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Insulin-like Growth Factor-binding Protein-3 Gene -202 *A/C* Polymorphism Is Correlated with Advanced Disease Status in Prostate Cancer[1]

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ABSTRACT

The circulating level of insulin-like growth factor-binding protein-3 (IGFBP-3) is inversely associated with the risk of prostate cancer (PCa) and its progression and may be modulated by the *A/C* polymorphism at position -202 in the promoter region of *IGFBP-3*. This study was conducted to evaluate the role of the *A/C* polymorphism as a genetic modifier in the etiology of PCa and its disease status. The polymorphism was analyzed by a PCR restriction fragment-length polymorphism technique in 307 PCa patients, 221 benign prostatic hyperplasia (BPH) patients, and 227 male controls. No significant difference in the genotype frequency was found between the PCa or BPH patients and controls (PCa *versus* control, $P = 0.316$; BPH *versus* control, $P = 0.964$). Regarding the tumor stage, the *C* allele was more frequently observed in patients having tumors with higher stage (P for trend $= 0.002$). When the PCa patients with localized disease (stage A + B + C) were considered as reference, those with *CC* and *AC* genotype had a significantly increased risk of metastatic disease (stage D) compared with those with *AA* genotype [age-adjusted odds ratio (aOR) = 3.89, 95% confidence interval (CI) = 1.42–10.64, $P = 0.008$, and aOR = 1.68, 95% CI = 1.01–2.79, $P = 0.044$, respectively]. The presence of the *C* allele appeared to be associated with an increased risk of metastatic PCa with a gene dosage effect (aOR = 1.82, 95% CI = 1.23–2.68, $P = 0.002$). Similarly, significant findings were also observed when PCa patients were compared between those with organ-confined disease (stage A + B) and those with extra-prostatic extension (stage C + D). Furthermore, the *C* allele was present more frequently in patients with higher tumor grade. In conclusion, the *IGFBP-3* -202 *A/C* polymorphism was not associated with susceptibility to PCa and BPH in Japanese men, but the presence of the *C* allele may cumulatively increase the risk for tumor metastasis and for having tumors with a biologically more aggressive phenotype. Because of the significant differences in incidence of clinically evident PCa according to racial backgrounds, the conjecture should be further examined in different racial populations.

INTRODUCTION

The incidence of PCa[4] has been rapidly increasing during the last decade in East Asia (1). However, the incidence rates remain significantly higher in African-Americans or in American Caucasians than Japanese (2–4). Although Japanese immigrants in the United States have experienced a marked increase in PCa incidence, the incidence is less than half of that of American Caucasians (3, 4). These epidemiological studies suggested that the occurrence of PCa is influenced by the multitude of genetic and environmental factors.

Deregulation of the IGF system was suggested to be specifically implicated in PCa. IGFBP-3, which is a major circulating IGFBP (5), binds to IGF-I, forming a complex that limits the IGF-I bioavailability for binding to the IGF-I receptor (6). IGFBP-3 suppressed the mitogenic and antiapoptotic action of IGF-I (7, 8) and was inversely associated with malignant transformation of the prostate (9, 10) and the progression of PCa (11–14). Studies have revealed that the circulating IGF-I and IGFBP-3 levels may be altered in PCa patients. In these patients, the circulating IGF-I level was shown to be often increased (15, 16), whereas the circulating or prostate tissue levels of IGFBP-3 were often decreased (9–11, 15, 17–19). Moreover, a series of studies have demonstrated that the plasma IGFBP-3 levels were significantly lower in African-American men than those in American Caucasian men and the highest in Japanese men (20–22). Because the lower IGFBP-3 levels could result in a greater IGF-I bioavailability (5), these findings may partly explain why the African-American men have a greater incidence of PCa than the American Caucasian and Japanese men.

A recent Physicians' Health Study revealed the presence of *A/C* polymorphism at position -202 in the promoter region of *IGFBP-3* and reported that the polymorphism was correlated with the circulating IGFBP-3 level in men and circulating IGFBP-3 levels were higher when the subjects possessed at least one *A* allele (23). They suggested that the circulating IGFBP-3 level may be modulated by the *A/C* polymorphism. To assess the role of the *A/C* polymorphism as a genetic modifier in the etiology of PCa and its disease progression, we investigated the *IGFBP-3* genotype distribution in men with or without PCa and in PCa patients with or without metastatic disease.

MATERIALS AND METHODS

Subjects. We studied a consecutive series of 800 subjects, including 307 PCa patients, 221 BPH patients, and 272 male controls at the Akita University Medical Center and its related community hospitals in Akita prefecture, who agreed to participate in this study. Most subjects were enrolled in previous studies (24–26). The subjects were selected between April 1997 and November 2001 for the PCa patients, between August 1997 and November 2000 for the BPH patients, and between March 1998 and September 2001 for the male controls.

All PCa patients were diagnosed histologically with specimens obtained from transrectal needle biopsy or transurethral resection of the prostate for voiding symptoms. The clinical or pathological stage of PCa at the time of diagnosis was determined by reviewing the medical records based on the Tumor-Node-Metastasis system (27). PCa was classified into stage A ($T_{1a-b}N_0M_0$), stage B ($T_{1c-2}N_0M_0$), Stage C ($T_{3-4}N_0M_0$), and Stage *D* ($T_{1-4}N_1M_{0-1}$ or $T_{1-4}N_{0-1}M_1$) by the modified Whitmore-Jewett system (28). In 120 cases in whom radical prostatectomy was performed, final pathological stage was applied, and in other 187 cases without radical prostatectomy, clinical stage was applied. Pathological grading of PCa was determined according to the General Rule for Clinical and Pathological Studies on Prostate Cancer by the Japanese Urological Association and the Japanese Society of Pathology (29), which is based on the WHO criteria (30) and according to the Gleason score (31). All pathological grading was based on needle biopsy specimens in stage B–D patients and surgical specimens in stage A patients. Well-, moderately, and poorly differentiated carcinoma generally correspond to Gleason scores of 2–4, 5–7, and 8–10, respectively (29, 32). In the present study, because the two grading systems were individually used by local

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[4] The abbreviations used are: PCa, prostate cancer; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; aOR, adjusted odds ratio; CI, confidence interval.

pathologists, the tumor grade system was newly categorized as follows: (*a*) the low-grade cancer included the well-differentiated or Gleason 2–4 carcinomas; (*b*) the intermediate grade cancer included the moderately differentiated or Gleason 5–7 carcinomas; and (*c*) the high-grade cancer included the poorly differentiated or Gleason 8–10 carcinomas. In 3 patients, the final pathological grade was not determined because the endometrioid carcinoma, whose grading system has not been established, was pathologically diagnosed.

All BPH patients had various degrees of lower urinary tract symptoms and an apparent prostatic enlargement by digital rectal examination. The serum total PSA levels were measured in all of the patients, and men with an elevated total PSA level (4 ng/ml or greater by the Tandem-R assay; Hybritech, Inc., San Diego, CA) were confirmed not to have PCa by transrectal sextant biopsies. Serum total PSA was measured using the Tandem-R assay in most cases. When serum total PSA was measured using kits other than the Tandem-R, the measured total PSA level was adjusted to that of the Tandem-R assay using a formula published elsewhere (33). The male controls, comprising 272 volunteers without any apparent voiding symptoms, were selected randomly from a natural Japanese population attending a medical check-up. They were all tested for serum total PSA levels (the Tandem-R assay), and those with abnormal total PSA levels (≥4 ng/ml, the Tandem-R assay) were omitted from the normal controls. Written informed consent was obtained from all of the control subjects. The present study was approved by the Institutional Review Board of the Akita University School of Medicine.

PCR Restriction Fragment-length Polymorphism Analysis. DNA was extracted from blood samples collected from all subjects using a QIAamp Blood Kit (Qiagen, Hilden, Germany) or by the standard method with proteinase K digestion followed by phenol/chloroform extraction. The 244-bp fragment encompassing the *A* to *C* polymorphic site in the *IGFBP-3* promoter region was amplified using specific primers 5′-CCGAGAGCGGAAGGGG-TAAG-3′ in sense and 5′-TGCTCAGGGCGAAGCACGGG-3′ in antisense. PCR reactions were carried out in a 25-μl volume containing ~20 ng of genomic DNA, 1 × PCR buffer supplied by a manufacturer, 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1 mM $MgCl_2$, 50 pmol of each primer, and 1 unit of Ampli-Taq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ). After a 10-min initial denaturation step at 95°C, 35 cycles of PCR reaction consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s were carried out, followed by a 7-min final extension step at 72°C in a thermal cycler (GeneAmp PCR System 9700; Perkin-Elmer). After confirmation of successful PCR amplification by 1.5% agarose gel electrophoresis, each PCR product was digested overnight with 5 units of *Fsp*I enzyme at 37°C (New England Biolabs, Inc., Beverly, MA) and was electrophoresed on 2.5% agarose gel.

The 244-bp PCR fragment was divided into 164- and 80-bp fragments when the *Fsp*I site was present. The genotype was designated as *C* or *A* when the *Fsp*I restriction site was present or absent, respectively (Fig. 1*A*). The validity of the PCR restriction fragment-length polymorphism analysis was confirmed by direct sequencing of several PCR samples with each genotype using the BigDye FN Sequencing kit (PE Applied Biosystems, Foster City, CA; Fig. 1*B*).

Statistical Analysis. χ^2 test for Hardy-Weinberg equilibrium was evaluated in each group (PCa, BPH, and control; degrees of freedom = 1). Pearson's χ^2 test was used to compare allele frequencies and genotype frequencies. The OR and 95% CI with respect to *IGFBP-3* genotypes were calculated from a multivariate logistic regression model. We hypothesized the *C* allele as an inherent genetic risk factor for PCa and its disease progression. Statistical modeling was performed on the relative risk of the *CC* or *AC* genotype against the *AA* genotype independently using the model adjusted by age as a potential confounding factor (in years). The relation between genotype distributions with tumor grade or stage was also examined using a multivariate logistic regression analysis adjusted by age as a potential confounding factor. The Cochran-Armitage trend test was used to examine the relation between the allele frequency and increasing tumor stage or grade. In addition, the gene dosage effect of the *C* allele was assessed by modeling a linear effect on the log odds scale for each *C* allele in a multivariate logistic regression, such as the genotypes *CC, AC, AA,* which were valued as "2," "1," and "0," respectively. The mean age of the subjects among the three groups was examined using the unpaired two-tailed *t* test. All data were entered into an access database and analyzed using the Excel 2000 and SPSS (version 10.0J; SPSS, Inc.) software.

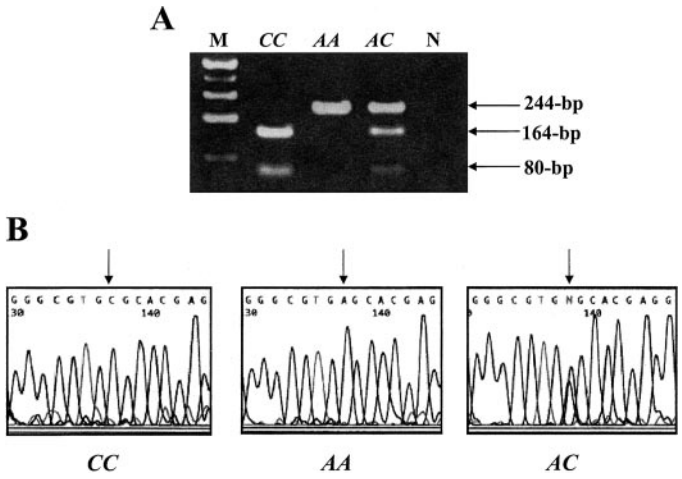

Fig. 1. Representative screening for the *IGFBP* genotypes. *A*, patterns for each of the genotypes *CC, AA,* and *AC* (*Lanes 2–4*) screened by PCR restriction fragment-length polymorphism analysis. *Numbers* in *parentheses* are the size of each fragment (*bp*). *M*, molecular size marker (*Lane 1*). *N*, negative control (*Lane 5*). *B*, results of nucleotide sequence analysis for each of the genotypes. *Arrows*, the location of nucleotides at which the polymorphism occurs.

RESULTS

Subject Characteristics. The mean age (±SD) was 71.77 ± 7.96 years for the PCa patients, 70.94 ± 9.43 years for the BPH patients, and 70.79 ± 8.06 years for the controls. No significant differences in the mean age were found between the PCa patients and controls ($P = 0.143$) or between the BPH patients and controls ($P = 0.853$). There were 27 stage A patients whose PCa was diagnosed incidentally by specimens removed for BPH treatment. Eighty-six PCa patients had clinical stage D2 disease, and 9 had clinical D1 disease, which was judged by radiological studies, whereas 15 patients were pathologically confirmed as having lymph node metastatic (D1) disease. In total, 110 patients were classified into having metastatic PCa; 106 and 64 PCas were classified into stage B and C disease, respectively, by clinical or pathological findings (Table 1).

Genotypes of *IGFBP-3* -202 *A/C* Polymorphism and Risk of PCa and BPH. The frequencies of the *IGFBP-3* genotype in the PCa, BPH, and control groups are shown in Table 1. No significant differences in the allele frequencies were found when the PCa patients (*A* 0.78, *C* 0.22) and BPH patients (*A* 0.75, *C* 0.25) were compared with the controls (*A* 0.75, *C* 0.25), respectively. The allele frequencies of the controls were significantly different from those of the American men reported by the Physicians' Health Study (*A* 0.4, *C* 0.6; $P < 0.001$; Ref. 23). The *IGFBP-3* genotype frequency in each group (PCa, BPH, and control) was in Hardy-Weinberg equilibrium ($P > 0.05$, data not shown).

Statistical analyses of the genotype prevalence showed that no significant differences were found between the PCa patients and controls ($P = 0.316$), between the BPH patients and controls ($P = 0.964$), and between the PCa patients and BPH patients ($P = 0.482$), respectively (Table 1). To evaluate the risk of PCa and BPH according to the *IGFBP-3* genotypes, the logistic regression analysis was conducted with adjustment for age at the time of diagnosis (Table 1). Compared with the men with the *AA* genotype, no significant increased risk of PCa and BPH was found in men with the *AC* or *CC* genotype (Table 1).

***IGFBP-3* Genotype and PCa Disease Status.** We examined the relation between the *IGFBP-3* -202 *A/C* polymorphism and tumor stage or grade at the time of diagnosis. Regarding the tumor stage, the frequency of the *AA* genotype decreased as the tumor stage increased. The *C* allele was more frequently observed in patients having tumors

Table 1 *IGFBP-3 genotype frequencies in PCa patients, BPH patients, and control males*

Study group	Total no.	Genotype (%)			*C* allele frequency (%)
		AA	*AC*	*CC*	
PCa[a]	307	189 (61.6)	100 (32.6)	18 (5.9)	22.1
Stage[b]					
A	27	20 (74.1)	6 (22.2)	1 (3.7)	14.8
B	106	70 (66.0)	35 (33.0)	1 (0.9)	17.5
C	64	42 (65.6)	17 (26.6)	5 (7.8)	21.1
D	110	57 (51.8)	42 (38.2)	11 (10.0)	29.1
Grade[c]					
Low	45	29 (64.4)	15 (33.3)	1 (2.2)	18.9
Intermediate	142	89 (62.7)	45 (31.7)	8 (5.6)	21.5
High	117	68 (58.1)	40 (34.2)	9 (7.7)	24.8
BPH[d]	221	125 (56.6)	83 (37.6)	13 (5.9)	24.7
Control (reference)	272	152 (55.9)	105 (38.6)	15 (5.5)	29.7

[a] PCa *versus* control, $P = 0.316$ by χ^2 test.
[b] According to the Whitmore-Jewett system. Stage A = $T_{1a-b}N_0M_0$, Stage B = $T_{1c-2}N_0M_0$, Stage C = $T_{3-4}N_0M_0$ and Stage D = $T_{1-4}N_1M_{0-1}$ or $T_{1-4}N_{0-1}M_1$. *C* allele frequency, $P = 0.002$ by the Cochran-Armitage trend test. Stage A + B + C *versus* Stage D, $P = 0.010$ by χ^2 test. Stage A + B *versus* Stage C + D, $P = 0.010$ by χ^2 test.
[c] Low grade = well-differentiated carcinoma (WHO) or Gleason score 2–4 carcinoma, Intermediate grade = moderately differentiated carcinoma (WHO) or Gleason score 5–7 carcinoma, High grade = poorly differentiated carcinoma (WHO) or Gleason score 8–10 carcinoma. *C* allele frequency, $P = 0.215$ by the Cochran-Armitage trend test.
[d] BPH *versus* control, $P = 0.964$ by χ^2 test.

with higher stage (P for trend = 0.002; Table 1). A significant difference in the genotype frequency was found between the localized PCa patients (stage A + B + C) and metastatic PCa patients (stage D; $P = 0.01$) and the organ-confined PCa patients (stage A + B) and extraprostatic PCa patients ($P = 0.01$; Table 1). Compared with the *AA* genotype, the PCa patients with *CC* genotype had a 3.89-fold increased risk of metastatic disease, and those with the *AC* genotype had a 1.68-fold increased risk of metastatic disease (Table 2). When the *CC*, *AC*, and *AA* genotypes were valued as "2," "1," and "0" into the model, respectively, the presence of the *C* allele significantly increased the risk of metastatic disease with a gene dosage effect (aOR = 1.82, 95% CI = 1.23–2.68, $P = 0.002$). However, when the patients with localized and metastatic PCa were independently compared with the normal controls, no significant risk of localized PCa (aOR = 0.54, 95% CI = 0.23–1.38, $P = 0.198$) or metastatic PCa (aOR = 1.95, 95% CI = 0.85–4.5, $P = 0.118$) was found in men with the *CC* genotype against those with the *AA* genotype. Similar findings were found when the PCa patients were compared between those with organ-confined (stage A–B) disease and those with extraprostatic extension (stage C–D; Tables 1 and 2).

No significant difference in the genotype frequency was found among the three subgroups of grade (low, intermediate, and high grade; $P = 0.715$; Table 1). The frequency of *AA* genotype was decreased, whereas that of *CC* genotype was increased as the tumor grade rose (Table 1). Although not statistically significant by the linear trend test, the *C* allele was more frequently observed in patients with higher tumor grade (P for trend = 0.215; Table 1).

DISCUSSION

A relatively small number of studies concerning the *IGFBP-3* -202 *A*/*C* polymorphism on common diseases has been reported, and the genotype frequency of this polymorphism in the normal population has not been fully clarified. The present study revealed that the *A* allele appeared to be significantly more common in the Japanese men (*A* 0.75, *C* 0.25) than American men (*A* 0.4, *C* 0.6) reported by the Physicians' Health Study (23). It was reported previously that the *A* allele was correlated with a higher plasma level of IGFBP-3 in men (23) and that the mean plasma IGFBP-3 level was significantly higher in Japanese men than that in African-American and American Caucasian men (21). We found no association between the *IGFBP-3* genotype and susceptibility to PCa or BPH in Japanese men. However, it should be noted that, because the control subjects were a cohort of aged men with normal PSA levels and no significant voiding symptoms, it is possible that the control subjects might include substantial cases with BPH and some PCa cases as well.

The present findings showed a significant association between the *IGFBP-3* -202 *A*/*C* polymorphism and risk of advanced disease in PCa patients. Furthermore, the presence of the *C* allele appeared to increase the risk with a gene dosage effect. However, the conjecture should be interpreted with a caution because the frequency of the *CC* genotype was relatively low in Japanese men, leaving the possibility that such significant findings were caused by chance. In addition, there was no significant difference in the *IGFBP-3* genotype frequency between the metastatic PCa patients and normal controls, and the effect of the *C* allele was not evident when compared with the normal controls. However, if the IGHBP-3 system as influenced by the *IGHBP-3* genotype was genuinely involved in PCa progression but not in its early carcinogenesis, and if the *C* allele had a deteriorating effect, whereas the *A* allele had a protective effect in its progression, it would be reasonable that the *IGHBP-3* allelic frequency was associated with disease status but not distinct between normal and PCa subjects. It remains to be verified if the effect of the *C* allele (or the *A* allele) was only biologically significant under a certain condition in PCa patients.

Table 2 *aOR according to IGFBP-3 genotype*

		aOR (95% CI, *P*) according to IGFBP-3 genotype[a]		
		AA	*AC*	*CC*
Study group				
	PCa against control	1.00	0.77 (0.55–1.09, 0.144)	0.97 (0.48–2.00, 0.942)
	BPH against control	1.00	0.96 (0.66–1.40, 0.840)	1.06 (0.48–2.30, 0.892)
Tumor stage[b]				
	Stage D against Stage A + B + D	1.00	1.68 (1.01–2.79, 0.044)	3.89 (1.42–10.6, 0.008)
	Stage D + C against Stage A + B	1.00	1.31 (0.80–2.15, 0.279)	7.82 (1.74–35.2, 0.007)
Tumor grade[b]				
	High against low + intermediate	1.00	1.16 (0.70–1.91, 0.567)	1.84 (0.69–4.90, 0.221)

[a] These data were adjusted for age.
[b] Tumor stage and grade systems are the same as Table 1.

Smith *et al.* (34) hypothesized that IGFBP-3 is directly involved in generating osteoblastic bony metastases, perhaps via a PSA-dependent paracrine loop involving both the PCa and bone cells. A recent large-scaled study has demonstrated that a lower plasma IGFBP-3 level was associated with a significantly higher risk of advanced-stage PCa (13). Kanety *et al.* (11) suggested that a lower level of serum IGFBP-3 was detected in PCa patients with metastatic disease compared with healthy controls. Furthermore, the preoperative plasma IGFBP-3 level has been shown to be a useful predictor of treatment failure after radical prostatectomy (14). Miyata *et al.* (35) recently also mentioned that the serum IGFBP-3:PSA ratio might be a useful prognostic marker of advanced PCa in Japanese PCa patients, and a lower level of plasma IGFBP-3 might be correlated with the presence of the *C* allele with a gene dosage effect (23). These findings may support our present finding that the presence of the *C* allele was significantly associated with an increased risk of metastatic disease in PCa patients with a gene dosage effect. On the other hand, Wolk *et al.* (16) found no association between the serum IGFBP-3 levels and disease status of PCa. However, the age, energy intake, nutrient status, and body mass index of individuals can profoundly affect the circulating IGFBP-3 level, and the hormones, growth factors, and cytokines are related to the *IGFBP-3* mRNA expression (8). The presence of prostatic disease, especially PCa, may alter the circulating IGFBP-3 level and other IGF-related protein, therefore making the interpretation of results of retrospective case control studies more difficult. Furthermore, the levels and activity of IGF-I and IGFBP-3 in the prostate cells appear to be more complicated (36). Additional studies on the biological role of the *IGFBP-3* -202 *A*/*C* polymorphism in the context of the IGF-I and IGFBP-3 axis should take many confounding factors into account.

The promoter region, where the *IGFBP-3* -202 *A*/*C* polymorphism is located, may harbor the response elements for various hormone receptors and transcription factors, including insulin, growth hormone, retinoic acid, vitamin D, estrogen, thyroid hormone, glucocorticoids, tumor necrosis factor-α and β, and epidermal growth factor (8, 37). As shown by an *in vitro* expression assay (23), the *IGFBP-3* mRNA synthesis may be altered in correlation with the presence of the *C* or *A* allele, which might have a significant impact on the disease status in PCa patients.

In conclusion, the *IGFBP-3* -202 *A*/*C* polymorphism was not associated with the susceptibility to PCa and BPH in Japanese men. However, the presence of *C* allele may be a genetic risk factor for metastasis or advanced disease status in PCa patients with a gene dosage effect and may also be associated with a biologically more aggressive tumor. However, the results should be interpreted with caution because of the relatively small number of study subjects and absence of significant difference in the genotype frequency between the metastatic PCa patients and normal controls. The proposed biological mechanism for the role of *A*/*C* polymorphism in progression of PCa will require further exploration and validation.

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