A G/A Polymorphism in the Androgen Response Element 1 of Prostate-specific Antigen Gene Correlates with the Response to Androgen Deprivation Therapy in Japanese Population

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Abstract. *Prostate-specific antigen (PSA) gene expression is regulated by androgen receptor (AR) through androgen response elements (AREs) in the promoter region of the PSA gene. A single nucleotide polymorphism with guanine (G) to adenine (A) substitution is identified at position –158 in the ARE of the PSA gene. The purpose of this study was to investigate the allelic differences in the PSA promoter activity in vitro and the relation to several clinical factors of prostate cancer patients in the Japanese population. No significant differences of promoter activity in luciferase assay and binding activity of androgen receptor were noted between the two alleles in vitro. The PSA –158 G/A polymorphism was determined by PCR amplification and restriction digestion assays in 101 organ-confined prostate cancer (PC) patients who underwent radical prostatectomy and 52 controls with benign prostatic hyperplasia. The results revealed that homozygosity for the A allele in Japanese is less common (only 8.5%) than in ethnic populations. There were no significant differences in serum PSA value at the time of diagnosis, differentiation of cancer, pathological stage, cancer volume or ratio of serum PSA/cancer volume. However, cancer volume after neoadjuvant endocrine therapy was significantly smaller in GG genotype than in AA + AG genotypes. Our data indicate that the PSA –158 G/A polymorphism has no effect on the PSA promoter activity in vitro and no association with the serum PSA level in Japanese men, however suggest that the patients with GG genotype of ARE1 may be more sensitive to androgen ablation therapy. Taken together, the ARE1 polymorphism in the PSA gene promoter may be one of the biomarkers for response to androgen deprivation therapy.*

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The diagnosis of prostate cancer is improving, especially because of the widespread use of its tumor marker, prostate-specific antigen (PSA). Serum PSA concentration is a useful tool for early diagnosis and monitoring in prostate cancer progression (1, 2). The PSA gene codes for a serine protease of 240 amino acid glycoprotein (33 kDa) and is found in the serum as a 33 kDa monometer, as an 100 kDa complex with α1-antichymotrypsin and as an 800 kDa complex with α2-macroglobulin (1). The PSA gene is regulated by steroid hormones and its transcription arises from androgen receptor (AR) interaction with androgen response elements (AREs) in the PSA promoter gene (3-7).

The AR gene contains two polymorphic polynucleotide (CAG and GGC) repeats in the cording region. *In vitro* studies suggested that the transactivation activity of the AR is inversely related to the length of the CAG repeat (8-10). Several reports have shown the association of short AR CAG allele (less than 21 or 22 CAG repeats) with an increase in prostate cancer risk (11-13). Three AREs were identified in the PSA promoter at position –170 bp (ARE1), –394 bp (ARE2) (7) and –4200 bp (ARE3) in the 5' upstream enhancer region from the transcription start site (3-6). Many studies have shown that ARE polymorphisms correlate with serum PSA levels, differentiation, clinical stage and prostate

carcinogenesis (14-16). The polymorphism –158G/A (guanine to adenine substitution at position of –158 of the PSA promoter region within ARE1) (17) was proposed to interact differently with AR and, thereby, associate with the prostate cancer risk (16).

It has been reported that patients with GG genotype were at significantly increased risk for advanced prostate cancer and subjects with both GG genotype and short CAG allele in AR had a more than 5-fold increase in prostate cancer risk (16). We focused on ARE1 polymorphism and investigated whether ARE1 polymorphism correlated with clinicopathological features of prostate cancer in Japanese cases. The allelic differences in PSA promoter activity were examined by luciferase assay *in vitro*. Moreover, the association between the polymorphism and clinicopathological characteristics, including serum PSA levels, grades, differentiations, cancer volumes and effect of neoadjuvant endocrine therapy, were characterized.

Materials and Methods

PSA ARE1 expression constructs. Genomic DNA extracted from individuals homozygous for the A/A or G/G genotypes (identified by PCR and DNA sequencing) were used as a template. Two kinds of 6-kb PSA promoter fragments containing ARE1 (ARE1-A or ARE1-G), ARE2, ARE3 and the transcription start site of the PSA gene were obtained by PCR. The sequences of primers were as follows: forward primer 5'-ACGTGACTCTTTTCTTTTCCCGGTG ACATCGTG-3' and reverse primer 5'-ACGTCTCGAGTGGGGA GAAAGTTCTGGGGTAAGT-3'. The PCR fragments were subcloned into the luciferase reporter vector pGL3 Basic (Promega, Madison, WI, USA) (ARE1-A Luc and ARE1-G Luc).

Transient transfection and reporter gene activity assay. LNCaP cells ($5x10^5$) were plated on 24-well plates and incubated in RPMI 1640 with 10% fetal bovine serum for 24 h before transfection. Cells were transfected with ARE1-A Luc or ARE1-G Luc and 1 μg of reporter plasmid of pRL-TK vector containing cDNA encoding Renilla luciferase (Promega) for normalization, using the Tfx transfection reagent (Promega) in phenol red-free RPMI 1640 containing 2% charcoal-stripped fetal bovine serum. Twenty-four h after transfection, the cells were incubated in RPMI 1640 with 2% charcoal-stripped fetal bovine serum containing 1 nM testosterone analog R1881. After 24 h incubation in experimental medium, the cells were washed and lysed. Luciferase activity was assayed using the Dual-Luciferase Reporter assay system (Promega). All values in transfection were normalized by co-transfection with the pRL-TK vector. Each experiment was performed at least in triplicate.

Nuclear extract preparation. Nuclear extracts were prepared as described previously (18). All the following steps were performed at 4˚C. The cells were resuspended in sucrose buffer [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 0.5 mM PMSF and 0.5% NP-40] and then the lysate was centrifuged at 500 g for 5 min to obtain the nuclei pellet, which were washed with sucrose buffer without NP-40. The nuclei were resuspended in low salt buffer [20 mM HEPES (pH7.9), 25% glycerol, 0.02 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP-40, 0.5 mM DTT and 0.5 mM PMSF] followed by addition of high salt buffer [20 mM HEPES (pH7.9), 25% glycerol, 0.8 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP-40, 0.5 mM DTT and 0.5 mM PMSF] with incubation for 20 min on a rotatory platform. The samples were diluted in diluent buffer [2.5 volumes of HEPES (pH7.6), 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF] and were then centrifuged at 13.700-g for 20 min. Five-microgram aliquots of supernatant (nuclear extract) were stored at –70˚C.

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides spanning the region between –156/–180 of ARE1 were prepared as probes: AA genotype 5'-CAG AAC AGC AAG TAC TAG CTC TCC-3' and GG genotype 5'-CAG AAC AGC AAG TGC TAG CTC TCC-3'. The binding reactions were performed by incubating ^{32}P-labeled double-stranded oligonucleotides with nuclear extract in binding buffer [10 mM Tris-HCL (pH7.6), 5% glycerol, 1 mM EDTA, 50 mM NaCl, 1 mM DTT and 0.1 μg/ml poly (dI-dC). For oligonucleotide competition assays, 25- or 100-fold molar excesses of unlabelled oligonucleotides were also added to the binding reaction. After incubation at 25˚C for 30 min, the reaction mixtures were separated on 4% nondenaturing polyacrylamide gel. For antibody supershift assays, 1 μl of AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the mixture before adding the probes and incubating at 4˚C for 1 h. Protein-DNA complexes were separated on 4% nondenaturing polyacrylamide gels in 0.5xTris-Borate-EDTA buffer at 4˚C and were visualized by autoradiography.

Study population. From January 2000 to July 2004, a total of 101 clinically localized prostate cancer patients, who underwent radical prostatectomy at the Mie University, Saiseikai Matsusaka General Hospital and Suzuka General Hospital, Japan, were analyzed. Among them, fifty-one patients were administered neoadjuvant endocrine therapy (either monotherapy with LH-RH analog or maximam androgen blockade with LH-RH analog and antiandrogen). Fifty-two symptomatic benign prostate hyperplasia (BPH) patients who underwent retropubic prostatectomy and had no pathological evidence of prostate cancer served as controls to be compared with prostate cancer patients. All serum PSA levels were measured using E-test Tosoh II assay with the AIA-600 machine (Tosoh, Tokyo, Japan). Written informed consent was obtained from all patients and the work was conducted according to the procedures approved by the institutional review board.

Genotyping of polymorphism. Genomic DNA was obtained from peripheral blood leukocytes (prostate cancer patients) and from prostatectomy tissue samples (BPH patients) with a DNeasy Tissue Kit (QIAGEN, Germany). The genotypes were determined using a PCR amplification and digestion assay. PCR amplification spanning the G/A substitution at position –158 in the promoter region of the PSA gene was performed in a total volume of 50 μl reaction containing 50 ng genomic DNA, 800 mM dNTP, 2.5 mM $MgCl_2$, 2.5 units Ampli Taq (Applied Biosystems, Foster City, CA, USA) and 0.5 mM for each primer, forward primer (5'-TTG TAT GAA GAA TCG GGG ATC GT-3') and reverse primer (5'-TCC CCC AGG AGC CCT ATA AAA-3') as described previously (16). Cycle conditions were 94˚C for 5 min, followed by 35 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec and a final

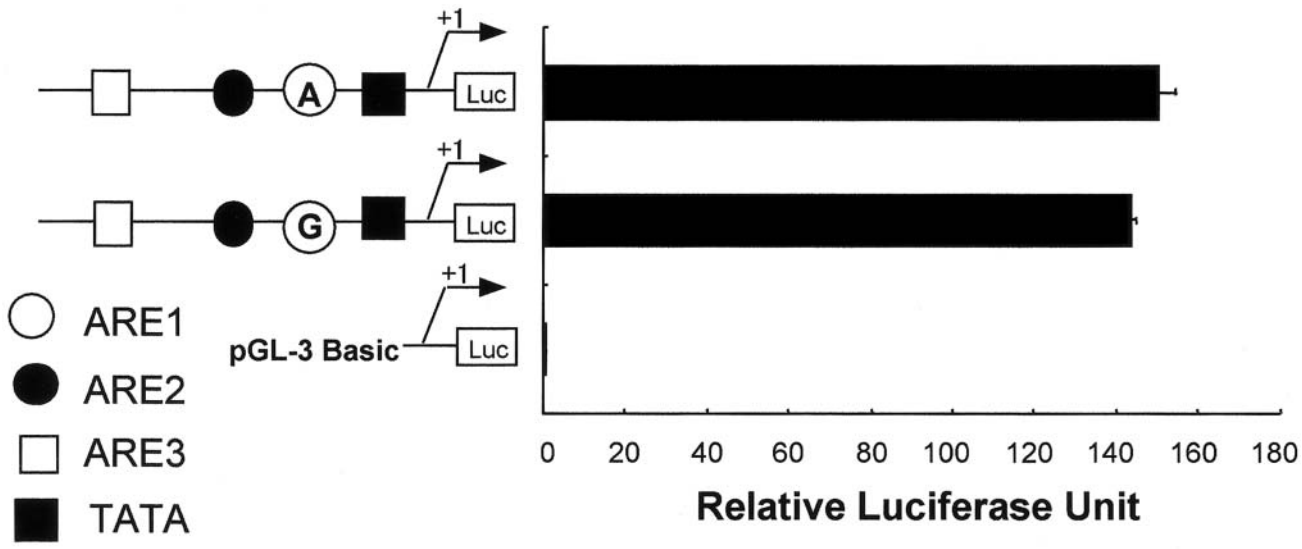

Figure 1. *Promoter activity of the PSA –158 G/A polymorphism. LNCaP cells were transiently transfected with ARE1 constructs (ARE1-A Luc or ARE1-G Luc) in the presence of 1 nM R1881. The diagram on the left is a map of 6.0 kb PSA promoter in the Luc plasmid. Consensus binding sites for ARE1 (○), ARE2 (●), ARE3 (❒) and TATA (■) are indicated. Normalized luciferase activities are depicted on the right. The data are mean±SD.*

cycle at 72˚C for 10 min. Twenty microliters of the PCR product were digested with *Nhe* I restriction enzyme (Toyobo, Japan) at 37˚C for 4 h. Digested products were separated on 2% agarose gel and defined by three distinct banding patterns: AA (300 bp), AG (150, 300 bp), GG (150 bp).

Measurement of cancer volume. Five-mm cut specimens from radical prostatectomy were processed for HE staining and 5-micron thick serial sections were measured. The tumor surface area was marked with a pen on each slide and the volume was calculated using the NIH image 1.62 software.

Statistical analysis. Results are described as the mean±SEM. Categorical variables of genotype, differentiation and pathological stage were compared by Chi-squared analysis and statistical analyses of variance for prostate volume, and PSA levels were compared by Bonferroni-Dunn *post hoc* test using the Stat View software (Abacus Concepts Inc., Berkeley, CA, USA). AA and AG genotypes were pooled together to compare with GG genotype because the case number of AA genotype was too small. $P<0.05$ for Chi-squared test and $p<0.01$ for Bonferroni-Dunn tests were considered statistically significant.

Results

Effect of ARE1 allels on PSA promoter and binding of androgen receptor. To investigate whether the two ARE1 alleles differed in the promoter activity, LNCaP cells were transfected with ARE1-A Luc and ARE1-G Luc constructs and the luciferase assay was performed. As shown in Figure 1, no significant differences were observed in promoter activities between the two reporter constructs. The binding activity of androgen receptor to ARE1-A and ARE1-G also did not show any difference in the gel mobility shift assay (Figure 2).

Genotypes of prostate cancer and BPH patients. The subjects included 101 cases of prostate cancer and 52 cases of benign prostatic hyperplasia (BPH). The genotype frequencies for ARE1 polymorphism were AA (7.9%), AG (34.7%) and GG (57.4%) in the prostate cancer patients, and AA (9.6%), AG (32.7%) and GG (57.7%) in the BPH patients (Table I). A significant difference in genotype distribution was not seen between the two groups. The AA genotype was less frequent in both prostate cancer and BPH patients. Serum PSA levels were measured at diagnosis with prostate cancer and the levels of AA, AG and GG genotypes were 16.6±6.9, 16.5±3.6 and 15.9±1.6 ng/ml, respectively. Significant differences were not seen among the three genotypes. The distribution of cancer differentiation was categorized as well, moderate and poor. Although statistically not significant, a higher ratio of well-differentiated prostate cancers and a lower ratio of poorly-differentiated prostate cancers were observed in AA + AG genotypes than in the GG genotype (42.9% *vs.* 24.1%, and 19.0% *vs.* 33.3%, respectively) (Table II). No association of pathological stage with PSA –158 G/A polymorphism was found (Table II). The allelic differences of the effect of neoadjuvant endocrine treatment prior to surgery was subsequently evaluated. There was no significant difference in cancer volume between the AA+AG genotypes and the

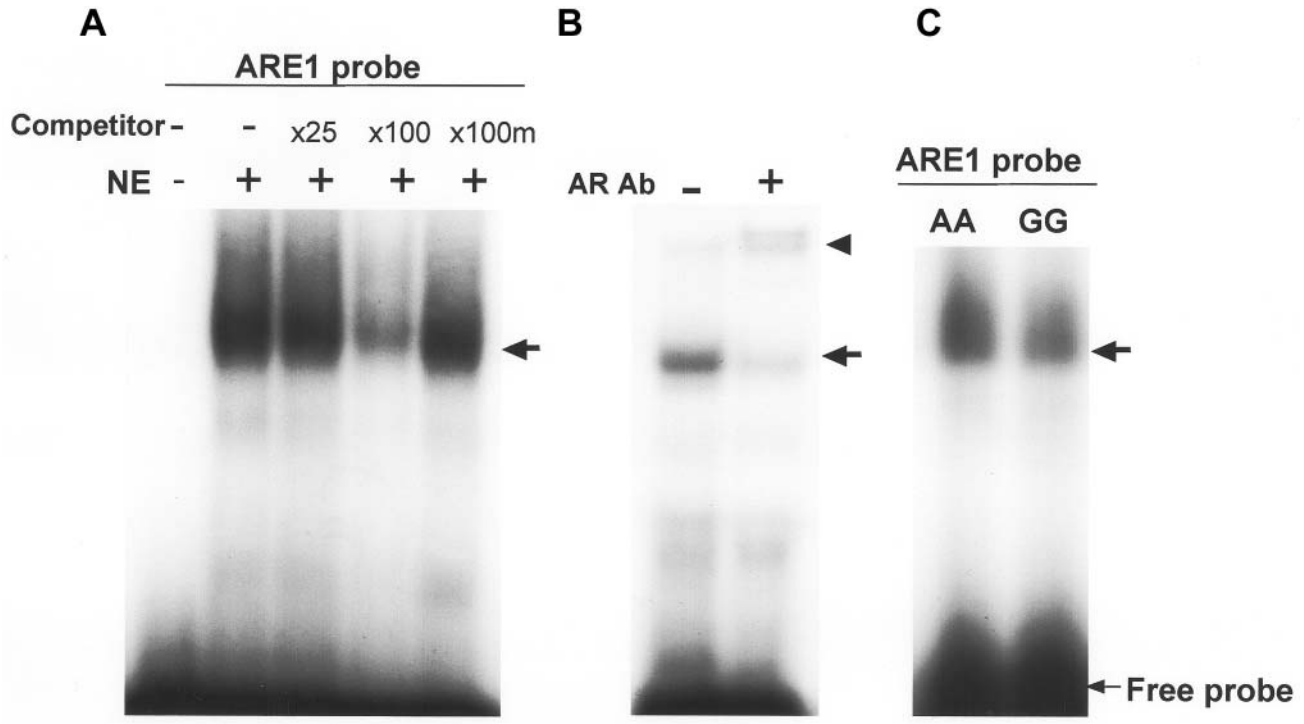

Figure 2. *Formation of DNA-protein complexes on the ARE1 in PSA gene promoter. Nuclear extracts (NE) from LNCaP cells were incubated with a ^{32}P-labeled fragment of the PSA promoter containing the ARE1 in the absence or presence of 25- or 100-fold molar excess, or the mutant oligonucleotide (100m). Nucleo-protein complexes were analyzed by electrophoresis on a 4% polyacrylamide gel (A). To identify proteins bound to the ARE1 consensus site, the reaction mixture was incubated with an anti-AR antibody (B). The probes containing the A or G genotype of ARE1 were used in EMSA (C). Arrows indicate the specific DNA-AR complexes and the arrowhead shows supershifted band in the presence of AR antibody.*

GG genotype in the patients without neoadjuvant endocrine therapy (697.4±161.3 *vs.* 784.8±160.1 mm^3, respectively) (Table III). Cancer volume after neoadjuvant endocrine treatment was significantly smaller in the GG genotype than in the AA+AG genotypes (644.7±189.7 *vs.* 246.0±55.5 mm^3, respectively, p=0.008) (Table III). There was no significant difference in the periods of neoadjuvant endocrine treatment between GG and AA+AG. No significant difference in the ratio of serum PSA level/cancer volume was observed between the two groups in the patients without neoadjuvant endocrine therapy (Table III).

Discussion

PSA is a useful biomarker for prostate cancer. It has been shown that serum PSA level is proportional to tumor volume and correlates positively with the clinical stage of the disease (19). PSA gene expression is tightly regulated by androgens, as mediated by the AR (3-7). The AR modulates transcription, through its interaction with consensus DNA binding site, termed androgen response element (ARE).

At least three AREs are present in the PSA promoter, at positions –170 (ARE1), –394 (ARE 2) (7) and –4200 (ARE3) (3-6). We studied the ARE1, which was located at the most proximal of AREs. A G/A substitution was verified at position –158 of ARE1: AGAACAnnnAGTACT and AGAACAnnnAGTGCT (17). We initially evaluated the allelic frequencies of PSA polymorphism at position –158 in Japanese men. The AA genotype of ARE1 in PSA polymorphism was less frequent in both the Japanese prostate cancer (7.9%) and BPH groups (9.6%) than in African-American and non-Hispanic Caucasian populations (approximately 25%) (20). It is also reported that the G allele was most frequent among healthy Japanese Americans (20) and Japanese people (15). According to these results, the Japanese may have the hereditary characteristic of A to G substitutions of ARE1.

Previous studies have shown that homozygosity for the A allele is associated with higher serum PSA levels in men with or without prostate cancer, including both localized and advanced disease (14). However, we did not observe a significant association between ARE1 polymorphism and serum PSA levels at the time of diagnosis. We only

Table I. *The genotypic frequencies of PSA polymorphism in patients with prostate cancer and controls with BPH.*

	PC	BPH
Number of patients	101	52
age	66.3±5.5	69.9±7.2
Genotype*		
AA	8 (7.9%)	5 (9.6%)
AG	35 (34.7%)	17 (32.7%)
GG	58 (57.4%)	30 (57.7%)

*p=0.925.

Table II. *Tumor differentiation and pathological stage in patients with prostate cancer with different PSA-158G/4 genotypes.*

	AA+AG	GG	p value
Number of patients	42	54	
well	18 (42.9%)	13 (24.1%)	
mod.	16 (38.1%)	23 (42.6%)	
poorly	8 (19.0%)	18 (33.3%)	0.107
Number of patients	43	58	
pT2	25 (58.1%)	36 (62.1%)	
pT3	17 (39.5%)	18 (31.0%)	
pT0	1 (2.4%)	4 (6.9%)	0.445

*AG genotype.
well=well-differentiated.
mod=moderately-differentiated.
poorly=poorly-differentiated.

Table III. *Serum PSA, cancer volume, and PSA/cancer volume ratio in prostate cancer patients with or without neodjuvant endocrine therapy.*

	AA+AG	GG	p value
Without NET			
Number of patients	25	25	
PSA (ng/ml)	10.1±3.2	12.6±1.6	0.240
Ca vol (mm^3)	697.4±161.3	784.8±160.1	0.485
PSA/Ca vol	91.9±42.3	58.9±23.2	0.939
With NET			
Number of patients	18	33	
NET periods (weeks)	18.1±4.4	13.8±1.8	0.912
PSA (ng/ml)	25.4±6.4	18.3±2.3	0.548
Ca vol (mm^3)	644.7±189.7	246.0±55.5	0.008
PSA/Ca vol	58.3±16.8	185.5±47.4	0.037

NET: Neoadjuvant endocrine therapy.
Ca vol: cancer volume.

investigated the clinically organ-confined prostate cancers, which had good indications for radical prostatectomy. High levels of expression of mitogen-activated protein (MAP) kinases were detected in more advanced, metastatic and androgen-independent prostate cancers (21). We reported that the MAP kinase pathway is involved in the androgen-independent PSA expression in LNCaP cells (22). Our preliminary data show that the promoter activity of ARE1-G Luc is 2-fold higher than that of ARE-1A Luc, when LNCaP cells were co-transfected with MEK1 expression vector (data not shown). Thus, there could be differences in the regulation of PSA expression between early and advanced prostate cancers. We might not fully observed obvious differences in PSA levels, since advanced cases were excluded from the present study. A recent functional study indicated that ARE1 polymorphism had no significant effects on the activity of the PSA promoter *in vitro* and serum PSA levels *in vivo* (23). We also examined the activities of the PSA promoter and found no significant difference between AA and GG genotypes. It was hypothesized that AR binds these two alleles with different affinities, producing quantitative differences in PSA gene expression (16). However, our data in EMSA indicated that G/A substitution of ARE1 made no difference in the binding activity of AR to ARE1. Supporting our results, a recent study indicated that the ARE1 polymorphism is not associated with serum PSA levels and the PSA polymorphisms at positions –4643, –5412 and –5429 have strong association with serum PSA level (24).

It was also reported that PSA polymorphism at position –158 is associated with the risk of prostate cancer or its disease progression in Caucasian populations (14, 16, 25). The G allele increases the risk of prostate cancer and the GG genotype is associated with higher pathological stage in the Chinese population (26). On the contrary, there is a conflicting report that PSA polymorphism at position –158 was not associated with the prostate cancer development and its disease progression in Japanese population (15). Our results also showed no differences in pathological stage and cancer differentiation in ARE1 polymorphism. Next, we examined whether the ARE1 polymorphism influenced cancer volume and PSA production per cell. Cancer volumes were measured using prostatectomy specimens and serum PSA levels were normalized by cancer volume, namely the ratio of serum PSA levels/cancer volume was calculated in prostate cancer patients without neoadjuvant endocrine therapy. There were no significant differences in either of them between the two groups. These results are in discordance with a recent study in a Chinese population showing that the GG genotype was associated with larger tumors. Furthermore, these authors observed more advanced tumors with higher Gleason scores in the GG group (26). In the present study, there was no association of ARE1 polymorphism with Gleason scores (data

not shown). The discrepancy, despite the same Asian populations, could be due to the difference of subjects (only organ-confined prostate cancer cases were included in this study), as well as the limited number of patients in the AA group (only 8 cases).

Many studies have shown that neoadjuvant endocrine therapy prior to radical prostatectomy significantly decreased the positive surgical margin rates. However, no definite evidence of improving progression-free survival was reported (27). Whether neoadjuvant endocrine therapy is significant for localized prostate cancer and for which patients the therapy is useful requires further examination. To address these questions, we investigated whether ARE1 polymorphism had any association with cancer volumes and the serum PSA level/cancer volume ratio in prostate cancer patients with or without neoadjuvant endocrine therapy. Interestingly, cancer volume was significantly smaller in the GG genotype than in the AA+AG genotypes in the neoadjuvant group. In addition, four patients with the GG genotype and one patient with the AG genotype had no residual cancer (pT0) after neoadjuvant endocrine therapy. These results might suggest that the patients with the G allele in ARE1 are more sensitive to androgen deprivation therapy. It has been reported that the prognosis after hormone treatment for prostate cancer is better in Japanese-American than in Caucasians treated at the Queen's Medical center in Hawaii (28). The evidence in the report strongly supports our results that androgen ablation therapy could be more effective in the prostate cancer patients with GG genotype (or G allele) which is abundant in Japanese men. The molecular mechanisms might be explained by the GG genotype in ARE1 being associated more strongly with other important genes related to the responsiveness of androgen, such as the number of CAG repeats and/or cofactors of AR.

This is the first report to show an association between ARE1 polymorphisms at positions –158 of the PSA promoter and the effect of endocrine therapy of prostate cancer. ARE1 polymorphism in PSA gene promoter may be one of the important biomarkers for response to androgen deprivation therapy, including neoadjuvant endocrine therapy prior to radical prostatectomy.

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