cannot explain the persistence of PIN. Since our prior study showed persistent PIN despite 40 weeks of bicalutamide therapy,[4] it is possible that the duration of bicalutamide therapy in the current study was not enough to exert the antiandrogen effect on PIN. Alternatively persistent PIN might be explained by androgen resistant clones but further investigations are needed to understand the mechanism responsible for persistent PIN.[15,16] Currently the clinical importance of PIN after chemoprevention treatment is not clear because PIN in humans has a long-term natural history.[17] Therefore, it should be noted that the results of the current animal study cannot be directly transferred to human prostate cancer.

CONCLUSIONS

The results of the current study and our previous study clearly demonstrate that the effects of bicalutamide on DMAB induced rat prostate carcinogenesis are complicated. Bicalutamide started in the later period efficiently eradicated existing microscopic cancer. However, it exerted a promotional effect when initiated in the early phase and continued. In the latter case a difference in the reactivity of the early initiated cell rather than increased serum testosterone might have a role in the promotional effect. As another possible explanation, the low dihydrotestosterone environment induced by bicalutamide, especially during the early oncogenic process, might lead to tumor growth potentiation.[18] Further investigations focusing on the mechanism of resistance to antiandrogen therapy are needed. Recently novel mutations and upregulation of androgen receptors was proposed as a mechanism of antiandrogen resistance.[19,20] The persistence of PIN that we report also indicates that it will be important to identify the mechanism of the antiandrogen resistance of PIN.

Abbreviations and Acronyms

CAD	=	complete androgen deprivation
DMAB	=	3,2′-dimethyl-4-aminobiphenyl
LH	=	luteinizing hormone
PIN	=	prostatic intraepithelial neoplasia

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Association of the polymorphisms of genes involved in androgen metabolism and signaling pathways with familial prostate cancer risk in a Japanese population

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Abstract

Background: Androgen plays a central role in the normal and malignant development of prostate glands. Genetic polymorphisms of genes involved in androgen metabolism and signaling might be associated with the risk of prostate cancer. Methods: One hundred and two patients with prostate cancer with a family history and 117 healthy age- and residence-matched male controls were enrolled. Genotypes of the CAG repeat length of androgen receptor (AR), CYP17, 5α-reductase type II (SRD5A2), UDG-glucuronosyltransferase (UGT) 2B15, PSA promoter genes were analyzed. Results: For single polymorphisms, the presence of Y alleles showed a significantly lower risk of prostate cancer in comparison with the D/D genotype in UGT2B15 (odds ratio [OR] = 0.41, 95% confidence interval [CI] = 1.40–4.28, $p = 0.0015$), and the presence of A2 alleles showed a weak tendency to decrease prostate cancer risk in comparison with the A1/A1 genotype in CYP17 (OR = 0.69, 95% CI = 0.39–1.23, $p = 0.21$). The stratification of cases according to clinical stage and pathological grade showed that the A2/A2 genotype was significantly associated with localized stage cancer in comparison with metastatic stage cancer (OR = 5.18, 95% CI = 1.49–17.95, $p = 0.007$). The combination of UGT2B15 and CYP17 genotypes could identify higher risk subjects even in subjects with low-risk UGT2B15 genotypes, i.e., Y/Y + D/Y genotypes (OR = 1.97, 95% CI = 0.92–4.22, $p = 0.079$). Conclusion: Genetic polymorphisms of the genes involved in androgen metabolism and signaling were significantly associated with familial prostate cancer risk. Single nucleotide polymorphisms of low-penetrance genes could be targets to understand genetic susceptibility to familial prostate cancer.

Keywords: AR CAG repeat; Single nucleotide polymorphisms; Genetic susceptibility; Risk factors; Genotyping; Genomic DNA; SRD5A2; UGT2B15; CYP17; PSA promoter; 5α-Reductase; Genetic polymorphism; Prostate cancer; Family history

1. Introduction

The incidence of prostate cancer is lower in Japan than in western countries [1]; however, it has been increasing every year in the prostate-specific antigen (PSA) era [2]. Understanding the etiopathophysiology of prostate cancer would provide important information for a screening program and the development of new treatment modalities. Androgen plays a central role in the normal and malignant growth and function of prostate glands, and differences in androgen metabolism and signaling pathways might influence the carcinogenesis of prostate cancer [3]. Genetic polymorphisms of the genes related with these pathways have attracted the attention of researchers in relation to genetic susceptibility to prostate cancer.

In androgen metabolism, key enzymes for androgen synthesis, activation and deactivation were elucidated. Cytochrome P-450 (CYP) 17 catalyzes steroid 17α-hydroxylase and 17–20 lyase activities in testosterone biosynthesis in both testes and adrenal glands [4]. A polymorphic T to C substitution in the 5′-untranslated region of the CYP17 gene creates a recognition site for the MspAI restriction enzyme. This polymorphic substitution also creates an SP-1 (CCACC box) promoter site, and this might increase transcriptional activities [5]. Steroid 5α-reductase type II (SRD5A2)

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catalyzes the conversion of testosterone to dihydrotestosterone (DHT). SRD5A2 contains two well-characterized polymorphic sites: an alanine to threonine substitution at codon 49 (A49T) and a valine to leucine substitution at codon 89 (V89L) [6,7]. The mutated enzyme resulting from T49 showed about 3.6-fold higher activity than the wild-type A49 enzyme, and the association of T49 with prostate cancer risk was reported [6]. Although the association of V89L genotypes in SRD5A2 with prostate cancer risk is controversial, a recent study from a Japanese group showed that the presence of the V allele significantly increased prostate cancer risk [8]. UDP-glucuronosyltransferases (UGTs) are phase II detoxification enzymes catalyzing the inactivation of endogenous and exogenous compounds by transfer of the glucuronyl group from 5′-diphosphoglucuronic acid [9]. UGT2B enzymes glucuronidate the primary steroid and UGT2B15 catalyzes the biotransformation of DHT in prostate glands [10]. Differences of the UGT2B15 enzyme could alter the intraprostatic DHT concentration.

Androgen signaling is mediated by AR, and the association of the genetic polymorphism of the CAG repeat length in exon 1 with prostate cancer risk was extensively examined. The transcriptional activity of AR was negatively correlated with the CAG repeat length of AR [11,12]. The shorter CAG repeat length could be associated with prostate cancer risk [13–17]. The PSA gene is the androgen-regulated gene and might influence the biology of prostate cancer [18,19]. The PSA promoter gene contains several genetic polymorphisms and the association of these polymorphisms with prostate cancer was examined [20,21].

We have extensively collected familial prostate cancer pedigrees in Japan [22,23]. Family history is one of the important risk factors for prostate cancer [24]. Several susceptibility loci or candidate genes have been reported to explain the genetic susceptibility of prostate cancer [25,26]; however, these genetic factors could explain the susceptibility of a very small percentage of familial/hereditary prostate cancer pedigrees [27,28]. Thus, we considered familial prostate cancer as a high-risk group for prostate cancer development, and reported the association of prostate cancer risk with the genetic polymorphism of high-penetrance genes including *HPC2/ELAC2* [29] and *RNASEL* [30] or low-penetrance genes encoding Vitamin D receptor gene [31], CYP1A1 [32], glutathione *S*-transferase [33] and p53 [34]. In this study, we examined the association of gene polymorphisms involved in androgen metabolism and signaling pathways with familial prostate cancer risk in a Japanese population.

2. Materials and methods

2.1. Patients

This case-controlled study included 102 prostate cancer patients with a family history of prostate cancer in first-degree relatives. The numbers of affected family members were two in 73 patients, three in 14 patients and four in 15 patients. Seventy-three patients with 2 affected family members were categorized into familial/not-hereditary prostate cancer, and 29 patients with 3 or more affected family members were categorized into hereditary prostate cancer according to Carter et al.'s definition [35]. All prostate cancer patients were confirmed histologically at Gunma University Hospital and its affiliated hospitals. Their ages ranged from 40 to 88 years old with a mean age of 69.9 years. Clinical stages were A in 2, B in 41, C in 32, D in 25 and unknown in 2 patients according to Jewett's staging system. Gleason scores were less than 7 in 25 and equal or more than 7 in 77 patients. One hundred seventeen non-cancer controls were recruited from clinics at Gunma University Hospital. Controls were excluded if they had an abnormal PSA level (i.e., $\geq$4.0 ng/ml), and abnormal digital rectal examination, and previous diagnosis of cancer. Their ages ranged from 51 to 88 years old with a mean of 71.0 years. No significant differences in age were observed between cases and controls. All cases and controls enrolled in this study under informed consent. The Ethical Committee of Gunma University approved this study.

2.2. Genotyping

Genomic DNA was isolated from whole blood using a GENOMIX kit (Talent srl. Trieste, Italy). Samples were diluted to 10 μg/μl and stored at −20 °C. To analyze the CAG repeat length of the AR gene, CAG repeat containing regions were amplified according to the method described by Yoshida et al. [36], and CAG repeat length was measured by direct sequencing of PCR products. Sequencing analysis was performed by the method described previously [37]. For the detection of UGT2B15 variants, allele-specific PCR was performed according to the method described by MacLeod et al. [38]. The G allele and T allele corresponded to aspartate (D) and tyrosine (Y) as encoded amino acids, respectively. Genotypes of CYP17, SRD5A2 and PSA genes were determined by the PCR-based restriction fragment length polymorphism method. For T/C polymorphism of the CYP17 gene, the wild-type allele, A1, and mutated allele, A2, were determined by the method described by Gsur et al. [39]. For SRD5A2, V89L polymorphisms were determined by the modified method described by Yamada et al. [40] Genomic DNA (40 ng) was mixed with dNTP (300 μM), 1 μM forward and reverse primers and 2.5 units of ProofStart DNA polymerase (Qiagen, Valencia, CA, USA) in the presence of 1× ProofStart PCR buffer and 1× Q-Solution according to the manufacturer's protocol (Qiagen). Cycling conditions were: 95 °C for 5 min for 1 cycle; 94 °C for 30 s; 73 °C for 60 s for 35 cycles, followed by an elongation cycle of 72 °C for 7 min. Obtained PCR products were digested with *Rsa*I for detection of the V89L polymorphism of SRD5A2. For the PSA gene, the G/A polymorphism in the promoter region was determined by the method described by Xue

Table 1
Primer list

Gene name	Forward	Reverse
AR	5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′	5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′
CYP17	5′-CAT TCG CAC CTC TGG AGT C-3′	5′-GGC TCT TGG GGT ACT TG-3′
SRD5A2	5′-GCA GCG GCC ACC GGC GAG G-3′	5′-AGC AGG GCA GTG CGC TGC ACT-3′
UGT2B15		
First PCR	5′-GAC TGT GTT GAC ATC TTC GGC TTC T-3′	5′-CCA GTA GCT CAC CAC AGG GAT TAA G-3′
Second PCR		
G allele	5′-CCT ACA TCT TTA ACT AAA AAT G-3′	5′-CCA GTA GCT CAC CAC AGG GAT TAA G-3′
T allele	5′-CCT ACA TCT TTA ACT AAA AAT T-3′	5′-CCA GTA GCT CAC CAC AGG GAT TAA G-3′
PSA	5′-TTG TAT GAA GAA TCG GGG ATC GT-3′	5′-TCC CCC AGG AGC CCT ATA AAA-3′

et al. [20]. To assess the accuracy of genotyping, 10% of randomly selected samples were re-analyzed, and the concordant results were confirmed (Table 1).

2.3. Statistical analysis

The χ^2 test was performed to evaluate whether the distribution of genotype frequencies of genes varied among cases and controls. Odds ratios (ORs) were calculated as an estimate of relative risk and 95% confidence intervals (CIs) were calculated from unconditional logistic regression models. We also performed logistic regression analysis to establish whether the pathological grade and clinical stage were associated with genotypes. The clinical stage categories were localized (Stages A, B and C) or metastatic (Stage D), and the pathological grade was Gleason score <7 or ≥7. SPSS Professional Statistics™ (SPSS Inc., Chicago IL, USA) was used for all statistical analysis.

3. Results

We examined five polymorphisms of five genes related with androgen metabolism and signaling pathways in this study, and Table 2 summarizes the distribution of genotypes among cases and controls. We first evaluated the association of individual genotypes with prostate cancer risk. In controls, a short AR CAG repeat and long AR CAG repeat were observed in 50.4 and 49.6%, respectively, and in cases, in 55.4 and 44.1%, respectively. The CAG repeat length of AR showed no significant association among cases and controls. As for the distribution of the CYP17 genotype, A1/A1, A1/A2 and A2/A2 genotypes were observed in 27.3, 51.3 and 21.4% of controls, and in 35.3, 47.1 and 17.6% of cases, respectively. The presence of the A2 allele showed a slight tendency to reduce prostate cancer risk (OR = 0.69, 95% CI = 0.39–1.23, $p = 0.21$). As UGT2B15 genotypes, D/D, D/Y and Y/Y genotypes were observed in 28.3, 60.7 and 11.1% of controls,

Table 2
Association of genetic polymorphisms of cag repeat length of AR, UGT2B15, CYP17 and PSA promoter with prostate cancer risk

Gene	Genotype	No. of subjects (%)		OR (95% CI)	p-Value
		Cases	Controls		
AR					
Cag repeat	22<	57 (55.4%)	59 (50.4%)	Ref.	
Length	≤22	45 (44.1%)	58 (49.6%)	0.80 (0.47–1.37)	0.42
CYP17	A1/A1	36 (35.3%)	32 (27.3%)	Ref.	
	A1/A2	48 (47.1%)	60 (51.3%)	0.71 (0.39–1.31)	0.27
	A2/A2	18 (17.6%)	25 (21.4%)	0.64 (0.30–1.38)	0.26
	A1/A2 + A2/A2	66 (64.7%)	85 (72.7%)	0.69 (0.39–1.23)	0.21
UGT2B15	D/D	50 (49.0%)	33 (28.2%)	Ref.	
	D/Y	39 (38.2%)	71 (60.7%)	0.36 (0.20–0.65)	0.0006
	Y/Y	13 (12.8%)	13 (11.1%)	0.66 (0.27–1.60)	0.36
	D/Y + Y/Y	52 (51.0%)	84 (71.8%)	0.41 (0.23–0.72)	0.0015
SRD5A2	V/V	33 (32.4%)	42 (35.9%)	Ref.	
	V/L	46 (45.1%)	50 (42.7%)	0.85 (0.47–1.57)	0.61
	L/L	23 (22.6%)	25 (32.4%)	0.85 (0.41–1.77)	0.67
	V/L + L/L	69 (67.6%)	75 (64.1%)	0.85 (0.49–1.50)	0.58
PSA promoter	G/G	66 (64.7%)	74 (67.2%)	Ref.	
	G/A	27 (26.5%)	32 (27.4%)	0.95 (0.51–1.74)	0.86
	A/A	9 (8.8%)	11 (9.4%)	0.92 (0.36–2.35)	0.86
	G/A + A/A	36 (35.3%)	43 (32.8%)	0.94 (0.54–1.63)	0.82

OR, odds ratio; CI, confidence interval; Ref., reference.

Table 3
Pathological grade and genetic polymorphisms of CYP17 genotypes

Genotype	Gleason score		OR (95% CI)	p-Value
	<7	≥7		
A1/A1	7 (28.0%)	29 (43.3%)	Ref.	
A1/A2	8 (32.0%)	30 (44.8%)	0.91 (0.29–2.82)	0.86
A2/A2	10 (40.0%)	8 (11.9%)	0.19 (0.06–0.67)	0.007
A1/A2 + A2/A2	18 (72.0%)	38 (56.7%)	0.51 (0.19–1.38)	0.18

OR, odds ratio; CI, confidence interval; Ref., reference.

Table 4
Combination analysis of CYP17 and UGT2B15 genotypes

UGT2B15	CYP17	No. of subjects (%)		OR (95% CI)	p-Value
		Cases	Controls		
Y/Y + D/Y	A1/A2 + A2/A2	33 (32/4%)	65 (55.6%)	Ref.	
Y/Y + D/Y	A1/A1	19 (18.6%)	19 (16.2%)	1.97 (0.92–4.22)	0.079
D/D	A1/A2 + A2/A2	33 (32.4%)	20 (17.1%)	3.25 (1.62–6.52)	0.0007
D/D	A1/A1	17 (16.7%)	13 (11.2%)	2.58 (1.11–5.94)	0.024
D/D	All	50 (49.1%)	33 (28.3%)	2.98 (1.65–5.48)	0.0004

OR, odds ratio; CI, confidence interval; Ref., reference.

respectively, whereas in 49.0, 38.2 and 12.8% of cases, respectively. The D/Y genotype was significantly associated with prostate cancer risk (OR = 0.36, 95% CI = 0.20–0.65, $p = 0.0006$), and the presence of the Y allele, i.e., D/Y + Y/Y, significantly decreased the risk of prostate cancer (OR = 0.41, 95% CI = 0.23–0.72, $p = 0.002$). We examined V89L polymorphism in SRD5A2. V/V, V/L and L/L genotypes were observed in 35.9, 42.7 and 32.4% of controls, respectively, and in 32.4, 45.1 and 22.6% of cases, respectively. No significant association was found in genotypic frequencies among cases and controls. Finally, we examined the genetic polymorphism of the PSA promoter. G/G, G/A and A/A genotypes were observed in 67.2, 27.4 and 9.4% of controls, respectively, and in 64.7, 26.5 and 8.8% of cases, respectively. No significant association was found in genotypic frequencies among cases and controls.

Analyses of individual genotypes showed a significant association of prostate cancer risk with UGT2B15 genotypes and a weak tendency for risk association with CYP17 genotypes, as shown in Table 2. We stratified cases according to clinical stage and pathological grade, and examined the association of these clinical parameters and genotypes of UGT2B15 and CYP17. As for UGT2B15 genotypes, no significant differences were observed in genotypic distribution according to the pathological grade and clinical stage (data not shown). As for CYP17 genotypes, A1/A1, A1/A2 and A2/A2 genotypes were observed in 28.0, 32.0 and 40.0% of cases with a Gleason score of less than 7, respectively, and in 43.3, 44.8 and 11.9% of cases with Gleason score of equal or more than 7, respectively, as shown in Table 3. Cases with high-grade cancer showed a significantly low frequency of the A2/A2 genotype in comparison with the A1/A1 genotype.

Finally, we assessed the effect of multiple genotypes on familial prostate cancer risk. Through individual investigations of genotypes, we combined the genetic polymorphisms of UGT2B15 and CYP17. Table 4 shows the relation of ORs with the genotype combinations. When any Y genotypes of UGT2B15 and any A2 genotypes of CYP17 were set as a reference, the D/D genotype and the A1/A2 + A2/A2 genotype significantly increased the risk of prostate cancer with OR = 3.25 (95% CI = 1.62–6.52, $p = 0.0007$). The D/D and A1/A1 genotype combination also significantly increased the risk of prostate cancer with OR = 2.58 (95% CI = 1.11–5.94, $p = 0.024$). In subjects with Y/Y + D/Y genotypes of UGT2B15, which were considered low-risk genotypes, subjects with the A1/A1 genotype tended to have an increased risk for prostate cancer (OR = 1.97, 95% CI = 0.92–4.22, $p = 0.079$).

4. Discussion

In this study, we examined the genetic polymorphisms of genes involved in androgen metabolism and signaling. We observed that the presence of the Y allele in UGT2B15 significantly decreased the prostate cancer risk, and that the presence of the A2 allele in CYP17 showed a weak tendency to decrease the risk. Although the clinical parameters did not have any significant association with UGT2B15 genotypes, the A2/A2 genotype of CYP17 showed a significant association with localized stage prostate cancer. These findings suggested that the D/D genotype and the A1/A1 genotype were high-risk genotypes in UGT2B15 and CYP17, respectively. Thus, we performed a combination analysis of genotypes of UGT2B15 and CYP17, as shown in Table 4. The D/D genotype significantly increased prostate cancer risk in combination with any CYP17 genotype, i.e., A1/A2 + A2/A2, A1/A1 and A1/A1 + A1/A2 + A2/A2. Furthermore, the stratification of subjects with any Y

genotype of UGT2B15 (low-risk group) according to CYP17 genotypes suggested that subjects with the A1/A1 genotype tended to have an increased prostate cancer risk (OR = 1.97, 95% CI = 0.92–4.22, $p = 0.079$).

UGT2B15 is a key enzyme for the inactivation of active androgen, DHT, in prostatic cells. Human prostate cancer cells, LNCaP, expressed UGT2B15, and the expression of the enzyme was regulated by growth factors, cytokine and so on [41,42]. Levesque et al. studied the kinetics of polymorphic enzymes of UGT2B15 [43]. The V_{max} of the Y allele was approximately two-fold greater than the enzyme resulting from the D allele, although no significant differences were observed in K_{max} of both enzymes. These findings suggested that the D enzyme inefficiently metabolized DHT, resulting in high DHT concentration in prostate tissues. Higher levels of DHT were suggested to be associated with prostate cancer development by several researchers [44,45]. Our findings that the D/D genotype was significantly associated with prostate caner risk supported the hypothesis of the association of high DHT and prostate cancer development.

Several case-controlled studies of the association of the genetic polymorphism of CYP17 suggested that the A2/A2 genotype significantly increased the prostate cancer risk [39,40,46]. The A2 allele creates an SP-1-type (CCACC box) promoter site [5], and this might increase transcriptional activity. In this circumstance, the biosynthesis of testosterone could be enhanced, and thus the risk of prostate cancer development might be increased. On the other hand, the association of the A1 genotype with prostate cancer risk was also reported [47,48]. Habuchi et al. suggested that the A1/A1 genotype contributed to the development of benign prostate hyperplasia as well as prostate cancer [48]. The authors also observed a higher frequency of the A1/A1 genotype in patients with metastatic cancer. This fining showed a tendency for prostate cancer risk to increase in subjects with the A1/A1 genotype. We also observed that the A1/A1 genotype was significantly frequently found in metastatic cancer cases. These findings were consisted with the finding reported by Habuchi et al. [48].

Combination analyses of multiple polymorphisms of genes involved in androgen metabolism have been scarcely performed [49], although individual multiple genotypes were examined in the same study. Modugno et al. examined the polymorphisms of genes encoding aromatase, and androgen and estrogen receptors [49]. The authors observed that only a short CAG repeat significantly increased prostate cancer risk (OR = 1.75, 95% CI = 1.05–2.94, $p < 0.05$) by individual analysis; however, they found a higher risk for prostate cancer in subjects with the C/T genotype of aromatase (OR = 2.84, 95% CI = 0.85–9.50), *Xba*I −/− genotype (OR = 3.80, 95% CI = 1.36–10.60) and *Pvu*II −/− genotype (OR = 2.67, 95% CI = 1.03–6.90), after the stratification of subjects according to AR CAG repeat length. This study suggested that the combination of genetic polymorphisms of UGT2B15 and CYP17 could select subjects at higher risk for prostate cancer. In this study, the D/D genotype was considered a high-risk genotype for UGT2B15. The D/D genotype significantly increased the prostate cancer risk in reference to Y genotypes of UGT2B15 and A2 genotypes of CYP17 (Table 4). The combination of UGT2B15 and CYP17 genotypes with a relatively high-risk group, A1/A1, was identified in a low-risk group of UGT2B15, i.e., any Y genotype group.

We did not find any significant associations or tendencies among the genetic polymorphisms of AR CAG repeat length, SRD5A2 and PSA, and prostate cancer risk. The selection criteria for subjects and ethnic/racial differences may affect the results of genetic polymorphisms. We recruited control subjects from an age-matched, residence-matched out-patient clinic without cancer. The controls in this study included patients with benign prostate hyperplasia. Several studies have suggested that the development of BPH was influenced by gene polymorphisms for steroid hormone metabolism [8,48,50,51]. This might be one of the reasons for null associations of genetic polymorphisms with prostate cancer risk. Another reason might be the selection of prostate cancer cases. Cases in this study were recruited from familial prostate cancer patients registered in Gunma University Oncology Group, Japan. The investigation of genetic susceptibility to prostate cancer is an important research area. We have examined the association of genetic polymorphisms of low-penetrance genes with familial prostate cancer risk [31–34]. Differences in genetic background between familial prostate cancer and sporadic prostate cancer might affect the distribution of allelic frequencies; however, the results in this study would supply important information on genetic susceptibility to prostate cancer in a high-risk group.

In summary, we examined the association of familial prostate cancer risk with genetic polymorphisms of AR CAG repeat, CYP17, UGT2B15, SRD5A2 and PSA promoter. Individual analysis of the genotype showed that the D/D genotype of UGT2B15 significantly increased prostate cancer risk, and the A1/A1 genotype of CYP17 tended to increase prostate cancer risk. The stratification of cases according to clinical parameters demonstrated that the frequency of the A1/A1 genotype was significantly higher in metastatic stage cancer in comparison with localized stage cancer. Combination analysis of genotypes of UGT2B15 and CYP17 could select higher risk subjects even in subjects with low-risk UGT2B15 genotypes, i.e., Y/Y + D/Y genotypes. Further, large-scale case–control studies are warranted to confirm the association of genetic polymorphisms of genes involved in androgen metabolism with familial prostate cancer risk.

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Significance of micrometastases in pelvic lymph nodes detected by real-time reverse transcriptase polymerase chain reaction in patients with clinically localized prostate cancer undergoing radical prostatectomy after neoadjuvant hormonal therapy

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OBJECTIVE

To clarify the significance of micrometastases in pelvic lymph nodes in patients treated by radical prostatectomy (RP) for prostate cancer after neoadjuvant hormonal therapy (NHT).

PATIENTS AND METHODS

The study included 52 patients with clinically localized prostate cancer who received NHT followed by RP. The expression of prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) in 989 lymph nodes isolated from the 52 patients were assessed by a fully quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR). We regarded specimens in which either PSA or PSMA mRNA were positive as showing the 'presence of micrometastasis'. Lymph node specimens were also stained immunohistochemically with an antibody against PSA.

RESULTS

Pathological examinations detected tumour cells in 11 lymph nodes from four patients, and real-time RT-PCR further identified micrometastasis in 40 lymph nodes from 19 patients with no pathological evidence of nodal involvement. The presence of micrometastatic cancer cells was confirmed by immunohistochemical staining in 19 lymph nodes from 11 patients with pathologically negative nodes. The presence of micrometastases was significantly associated with other conventional prognostic variables, including the pretreatment serum PSA level, biopsy Gleason score and surgical margin status. The biochemical recurrence-free survival rate in patients with no micrometastasis was significantly higher than that in those with micrometastasis. Furthermore, multivariate analysis identified the presence of micrometastasis as an independent factor predicting biochemical recurrence.

CONCLUSIONS

Although residual foci of atrophic prostate cancer cells in resected lymph nodes after NHT can be difficult to diagnose by routine pathological examination, the present results show the usefulness of quantitative real-time RT-PCR targeting PSA and PSMA genes for detecting micrometastatic tumour foci in pelvic lymph nodes from patients with localized prostate cancer treated by NHT followed by RP. Furthermore, the present findings suggest that micrometastases in pelvic lymph nodes might be, at least partly, important in the development of biochemical recurrence in some patients undergoing RP after NHT.

KEYWORDS

prostate cancer, radical prostatectomy, micrometastasis, neoadjuvant hormonal therapy, real-time reverse transcriptase chain reaction

INTRODUCTION

The goal of neoadjuvant hormonal therapy (NHT) before radical prostatectomy (RP) is to induce tumour regression and to subsequently improve long-term cancer control rates [1]. To date, several randomized trials showed the ability of NHT to significantly decrease serum PSA levels, prostate volume and margin positive rates; however, there has been no confirmation that NHT confers any prognostic benefit. These outcomes could be possibly explained by the difficulty in exactly diagnosing the distribution of cancer cells in specimens obtained after NHT by routine pathological examination [2]. Indeed, Gleave *et al.* [3] reported that microscopic cancer foci were detected by immunohistochemical staining in two of four patients pathologically staged as pT0 after NHT.

Pelvic lymph node metastasis is regarded as the most important predictive factor of disease recurrence after RP; however, routine microscopic examination of lymphadenectomy specimens can miss small cancer foci, suggesting the presence of histologically undetectable micrometastases in the pelvic lymph nodes. This hypothesis is supported by various studies showing a higher sensitivity for detecting micrometastatic prostate cancer cells in pelvic lymph nodes by several molecular and immunohistochemical techniques than with conventional haematoxylin and eosin staining [4,5]. Difficulty in the exact diagnosis of the presence of micrometastatic cancer foci in lymph nodes can be particularly true for specimens obtained from patients treated by NHT before surgery.

Recently, a real-time detection and quantitative PCR-based assay was developed. The advantage of this method is the specific

detection of rare events, i.e. its sensitivity is sufficient to detect of 10–100 pg of RNA from the target gene. In addition, it is highly reproducible and quantitative, significantly eliminating the risks of contamination encountered with other types of PCR-based assays [6].

Accordingly, the method has been widely used for accurately detecting occult micrometastatic tumour burden in resected lymph node specimens [7,8]. Two independent groups also showed the utility of real-time reverse-transcriptase (RT)-PCR targeting PSA and/or prostate-specific membrane antigen (PSMA) genes for detecting occult micrometastasis in the resected lymph node specimens from patients undergoing RP with no NHT [9,10]. Thus in the present study we used this assay in 989 lymph nodes isolated from 52 patients with clinically localized prostate cancer who had received NHT followed by RP, to clarify the significance of micrometastases in NHT-treated pelvic lymph node specimens.

PATIENTS AND METHODS

The study was approved by the research ethics committee of our institution, and informed consent was obtained from all patients at the time of enrolment. Lymph node specimens were obtained from 52 patients with clinically localized prostate cancer who had RP and pelvic lymphadenectomy after NHT between October 2001 and July 2004. At our institution NHT was generally used in patients with high-risk prostate cancer, defined as clinical stage T2c or T3, a serum PSA level before treatment of >20 ng/mL and/or a biopsy Gleason score of 8–10. Pelvic lymphadenectomy was performed targeting the obturator fossa and external iliac region by removing all fatty, connective, and lymphatic tissue.

All patients were treated with NHT consisting of LHRH analogue (leuprolide or goserelin) and antiandrogen (80 mg bicalutamide or 375 mg flutamide daily) for at least 8 months (median 12, range 8–20). Lymph node samples were also available from seven female patients with invasive bladder cancer who had a radical cystectomy. Each lymph node was bisected; one half was snap-frozen immediately and stored at −80 °C until assessed, and the remainder was fixed in formalin, embedded in paraffin wax, and stained with haematoxylin and eosin for histopathological examination. In this series, all pathological examinations were done by one pathologist according to the 2002 TNM tumour stage classification system. Biochemical recurrence was defined as a serum PSA level of ≥0.2 ng/mL, and none of the patients received any additional therapy until their serum PSA levels reached ≥0.4 ng/mL.

Total RNA was extracted from lymph node specimens using the acid-guanidinium isothiocyanate, phenol chloroform method, and was reverse transcribed using an Oligo dT

Variable	Group* A	B	C
No. of patients	4	19	29
No. of dissected lymph nodes	75	365	549
Histological examination			
No. of positive patients	4	0	0
No. of positive lymph nodes	11	0	0
Real-time RT-PCR assay for PSA			
No. of positive patients	4	15	0
No. of positive lymph nodes	19	25	0
Real-time RT-PCR assay for PSMA			
No. of positive patients	4	17	0
No. of positive lymph nodes	23	33	0
Micrometastasis			
No. of positive patients	4	19	0
No. of positive lymph nodes	28	40	0

TABLE 1 Outcomes of histological examination and real-time RT-PCR assay

**Group A, patients with histologically confirmed lymph node metastases; group B, patients with micrometastases despite the lack of histological evidence indicating nodal involvement; group C, patients with no findings of lymph node metastases.*

TABLE 2 Comparison of conventional prognostic indicators according to lymph node metastases detected by histological examination and real-time RT-PCR assay

Variable	Group* A	B	C	P A vs B	B vs C	C vs A
Number of patients	4	19	29			
Mean (SD) preop PSA level, ng/mL	21.8 (7.4)	20.1 (9.7)	10.2 (6.1)	0.75	<0.001	0.002
Biopsy Gleason score						
≤6	0	3	17	0.35	0.011	0.014
7	1	9	8			
≥8	3	7	4			
Clinical stage				0.41	0.44	0.21
T1c	1	9	17			
T2	3	10	12			
Pathological stage				0.88	0.23	0.64
pT2	2	8	18			
pT3	2	10	11			
pT4	0	1	0			
Surgical margin				0.62	0.027	0.038
Negative	2	12	26			
Positive	2	7	3			
Mean (SD) tumour volume, mL	1.45 (1.31)	1.36 (1.14)	1.12 (0.86)	0.89	0.41	0.50

**Group A, patients with histologically confirmed lymph node metastases; group B, patients with micrometastases despite the lack of histological evidence indicating nodal involvement; group C, patients with no findings of lymph node metastases.*

Variables	Hazard ratio (95% CI)	P
Pretreatment serum PSA (<10.0 vs ≥10.0 ng/mL)	2.91 (1.51–6.32)	0.190
Biopsy Gleason score (≤6 vs ≥7)	2.31 (1.02–2.49)	0.039
Clinical stage (T1c vs T2 or T2)	2.33 (0.77–6.90)	0.420
Pathological stage (pT2 vs pT3 or pT4)	1.19 (0.97–1.95)	0.230
Surgical margin (negative vs positive)	3.12 (0.85–9.31)	0.140
Tumour volume (<1.0 vs ≥1.0 mL)	2.66 (0.86–5.76)	0.092
Micrometastasis (negative vs positive)	1.09 (1.04–1.27)	0.014

TABLE 3
Association of the variables with BRFS

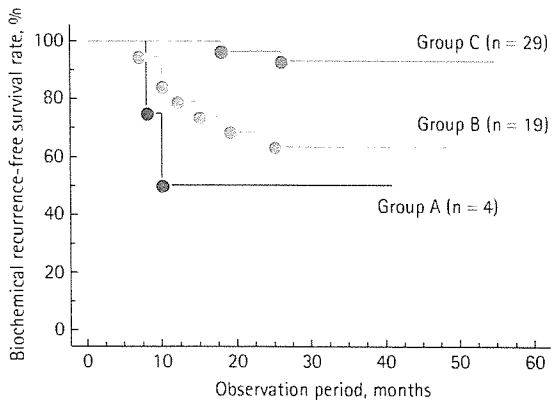

FIG. 1.
Comparison of BRFS rates in groups A, B and C by the Kaplan–Meier method. The BRFS rates in groups A and B were significantly lower than that in group C (P = 0.48, group A vs group B; P = 0.006, group B vs group C; P = 0.003, group C vs group A; by the log-rank test).

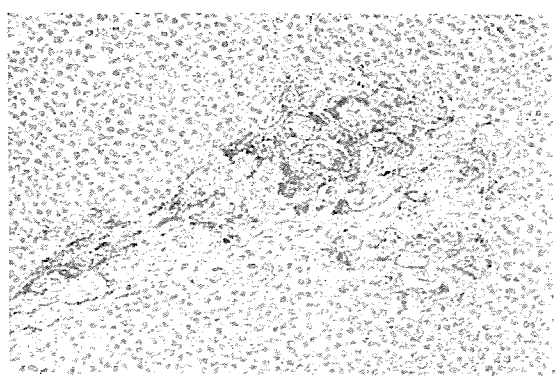

FIG. 2.
A representative result of immunohistochemically detected micrometastatic cancer foci using a monoclonal antibody against PSA in histologically uninvolved lymph node specimens.

and Superscript pre-amplification system (Life Technologies, Rockville, MD, USA). To analyse the expression levels of PSA, PSMA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, real-time quantitative PCR was performed using a Sequence Detector (ABI PRISM 7700, PE Applied Biosystems, Foster City, CA, USA). Selected sequences of forward and reverse (F and R respectively) primers and probes were as follows [10]; PSA-F: 5′CGTGGATTGGTGCTGCAC3′, PSA-R: 5′TGGGAATGCTTCTCGCACTC3′, PSA probe: 5′CCTGTCTCGGATTGTGGGAGGCTG3′, PSM-F: 5′TTCTGTTAAAAGCAGTGCTTCCAT3′, PSMA-R: 5′CGTATTTCGAGGGAGAGAATAGCTA3′, PSMA probe: 5′CACGGCCTCTCTCACGGATT ATAAGAACACA3′, GAPDH-F: 5′GAAGGT GAAGGTCGGAGTC3′, GAPDH-R: 5′GAAG ATGGTGATGGGATTTC3′, GAPDH probe: 5′CAAGCTTCCCGTTCTCAGCC3′. The probes used in the study consisted of an oligodeoxynucleotide with a 5′(6-carboxy-fluorescein) reporter dye and 3′(6-carboxytetramethylrhodamine) quencher dye.

Each cDNA was analysed by quantitative PCR using Master Mix (PE Applied Biosystems). The condition of thermal cycling was 50 cycles of amplification consisting of 15 s at 95 °C and 1 min at 60 °C.

Real-time quantification was based on the TaqMan assay according to the manufacturer's instructions, as described previously [11]. After the generation of a real-time amplification plot based on a normalized fluorescence signal, the threshold cycle (Ct), which is the fractional cycle number at which the amount of amplified target reached a fixed threshold, was determined. The Ct was then used for kinetic analysis and was proportional to the initial number of target copies in the sample. The starting quantity of a sample was calculated after comparison with the Cts of a serial dilution of a positive control, human prostate cancer LNCaP cells (American Type Culture Collection, Rockville, MD, USA). All specimens were analysed in triplicate and the mean values were used for quantification.

The quantification value of PSA or PSMA mRNA was described as each value relative to GAPDH mRNA. To exclude false-positives, we used the mean relative mRNA value plus 2 SDs of PSA or PSMA mRNA expression in 148 lymph nodes from female patients with bladder cancer as the threshold for PSA or PSMA, respectively, and values above the threshold for PSA or PSMA mRNA were defined as PSA or PSMA mRNA positive, respectively. We regarded specimens in which PSA and/or PSMA mRNA were positive as showing the 'presence of micrometastasis'. In cases diagnosed as having micrometastases by real-time RT-PCR despite the lack of positive findings on routine pathological examinations, sections next to the site of the original sections for haematoxylin and eosin staining were cut from the original formalin-fixed, paraffin-embedded blocks, and examined to determine whether occult foci of prostate cancer cells were present by immunohistochemical staining with a monoclonal antibody against PSA (Dako, Carpinteria, CA, USA) using standard immunohistochemical techniques, as reported previously [12]. All slides were reviewed by the same pathologist as described above, with no knowledge of any clinicopathological data.

Differences between the groups were compared using the chi-square test, unpaired

t-test, or Mann–Whitney *U*-test. The biochemical recurrence-free survival (BRFS) rates were calculated by the Kaplan–Meier method, and the difference was determined by the log-rank test. The significance of several factors in the time to biochemical recurrence was assessed by the Cox proportional hazards regression model, with statistical significance indicted at $P < 0.05$.

RESULTS

The expression of GAPDH mRNA in all lymph nodes was confirmed. In 148 lymph nodes from seven female patients with bladder cancer, the mean relative PSA and PSMA mRNA expression, plus 2 SDs, were 2.8 and 4.9, respectively, and these values were used as the threshold for the positive expression of PSA and PSMA mRNA in nodes from patients with prostate cancer in the subsequent study.

Eleven of the 75 lymph nodes from four patients with prostate cancer showed histopathological evidence of metastatic involvement, and real-time RT-PCR confirmed the expression of both PSA and PSMA mRNAs in all 11 nodes. In these four patients, positive PSA and/or PSMA mRNA expression was detected in an additional 17 histologically uninvolved lymph nodes; thus, in all, 28 lymph nodes were diagnosed as having occult micrometastases by the real-time RT-PCR assay. Of the 914 nodes from the remaining 48 patients with no histological evidence of pelvic lymph node metastases, positive PSA and PSMA mRNA expression was detected in 25 nodes from 15 patients and 33 nodes from 17 patients, respectively. Among these, 18 nodes from 13 patients were judged positive for both PSA and PSMA mRNA expressions; therefore, in all, 19 patients were regarded as having micrometastases to pelvic lymph nodes.

These outcomes are summarized in Table 1 by dividing the 52 patients into three groups, i.e. four with histologically detected lymph node metastases (group A), 19 with micrometastases despite the lack of histological evidence indicating nodal involvement (group B), and the remaining 29 with no findings of lymph node metastases on histological and real-time RT-PCR analyses (group C).

We then compared several clinicopathological features among these three groups. As shown in Table 2, despite the absence of significant differences between groups A and B in several factors examined, preoperative serum PSA level, biopsy Gleason score, and surgical margin status in groups A and B were significantly different from those in group C. During the observation period of the study (median 41 months, range 25–55) no patient had clinical progression; however, biochemical recurrence occurred in three, seven and two patients in groups A, B and C, respectively. The median interval between RP and biochemical recurrence in group A, B and C was 9, 12 and 22 months, respectively. As shown in Fig. 1, the BRFS rates in groups A and B were significantly lower than that in group C. Furthermore, multivariate analysis using the Cox regression hazard model showed that the presence of micrometastasis and the biopsy Gleason score were independently associated with BRFS irrespective of other factors examined in the study (Table 3).

To further confirm the presence of micrometastatic diseases in pelvic lymph nodes, we used immunohistochemical staining with a monoclonal antibody against PSA in 40 lymph nodes from 19 patients diagnosed as having micrometastases by the real-time RT-PCR assay despite the lack of pathological evidence of nodal involvement. Of the 40 nodes, 19 from 11 patients were evidently stained with PSA antibody; representative results are shown in Fig. 2.

DISCUSSION

Lymph node metastasis has been shown to be the most useful predictive factor for a poor prognosis in patients who have had RP for clinically localized prostate cancer with no NHT; however, ≈30% of such patients with no evidence of pathological nodal involvement will develop biochemical disease recurrence [13,14]. Although the cause of biochemical recurrence after RP is likely to be multifactorial, several investigators showed, using molecular and histochemical approaches, that a significant proportion of these recurrences might be due to occult metastases to pelvic lymph nodes that were undetected by routine pathological examinations [4,5]. Recently, two independent groups also reported the usefulness of quantitative real-time RT-PCR targeting the expression of PSA and PSMA genes for detecting occult micrometastatic tumour burden in the resected lymph node specimens from patients undergoing RP with no NHT [9,10]. To date, there has been no study investigating the micrometastatic features of prostate cancer cells to pelvic nodes after NHT; therefore, we used our previously reported assay on 989 lymph nodes dissected at RP from 52 patients with clinically localized prostate cancer who received NHT followed by RP, to clarify the significance of micrometastases in NHT-treated pelvic lymph node specimens.

Based on an autopsy study, ≈20 lymph nodes were shown to serve as a guideline for optimum and representative pelvic lymph node dissection [15]. In the present series, the mean number of pelvic lymph nodes removed at RP in the 52 patients was 19.0; therefore, the procedure for pelvic lymphadenectomy in this study, which met this requirement, would be suitable. We also examined 148 pelvic lymph nodes obtained from seven female patients undergoing radical cystectomy for invasive bladder cancer, to determine the appropriate thresholds for the positive expression of PSA and PSMA mRNAs on real-time RT-PCR. Although it is inevitable that extremely low levels of PSA and PSMA expression would be detected in lymph nodes from females, considering the principle of this assay, PSA and PSMA gene expressions are highly restricted to prostate epithelial cells [5], indicating that the thresholds used were appropriately determined. Furthermore, the expression levels of both PSA and PSMA mRNAs in each node, which were shown to be heterogeneously expressed in prostate cancers [5], were measured, and nodes diagnosed as positively expressing PSA and/or PSMA mRNA were judged to show the presence of micrometastatic cancer foci. Collectively, these findings suggest that the present study was conducted under ideal conditions for evaluating the significance of micrometastasis of prostate cancer cells in pelvic nodes.

In the present series, we diagnosed the presence of micrometastatic tumour foci in 68 nodes from 23 patients by real-time RT-PCR, including 11 histologically involved nodes from four patients. This proportion of occult micrometastasis to pelvic nodes is similar to that in patients undergoing RP with no NHT, evaluated by the same method as in the present study [9]. In addition, the

sensitivity of this assay for detecting micrometastases in pelvic nodes is higher than that reported in previous studies evaluated by conventional RT-PCR [4,5]. It has been widely recognized that it is difficult to exactly diagnose the distribution of cancer cells in specimens obtained after NHT, particularly in lymph nodes, by routine pathological examination [2,3]. Considering these findings, real-time RT-PCR targeting PSA and PSMA genes could accurately diagnose the presence of micrometastatic prostate cancer cells in NHT-treated pelvic nodes, although the residual foci of malignant diseases become atrophic after NHT.

The characterization of clinicopathological features according to nodal status showed that despite the lack of significant differences between patients with histologically detected nodal involvement and those with nodes positive for micrometastases, there were significant differences in some adverse factors, including the preoperative PSA level, biopsy Gleason score and surgical margin status, between patients with and with no micrometastases. Furthermore, these factors have been shown to be risk factors for biochemical recurrence in NHT-treated patients [16,17]. These findings strongly suggest that some of the micrometastatic disease diagnosed by the current real-time RT-PCR have biological characteristics similar to those of histologically positive nodal disease in patients who received NHT. The prognostic outcome also supports this hypothesis, i.e. the BRFS in patients with micrometastases was significantly lower than that in those with no micrometastases, while there was no significant difference between patients with histologically confirmed nodal involvement and those with micrometastases.

In addition, the presence of micrometastasis and the biopsy Gleason score were independently associated with biochemical recurrence, irrespective of other factors examined. These findings suggest that the presence of micrometastatic prostate cancer cells in pelvic lymph nodes might at least partly be involved in the development of biochemical recurrence in patients undergoing RP after NHT.

There were several problems associated with the present study; more patients must be assessed and the observation period prolonged to make definitive conclusions on the prognosis. In addition, whether histologically undetectable and/or dormant micrometastatic disease in the lymphatic system always results in clinically significant disease recurrence should be investigated. Also, it would be interesting to evaluate whether removing the micrometastatic nodes affects the prognosis, as several recent studies reported the possible effect of lymphadenectomy on the survival of patients with histologically positive nodes [18,19]. Finally, there are some technical matters to be addressed: targeting genes different from those used in the present study, e.g. human glandular kallikrein and cytokeratin [20]; establishing an efficient method for RNA extraction from paraffin sections of lymph nodes [21]; and simplifying the real-time RT-PCR procedures used here, considering the introduction of this assay into clinical practice. Assessing these issues might facilitate the development of a more suitable approach for lymphadenectomy for NHT-treated patients with prostate cancer.

In conclusion, residual foci of atrophic cancer cells in resected lymph node specimens after NHT can be difficult to identify with conventional pathological examination; however, the present results showed the utility of quantitative real-time RT-PCR targeting the expression of PSA and PSMA genes for detecting micrometastatic tumour foci in pelvic lymph nodes from clinically localized prostate cancer at RP after NHT. Furthermore, the present findings suggest that some micrometastases in pelvic lymph nodes might at least partly be involved in the development of biochemical recurrence after NHT followed by RP.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **NHT**, neoadjuvant hormonal therapy; **RP**, radical prostatectomy; **RT**, reverse-transcriptase; **PSMA**, prostate-specific membrane antigen; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **BRFS**, biochemical recurrence-free survival; Ct, threshold cycle.

Quantitative Detection of Micrometastases in Pelvic Lymph Nodes in Patients with Clinically Localized Prostate Cancer by Real-time Reverse Transcriptase-PCR

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Abstract **Purpose:** Routine pathologic examination can miss micrometastatic tumor foci in the lymph nodes of patients with prostate cancer, resulting in confusion during tumor staging and clinical decision-making. The objective of this study was to clarify the significance of micrometastases in pelvic lymph nodes in patients who underwent radical prostatectomy for prostate cancer.

Experimental Design: The expression of prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) in 2,215 lymph nodes isolated from 120 patients with clinically localized prostate cancer was assessed by a fully quantitative real-time reverse transcriptase-PCR. We regarded specimens in which either PSA or PSMA mRNAs were positive as proof of the "presence of micrometastasis." Immunohistochemical staining of lymph node specimens with an antibody against PSA was also done.

Results: Pathologic examinations detected tumor cells in 29 lymph nodes from 11 patients, and real-time reverse transcriptase-PCR further identified micrometastasis in 143 lymph nodes from 32 patients with no pathologic evidence of lymph node involvement. The presence of micrometastatic cancer cells was confirmed by immunohistochemical staining in 61 lymph nodes from 17 patients with pathologically negative lymph nodes. The presence of micrometastases was significantly associated with other conventional prognostic variables, including serum PSA value, pathologic stage, Gleason score, and tumor volume. Biochemical recurrence was detected in 32 patients, 17 of whom were negative for lymph node metastasis by pathologic examination (including 4 patients with pathologically organ-confined disease), but were diagnosed as having micrometastasis. Biochemical recurrence–free survival rate in patients without micrometastasis was significantly higher than in those with micrometastasis irrespective of the presence of pathologically positive nodes. Furthermore, only the presence of micrometastasis was independently associated with biochemical recurrence regardless of other factors examined.

Conclusions: These findings suggest that ~30% of clinically localized prostate cancers shed cancer cells to the pelvic lymph nodes, and that biochemical recurrence after radical prostatectomy could be explained, at least in part, by micrometastases in pelvic lymph nodes.

Pelvic lymph node metastasis has been considered the most important predictive factor of disease recurrence in patients with clinically localized prostate cancer who have undergone radical prostatectomy. Patients with organ-confined prostate cancer have a good prognosis and a low risk of disease recurrence following radical prostatectomy, whereas biochemical recurrence, characterized by an increasing serum prostate-specific antigen (PSA) value, occurs in ~10% of patients in this category (1, 2). Because routine microscopic examination of lymphadenectomy specimens can miss small cancer foci, this finding might partially account for the presence of histologically undetectable micrometastases in the pelvic lymph nodes. In fact, various investigators have shown that higher sensitivity for detecting micrometastatic cancer cells in surgically removed pelvic lymph nodes at radical prostatectomy can be achieved by several molecular and histologic techniques targeting prostate-specific gene expression, including reverse transcriptase-PCR (RT-PCR) and immunohistochemical staining (3–6). To date, however, none of these methods have been introduced into clinical practice due to various limitations, such as a high false-positive rate and complicated procedures. Collectively, these findings suggest that an improved approach for detecting micrometastatic prostate cancer cells in the lymph nodes needs to be identified.

Recently, a real-time detection and quantitative PCR-based assay was developed (7). The advantage of this assay is the

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specific detection of rare events; that is, sensitivity has been shown to allow for the detection of 10 to 100 pg of RNA from the target gene. Furthermore, it is highly reproducible and quantitative, significantly eliminating the risks of contamination encountered with other types of PCR-based assays, and requires no post-PCR product manipulation. Accordingly, the method has been widely used for accurately detecting occult micrometastatic tumor burden in resected lymph node specimens (8–12). For example, Van Trappen et al. used real-time RT-PCR targeting the cytokeratin 19 gene, and reported that ~50% of early stage cervical cancers shed tumor cells to the pelvic nodes, and the amount of cytokeratin 19 expression was related to the clinicopathologic features (8). To our knowledge, however, there has not been any study analyzing lymph node specimens obtained from patients with prostate cancer using real-time RT-PCR assay in order to clarify the significance of micrometastases in biochemical recurrence after successful radical surgery.

Expression of the PSA and prostate-specific membrane antigen (PSMA) genes is exclusively restricted to prostate epithelial cells (4), and this high specificity made it possible to identify metastatic prostate cancer cells among non–prostate cells. Moreover, these two genes are expressed heterogenously in prostate epithelial cells (4); thus, simultaneous targeting of these two specific antigens might promote the detection of metastatic prostate cancer cells with a wide phenotypic spectrum. Considering these findings, we did a fully quantitative real-time RT-PCR assay targeted against PSA and PSMA gene expression in 2,215 fresh pelvic lymph nodes obtained from 120 patients with clinically localized prostate cancer, then analyzed the clinical significance of occult micrometastasis of prostate cancer cells to pelvic lymph nodes.

Patients and Methods

Surgical specimens. This study was approved by the research ethics committee of our institution, and informed consent was obtained from all patients at the time of enrollment. Lymph node specimens were obtained from 120 patients with clinically localized prostate cancer who underwent radical retropubic prostatectomy and pelvic lymphadenectomy without neoadjuvant therapies between October 2001 and July 2004. Pelvic lymphadenectomy was done, targeting the obturator fossa and external iliac region by removing all fatty, connective, and lymphatic tissue. Lymph node samples were also available from seven female patients with invasive bladder cancer who underwent radical cystectomy. Each lymph node was bisected. One half was snap-frozen immediately and stored at −80°C until assessed, and the remainder was fixed in formalin, embedded in paraffin, and stained with H&E for histopathologic examination. In this series, all pathologic examinations were done by a single pathologist according to the Unio Internationale Contra Cancrum (tumor-node-metastasis) tumor stage classification (13). Biochemical recurrence was defined as a serum PSA level of ≥0.2 ng/mL; none of the patients received any additional therapies until their serum PSA levels reached ≥0.4 ng/mL.

Real-time RT-PCR assay. Total RNA was extracted from lymph node specimens using the acid guanidinium isothiocyanate, phenol chloroform method, and 1 μg of each total RNA was reverse-transcribed using an Oligo dT and Superscript preamplification system (Life Technologies, Rockville, MD). To analyze the expression levels of PSA, PSMA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, real-time quantitative PCR was done using Sequence Detector (ABI PRISM 7700; PE Applied Biosystems, Foster City, CA). The sequences of primers and probes for these genes were determined by Primer Express software (PE Applied Biosystems). Selected sequences of forward (F) and reverse (R) primers, and probes are as follows: PSA F, 5′ CGTGGATTGGTGCTGCAC 3′; PSA R, 5′-TGGGAATGCTTCTCGCACTC-3′; PSA probe, 5′-CCTGTCTCGGATTGTGGGAGGCTG-3′; PSMA F, 5′-TTCTGTTAAAAGCAGTGCTTCCAT-3′; PSMA R, 5′-CGTATTTCGAGGGAGAGAATAGCTA-3′; PSMA probe, 5′-CACGGCCTCTCTCACGGATTATAAGAACACA-3′; GAPDH F, 5′-GAAGGTGAAGGTCGGAGTC-3′; GAPDH R, 5′-GAAGATGGTGATGGGATTTC-3′; GAPDH probe, 5′-CAAGCTTCCCGTTCTCAGCC-3′. The probes used in this study consisted of an oligodeoxynucleotide with a 5′ FAM (6-carboxy-fluorescein) reporter dye and 3′ TAMRA (6-carboxy-tetramethylrhodamine) quencher dye. Each complementary DNA was analyzed by quantitative PCR in a 50 μL volume using Master Mix (PE Applied Biosystems). The thermal cycling conditions were composed of 50 cycles of amplification consisting of 15 s at 95°C and 1 min at 60°C.

Real-time quantitation was done based on TaqMan assay according to the manufacturer's instruction as described previously (14, 15). After the generation of a real-time amplification plot based on normalized fluorescence signals, the threshold cycle (Ct), which is the fractional cycle number at which the amount of amplified target reached a fixed threshold, was determined. The Ct was then used for kinetic analysis and was proportional to the initial number of target copies in the sample. The starting quantity of a sample was calculated after comparison to the Cts of a serial dilution of a positive control, human prostate cancer LNCaP cells (American Type Culture Collection, Rockville, MD). All serial dilutions were carried out in duplicate, and the reactions to generate standard curves were repeated twice (each time in triplicate). All clinical specimens were also analyzed in triplicate and the mean values were used for quantification. The coefficient of variation for triplicate reactions was <10%, and the coefficient of variation between assays was also <10%. In this series, except for samples in which PSA and/or PSMA were not amplified, the ranges of Ct values for PSA and PSMA were as follows: PSA, 17.2 to 43.4; PSMA, 13.3 to 41.1.

Both the precise amount and quality of total RNA added to each reaction mix are extremely difficult to assess; therefore, transcripts of the GAPDH gene were quantified as an internal reference according to a quantitative PCR assay. The quantification value of PSA or PSMA mRNA was described as each value relative to GAPDH mRNA. To exclude false positives, we used the mean relative mRNA value plus 2 SDs of PSA or PSMA mRNA expression in 148 lymph nodes from female patients with bladder cancer as the cutoff value for PSA or PSMA, respectively, and values above the cutoff value for PSA or PSMA mRNA were defined as PSA- or PSMA mRNA–positive, respectively. In this study, we regarded specimens in which PSA and/or PSMA mRNA were positive as proof of the "presence of micrometastasis."

Immunohistochemical staining. In cases diagnosed as having micrometastases according to real-time RT-PCR, despite the lack of positive findings on routine pathologic examinations, sections adjunct to the site of the original sections for H&E staining were cut from the original formalin-fixed, paraffin-embedded blocks, and examined to determine whether occult foci of prostate cancer cells were present by immunohistochemical staining with a monoclonal antibody against PSA (Dako, Carpinteria, CA) using standard immunohistochemical techniques as reported previously (16). After staining, the sections were counterstained with hematoxylin. All slides were reviewed by the same pathologist without knowledge of any clinicopathologic data, as described above.

Statistical analysis. Differences between the two groups were compared using the χ^2 test, unpaired t test, or Mann-Whitney U test. The biochemical recurrence–free survival rates were calculated by the Kaplan-Meier method, and the difference was determined by log rank test. Forward stepwise logistic regression analysis was used to determine the association between several variables and biochemical recurrence. The significance of several factors in the time to biochemical recurrence was assessed by the Cox proportional hazards regression model. All statistical calculations were done using StatView 5.0 software