TABLE 1. *Initial therapy at each stage in Japanese patients*

	No. T Stage (%)				
	T1c	T2a	T2b	T3a	T3b
PADT	202 (35.7)	255 (40.6)	181 (43.0)	202 (57.1)	108 (67.5)
Radiation + ADT	37 (6.5)	29 (4.6)	28 (6.7)	46 (13.0)	30 (18.8)
Radiation alone	11 (1.9)	31 (4.9)	12 (2.9)	12 (3.4)	3 (1.9)
Prostatectomy + ADT	110 (29.5)	126 (20.1)	105 (24.9)	63 (17.8)	17 (10.6)
Prostatectomy	167 (29.5)	173 (27.5)	88 (20.9)	29 (8.2)	2 (1.3)
Watchful waiting	39 (6.9)	14 (2.2)	7 (1.7)	2 (0.6)	0
Totals	566	628	421	354	160

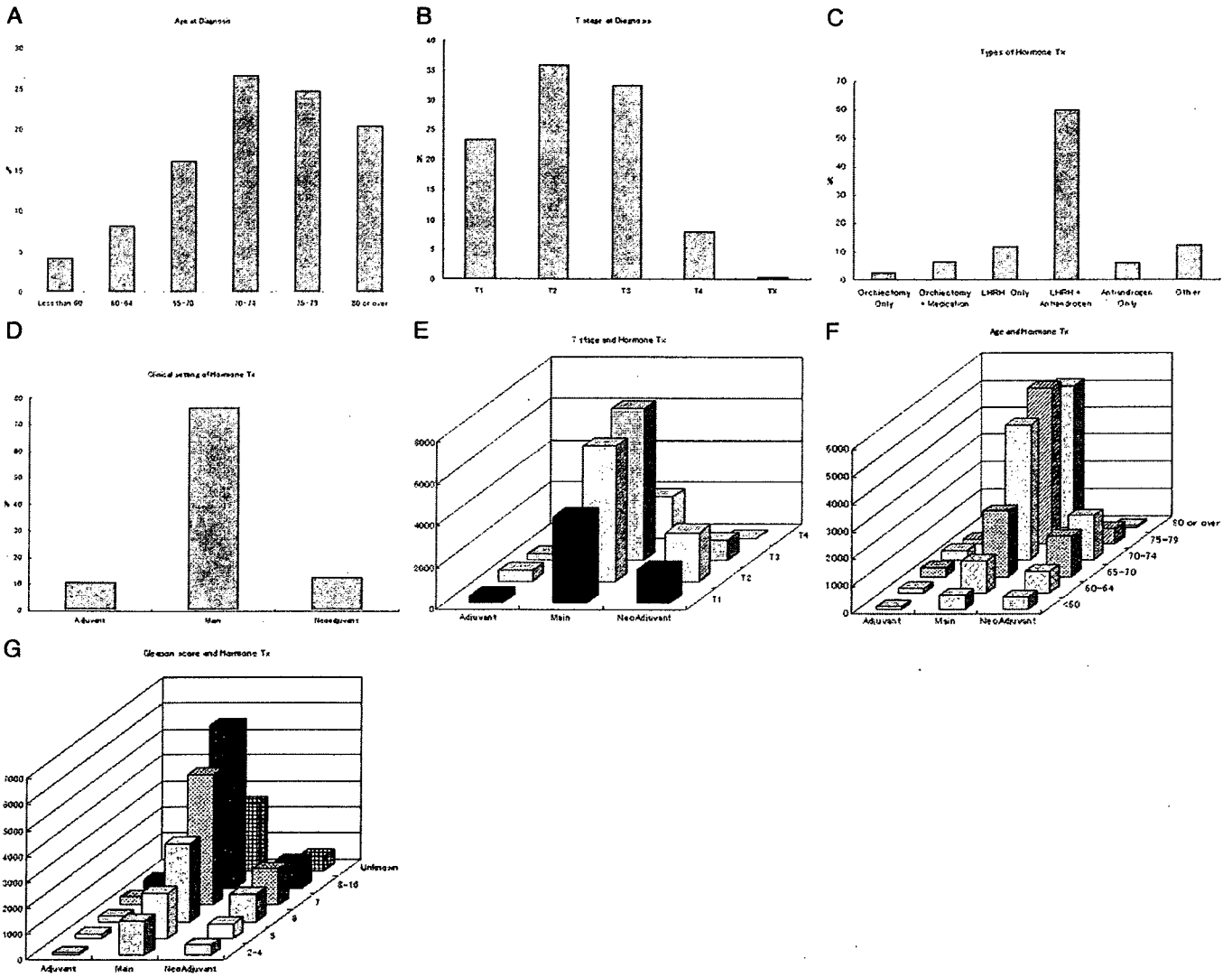
Japanese Urological Association surveillance.[4]

TABLE 2. *PADT time trend in patients with LPC in United States in 1989 to 2001*[5]

	% Risk Category		
Yrs	High	Intermediate	Low
1989–1992	4.6	8.9	32.8
1993–1995	8.0	11.4	39.8
1996–1998	16.7	21.3	49.7
1999–2001	14.2	19.7	48.2

and PADT, are also important. Of course, it is essential to include adverse effects and quality of life as additional end points, rather than focusing only on efficacy end points.

In view of the current situation in Japan, where a large number of patients with prostate cancer are receiving PADT irrespective of age or disease state, we organized the J-CaP Study Group.[12] During the 3 years from 2001 to 2003,



All patients treated with ADT as primary or adjuvant/neoadjuvant therapy in J-CaP database. *A*, patient age. *B*, T stage. *Tx*, treatment. *C*, hormone therapy. *D*, hormone therapy clinical setting. *Main*, primary androgen depletion therapy. *E*, correlation between T stage and hormone therapy. *F*, correlation between patient age and hormone therapy. *G*, correlation between Gleason score and hormone therapy.

26,153 patients from 395 domestic facilities were enrolled in this study. To be eligible for study inclusion a patient with any disease stage had to start receiving any type of ADT during this period. The figure shows patient age distribution, T stage, Gleason score, ADT clinical setting, ADT type, and the relationship of ADT type to T stage, age and Gleason score. The figure shows that many patients received PADT, particularly using a combination of an LHRH agonist and antiandrogen treatment, irrespective of age and Gleason score. We are following these enrolled patients and we plan to analyze progression-free, overall and cancer specific survival in relation to background variables, such as the clinical setting of ADT (primary, neoadjuvant or adjuvant) and the type of ADT (castration, LH-RH agonist and/or antiandrogen) with the ultimate goal of investigating the true significance of ADT for prostate cancer. At the same time we plan to prepare nomograms of the results of ADT for use in clinical practice.

CONCLUSIONS

Huggins et al first reported the effectiveness of ADT for advanced metastatic prostate cancer.[13] The Nobel Prize was awarded for that work. About 40 years have passed since then, during which the screening assay for prostate specific antigen has been widely used, as evidenced by a marked increase in cases of LPC at the time of detection. Clearly as more men are screened and at older ages, local therapies become less attractive. However, individuals desire therapy because they have been told that they have cancer. In this context active surveillance might be more popular, although in the United States CaPSURE™ surveillance has shown an increasing tendency toward electing PADT instead of active surveillance. Moreover, in Japan many patients are treated with PADT and men treated with active surveillance are rare, irrespective of age.

However, the role of ADT for the treatment of prostate cancer remains unchanged from that assigned to it 40 years ago. In other words, clinicians still often adhere to the view of ADT that prevailed in the age of Huggins et al of ADT as palliative therapy[13] without an adequate evaluation of the effectiveness of ADT for LPC or LAPC. Accurate evaluation of the significance of ADT for LPC and LAPC is urgently needed for better use to be made of this therapy in clinical practice.

Abbreviations and Acronyms

ADT	=	androgen deprivation therapy
EBRT	=	external beam radiotherapy
J-CaP	=	Japanese Prostate Cancer
LAPC	=	locally advanced prostate cancer
LHRH	=	luteinizing hormone-releasing hormone
LPC	=	localized prostate cancer
PADT	=	primary ADT

REFERENCES

1. American Urological Association Clinical Guidelines. Available at http://www.auanet.org/guidelines/. Accessed May 2, 2005
2. Prostate Cancer (PDQ®): Treatment. National Cancer Institute. Available at http://www.cancer.gov/cancertopics/pdq/treatment/prostate/healthprofessional/. Accessed May 2, 2005
3. Aus, G., Abbou, C. C., Bolla, M., Heidenreich, A., Schmid, H. P., van Poppel, H. et al: EAU guidelines on prostate cancer. Eur Urol, **48:** 546, 2005
4. Clinicopathological statistics on registered prostate cancer patients in Japan: 2000 report from the Japanese Urological Association. Cancer Registration Committee of Japanese Urological Association. Int J Urol, **12:** 46, 2005
5. Cooperberg, M. R., Grossfeld, G. D., Lubeck, D. P. and Carroll, P. R.: National practice patterns and time trends in androgen ablation for localized prostate cancer. J Natl Cancer Inst, **95:** 981, 2003
6. Kumamoto, E., Tsukamoto, T., Umehara, T., Shimazaki, J., Fuse, H., Ohshima, H. et al: Clinical studies on endocrine therapy of prostatic carcinoma (1): multivariate analyses of prognostic factors in patients with prostatic carcinoma given endocrine therapy. Hinyokika Kiyo, **36:** 275, 1990
7. Bolla, M., Collette, L., Bank, L., Warde, P., Dubois, J. B., Mirimanoff, R. O. et al: Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): a phase III randomised trial. Lancet, **360:** 103, 2002
8. D'Amico, A. V., Manola, J., Loffredo, M., Renshaw, A. A., DellaCroce, A. and Kantoff, P. W.: 6-Month androgen suppression plus radiation therapy vs radiation therapy alone for patients with clinically localized prostate cancer. JAMA, **292:** 821, 2004
9. Shipley, W. U., Desilvio, M., Pilepich, M. V., Roach, M., 3rd, Wolkov, H. B., Sause, W. T. et al: Early initiation of salvage hormone therapy influences survival in patients who failed initial radiation for locally advanced prostate cancer: a secondary analysis of RTOG Protocol 86-10. Int J Radiat Oncol Biol Phys, **64:** 1162, 2006
10. Akaza, H., Homma, Y., Okada, K., Yokoyama, M., Usami, M., Hirao, Y. et al: A prospective and randomized study of primary hormonal therapy for patients with localized or locally advanced prostate cancer unsuitable for radical prostatectomy: results of the 5-year follow-up. BJU Int, **91:** 33, 2003
11. Wirth, M. P., See, W. A., McLeod, D. G., Iversen, P., Morris, T. and Carroll, K.: Bicalutamide 150 mg in addition to standard care in patients with localized or locally advanced prostate cancer: results from the second analysis of the early prostate cancer program at median follow-up of 5.4 years. J Urol, **172:** 1865, 2004
12. Akaza, H., Usami, M., Hinotsu, S., Ogawa, O., Kagawa, S., Kitamura, T. et al: Characteristics of patients with prostate cancer who have initially been treated by hormone therapy in Japan: J-CaP surveillance. Jpn J Clin Oncol, **34:** 329, 2004
13. Huggins, C., Stephens, R. C. and Hodges, C. V.: Studies on prostate cancer:2. The effects of castration on advanced carcinoma of the prostate gland. Arch Surg, **43:** 209, 1941

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ORIGINAL REPORT

Impact of *IGF-I* and *CYP19* Gene Polymorphisms on the Survival of Patients With Metastatic Prostate Cancer

Norihiko Tsuchiya, Lizhong Wang, Hiroyoshi Suzuki, Takehiko Segawa, Hisami Fukuda, Shintaro Narita, Masaki Shimbo, Toshiyuki Kamoto, Kenji Mitsumori, Tomohiko Ichikawa, Osamu Ogawa, Akira Nakamura, and Tomonori Habuchi

From the Department of Urology and the Department of Medical Information Science Akita University School of Medicine, Akita; Department of Urology, Graduate School of Medicine, Chiba University, Chiba; and the Department of Urology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Address reprint requests to: Tomonori Habuchi, MD, Department of Urology, Akita University School of Medicine, 1-1-1 Hondo Akita 010-8543, Japan; e-mail: thabuchi@doc.med.akita-u.ac.jp.

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A B S T R A C T

Purpose
The prognosis of metastatic prostate cancer significantly differs among individuals. While various clinical and biochemical prognostic factors for survival have been suggested, the progression and response to treatment of those patients may also be defined by host genetic factors. In this study, we evaluated genetic polymorphisms as prognostic predictors of metastatic prostate cancer.

Patients and Methods
One hundred eleven prostate cancer patients with bone metastasis at the diagnosis were enrolled in this study. Thirteen genetic polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism or an automated sequencer with a genotyping software.

Results
Among the polymorphisms, the long allele (over 18 [CA] repeats) of insulin-like growth factor-I (*IGF-I*) and the long allele (over seven [TTTA] repeats) of cytochrome P450 (*CYP*) 19 were significantly associated with a worse cancer-specific survival ($P = .016$ and .025 by logrank test, respectively). The presence of the long allele of either the *IGF-I* or *CYP19* polymorphisms was an independent risk factor for death ($P = .019$ or .026, respectively). Furthermore, the presence of the long allele of both the IGF-I and CYP19 polymorphisms was a stronger predictor for survival ($P = .001$).

Conclusion
The prognosis of metastatic prostate cancer patients is suggested to be influenced by intrinsic genetic factors. The *IGF-I* (CA) repeat and *CYP19* (TTTA) repeat polymorphisms may be novel predictors in prostate cancer patients with bone metastasis at the diagnosis.

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INTRODUCTION

Prostate cancer is becoming one of the most common cancers in males in Japan.[1] The introduction of prostate-specific antigen (PSA) screening has increased the identification of early-stage prostate cancer, and the establishment of surgical and radiation techniques may improve the outcome in these patients. Meanwhile, the prognosis of metastatic prostate cancer patients remains poor, although approximately 80% of untreated patients respond to androgen deprivation therapy. However, it has also been acknowledged that the prognosis of metastatic prostate cancer differs significantly among individuals.[2] The prediction of prognosis and stratifying patients by their risk of progression are important for personalized treatments and follow-up strategies. To date, various clinical and biochemical parameters as well as tumor characteristics have been reported to predict the survival of patients with metastatic prostate cancer. As clinical and biochemical factors, lower performance status, pain score, extent of disease on bone scan, pretreatment serum testosterone, serum alkaline phosphatase (ALP) and acid phosphatase, PSA, and lower hemoglobin (HGB) were reportedly associated with treatment response or patient survival.[3-7] Pathologic and immunohistochemical analyses also demonstrated that nuclear texture, oligosaccharide sialyl Lewis (x), c-*erb*B-2 (Her2/*neu*), and tissue factor could be predictors of survival.[5,8,9]

The factors indicating the characteristics of cancer and polymorphisms as host genetic factors possibly influence the prognosis of cancer. Recently, various genetic polymorphisms were reportedly associated with a risk of prostate cancer although the

real influence of these polymorphisms remains controversial.[10-16] It has also been suggested that these polymorphisms may further modify the progression of cancer and define the response to therapy as well as cancer susceptibility. Since most prostate cancers show androgen-dependent growth, many previous analyses were performed with an emphasis on the polymorphisms of genes involved in biosynthesis and the metabolism of steroids and androgens. The association with susceptibility to prostate cancer has so far been demonstrated in vitamin D receptor (*VDR*), androgen receptor (*AR*), steroid-5-alpha-reductase, alpha polypeptide 2 (*SRD5A2*), cytochrome P450 (*CYP*) 11A1, *CYP17*, and aromatase (*CYP19*).[10-14,16] Other genetic polymorphisms such as transforming growth factor beta 1 (*TGF-β1*), cyclin D1 (*CCND1*), epidermal growth factor (*EGF*), human epidermal growth factor receptor 2 (*HER2/neu*), insulin-like growth factor (*IGF-I*), insulin-like growth factor binding protein-3 (*IGFBP-3*), and *PSA* have also been evaluated on the basis of their functions as regulators of cell cycle, differentiation, and apoptosis.[17-23] Most of these studies were designed to assess the association between polymorphisms and prostate cancer risk, tumor grade, or extension at the time of diagnosis using a case-control study model. To date, no definite host genetic factors influencing the prognosis of advanced prostate cancer patients have been reported. A long-term longitudinal study is required to evaluate the real impact of polymorphisms on disease progression, as well as treatment response or patient survival.

In this study, we analyzed 13 gene polymorphisms to examine the hypothesis that polymorphisms as host genetic factors are associated with the survival of metastatic prostate cancer patients.

PATIENTS AND METHODS

Patients

From July 1980 to July 2003, 111 native Japanese patients with prostate cancer with bone metastasis at diagnosis were enrolled in this study. The patients were diagnosed at Akita University Hospital (Akita, Japan) and its related community hospitals, Chiba University Hospital (Chiba, Japan) and Kyoto University Hospital (Kyoto, Japan), and only incident cases were included. This study was approved by the institutional review board (the ethical committee) of the Akita University School of Medicine, the Chiba University Graduate School of Medicine, and the Kyoto University Graduate School of Medicine. Written informed consent was obtained from all patients for the use of their DNA and clinical information.

All patients enrolled in this study had metastatic prostate cancer with no previous treatments. Prostate needle biopsy specimens provided material for pathologic diagnosis, and metastasis was identified by x-rays, computed tomography scans, or bone scintigraphy. After diagnosis, all patients underwent surgical castration or luteinizing hormone-releasing hormone (LH-RH) analogs with or without antiandrogens as the initial hormone therapy. Other optional therapies, including estrogens, antiandrogen agents, steroids, palliative radiation, or a combination of these were added to or replaced by the preceding therapies when treatment failure was noted. Pathologic grading of a biopsied specimen 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,[24] which is based on the WHO criteria and according to the Gleason score.[25] All pathologic grading was based on needle biopsy specimens judged by local pathologists with no designated primary pathologist. Well-, moderately-, and poorly differentiated carcinomas generally correspond to Gleason scores of 2 to 4, 5 to 7, and 8 to 10, respectively.[24,25] As both grading systems were used by local pathologists, the tumor grade system was newly categorized as follows: low-grade cancer included well-differentiated or Gleason 2 to 4 carcinomas; intermediate-grade cancer included moderately-differentiated or Gleason 5 to 7 carcinomas; and high-grade cancer included poorly-differentiated or Gleason 8 to 10 carcinomas. In 10 patients, the final pathologic grade was not determined because no grade information was described in the final report or a different grading system was applied by the local pathologists. In this study, performance status and extent of disease were excluded from the analysis because they are not objective and may not be suitable in an inter-institutional study. Pretreatment HGB, ALP, lactate dehydrogenase (LDH), and PSA levels were measured before the initial treatment of prostate cancer and PSA was measured every 3 months thereafter. An independent end point reviewer in each institution (H.S., T.S., and H.F.) determined the cause of death on the basis of standardized extractions from the patient files. For this determination, genotype data of patients were not revealed to the reviewers.

Genotyping Analysis

Blood samples were collected from each patient and DNA was extracted using a QIAamp Blood Kit (QIAGEN, Hilden, Germany) or standard phenol-chloroform. Polymorphisms of 13 genes consisting of *VDR*, *AR*, *SRD5A2*, *CYP11A*, *CYP17*, *CYP19*, *TGF-β1*, *CCND1*, *IGF-I*, *IGFBP-3*, *PSA*, *EGF*, and *HER2/neu* were genotyped. The polymorphisms were chosen based on literatures as the genes previously described to be associated with the increased risk for prostate cancer or considered important for prostate cancer biology.[11-14,16,26-33] The type, site, and primer sequences for each polymorphism analyzed in this study are summarized in Table 1. Single nucleotide polymorphisms (SNPs) and repeat polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism and by an automated sequencer (ABI PRISM 310 Genetic Analyzer; GMI Inc, Ramsey, MN) with GENESCAN software (Applied Biosystems, Foster City, CA), respectively.

Statistical Analysis

The end point of this study was cancer-specific death defined as death from prostate cancer or death from other causes strongly associated with progression of disease. Survival time was calculated from the date of prostate cancer diagnosis to the day of death due to cancer-specific death, with deaths from other causes censored. To compare survival rates, patients were dichotomized by the median value of age and PSA, by normal limits in HGB, ALP, LDH, by the tumor grade system (ie, high versus low/intermediate), and by criteria previously reported in each polymorphism. For repeat-length polymorphisms, the number of repeats was dichotomized and the long allele of the *AR*, *CYP19*, and *IGF-I* polymorphisms were defined as having more than 18 (CAG) repeats, more than seven (TTTA) repeats, and more than 18 (CA) repeats, respectively according to the previous reports.[11,13,16,29] Similarly, the repeat number of the *CYP11A1* polymorphism was dichotomized by the presence of four (TTTTA) repeats according to the previous study.[13] Differences in survival were tested using the logrank test. Hazard ratios (HRs) and 95% CIs for cancer death were assessed by the Cox proportional hazard regression models. Prostate specific antigen, HGB, ALP, and polymorphisms of the *IGF-I* and *CYP19* were employed as a variable set in the analysis, model A. Of the variable set, the *IGF-I* and *CYP19* polymorphisms were replaced by a combination of the two polymorphisms as an alternative analysis, model B.

Cancer-specific survival was estimated using the Kaplan-Meier method. Median follow-up time was computed among censored cases. All statistical analyses were performed using SPSS software version 12.0 (SPSS, Inc, Chicago, IL) and two-sided P values $< .05$ were considered to indicate statistical significance.

RESULTS

Patients and Treatment Characteristics

The mean age (± standard deviation) of the patients was 70.6 ± 8.7 years (range, 45 to 89; median, 71 years). The mean follow-up period was 46.3 ± 33.4 months (range, 1 to 153; median, 37 months). Pretreatment PSA, HGB, ALP, and LDH levels are shown in Table 2. Of 111 patients, bone metastasis alone, additional lymph node metastasis, and other visceral metastasis were seen in 60 patients

Table 1. List of Gene Polymorphisms Assessed in This Study

Genes	Type and Site of Polymorphisms		Methods of Analysis	Primers		Restriction Enzyme
				Forward	Reverse	
VDR[26]	*Bsm* 1	3′ UTR	PCR-RFLP	5′-*CAACCAAGACTACAAGTACCGCGTCAGTGA*-3′	5′-*AACCAGCGGGAAGAGGTCAAGGG*-3′	*Bsm* 1
AR[11]	(*CAG*)n	exon 1	GeneScan	5′-Hex-*TCCAGAATCTGTTCCAGAGCGTGC*-3′	5′-*ACTGCGGCTGTGAAGGTTGCTGT*-3′	—
SRD5A2[12]	V89L	exon 1	PCR-RFLP	5′-*GCCACCTGGGACGTTACTTCTG*-3′	5′-*ACTGCGGCTGTGAAGGTTGCTGT*-3′	*Rsa* 1
CYP11A1[13]	(*TTTTA*)n	promoter	GeneScan	5′-Hex-*GGTGAAACTGTGCCATTGCGCT*-3′	5′-*GTTATGGCCCCACTGTATAGACA*-3′	—
CYP17[14]	*T-34C*	promoter	PCR-RFLP	5′-*CCATTCGCACTCTGGAGTCAT*-3′	5′-*GACAGGAGGCTCTTGGGGTA*-3′	*Msp* A II
CYP19[16]	(*TTTA*)n	intron 4	GeneScan	5′-Hex-*TTATGAAAGGTAAGCAGGTACTTAG*-3′	5′-*GTCGTGAGCCAAGGTCACT*-3′	—
TGF-β1[27]	*T29C*	exon 1	PCR-RFLP	5′-*TTCAAGACCACCCACCTTCT*-3′	5′-*TCGCGGGTGCTGTTGTACA*-3′	*Msp* A II
CCND1[28]	*A870G*	exon 4	PCR-RFLP	5′-*GTGAAGTTCATTTCCAATCCGC*-3′	5′-*GGGACATCACCCTCACTTAC*-3′	*Scr* FI
IGF-1	(*CA*)*n*	promoter	GeneScan	5′-Hex-*GCTAGCCAGCTGGTGTTATT*-3′	5′-*ACCACTCTGGGAGAAGGGTA*-3′	—
IGFBP-3[30]	*A-202C*	promoter	PCR-RFLP	5′-*CCGAGAGCGGAAGGGGTAAG*-3′	5′-*TGCTCAGGGCGAAGCACGGG*-3′	*Fsp* 1
PSA[31]	*A-158G*	promoter	PCR-RFLP	5′-*ATCTCCTGAGTGCTGGTGTCTTA*-3′	5′-*GCAATTACTAGATCACCCTGGATG*-3′	*Rsa* 1
EGF[32]	*G61A*	exon 1	PCR-RFLP	5′-*TGTCACTAAAGGAAAGGAGGT*-3′	5′-*TTCACAGAGTTTAACAGCCC*-3′	*Alu* 1
Her2/neu[33]	1655V	exon 17	PCR-RFLP	5′-*AGAGAGCCAGCCCTCTGACGTCCAT*-3′	5′-*TCCGTTTCCTGCAGCAGTCTCCGCA*-3′	*Bsm* A1

Abbreviations: *VDR*, vitamin D receptor; *AR*, androgen receptor; *SRD5A2*, steroid-5-alpha-reductase, alpha polypeptide 2; *CYP* cytochrome; *TGF-β1*, transforming growth factor beta 1; *CCND1*, cyclin D1; *IGF-I*, insulin-like growth factor; *IGFBP-3*, insulin-like growth factor binding protein-3; *PSA*, prostate-specific antigen; *EGF*, epidermal growth factor; *HER2/neu*, human epidermal growth factor receptor 2.

(54.1%), 37 patients (33.3%), and 14 patients (12.6%), respectively. As the initial hormone therapy, surgical castration was selected in 57 patients (51.4%) and LH-RH analog was administrated in 54 patients (48.6%), while 42 patients (37.8%) and 69 patients (62.2%) were treated with monotherapy of surgical castration or LH-RH analog and combined androgen blockade, respectively. None of the patients were initially treated with anticancer drugs. Seventy patients (63.1%) had a decreased PSA level < 4 ng/mL, 36 patients (32.4%) did not achieve normalized PSA, and there was no information regarding the lowest PSA level in five patients (4.5%).

Survival Analysis

The 5- and 10-year cancer-specific survival of all patients was 40.1% and 22.2%, respectively, with a median survival time of 51 months. The survival was compared between two groups divided by the median value of age and PSA at diagnosis, by the normal limit for HGB, ALP, and LDH, and by tumor grade (high versus intermediate/low grade). Univariate analysis using the logrank test showed that a PSA level above 260 ng/mL ($P = .015$), HGB below ($P < .001$) and ALP above the normal limit ($P < .001$) were associated with poor survival (Table 2). Age ($P = .365$), LDH level at diagnosis ($P = .272$), and tumor grade ($P = .084$) were not influential factors of survival.

The genotype frequency of each SNP was indicated in Table 3. The repeat number of the *IGF-I* (CA) repeat polymorphism ranged from 15 to 21 and 18 genotypes were observed. The most frequent genotype was 17/19 (21 patients), followed by 16/17 (13 patients), 18/19 (11 patients), 17/18 (nine patients), 16/19 (nine patients), 18/18

Table 2. Association of Clinical Factors at Diagnosis With Cancer-Specific Survival in Metastatic Prostate Cancer

Category	No. of Patients	Mean ± SD	Range	Median	P*
Age, years		70.6 ± 8.7	45-89	71	.365
> 71	55				
≤ 71	56				
PSA, ng/ml		1,095.9 ± 2,174.8	15.0-12,490	260	.015
> 260	55				
≤ 260	56				
HGB, g/dl		13.1 ± 2.0	6.2-17.4	13.4	< .001
Low	26				
Normal	85				
ALP, IU		611.1 ± 778.6	17-4,486	279	< .001
High	50				
Normal	61				
LDH, IU		315.8 ± 180.8	97-956	277	.272
High	50				
Normal	61				
Tumor grade†		315.8 ± 180.8	97-956	277	.272
High	72				
Low/intermediate	29				.084

Abbreviations: SD, standard deviation; PSA, prostate-specific antigen; HGB, hemaglobin; ALP, alkaline phosphatase; IU, international unit; LDH, lactate dehydrogenase.
*Log-rank test.
†Low, intermediate, and high correspond to Gleason score 2-5, 6-7, and 8-10, respectively.

Table 3. Association of Gene Polymorphisms With Cancer-Specific Survival in Metastatic Prostate Cancer

Genes	No. of Patients	P*
VDR[10]		0.985
bb	76	
bB/BB	34/1	
AR[11]		0.984
With long allele	79	
Without long allele	32	
SRD5A2[12]		0.264
VV	32	
VL/LL	59/20	
CYP11A1[13]		0.948
With $(TTTTA)_4$ allele	54	
Without $(TTTTA)_4$ allele	57	
CYP17[14]		0.288
TT	26	
TC/CC	55/30	
CYP19[16]		0.025
With long allele	65	
Without long allele	46	
TGF-β1[27]		0.807
TT	45	
TC/CC	44/22	
CCND1[28]		0.999
AA	28	
AG/GG	54/29	
IGF-I[29]		0.016
With long allele	62	
Without long allele	49	
IGFBP-3[30]		0.819
AA	61	
AC/CC	40/10	
PSA[31]		0.771
GG	68	
GA/AA	54/14	
EGF		0.163
GG	43	
GA/AA	54/14	
Her2/neu[33]		0.274
II	82	
IV/VV	28/1	

Abbreviations: *VDR*, vitamin D receptor; *AR*, androgen receptor; *SRD5A2*, steroid-5-alpha-reductase, alpha polypeptide 2; *CYP* cytochrome; *TGF-β1*, transforming growth factor beta 1; *CCND1*, cyclin D1; *IGF-I*, insulin-like growth factor; *IGFBP-3* insulin-like growth factor binding protein-3; *PSA*, prostate-specific antigen; *EGF*, epidermal growth factor; *HER2/neu*, human epidermal growth factor receptor 2.

*Logrank test.

†Long allele: more than 23 (*CAG*) repeats.

‡Long allele: more than 18 (*CA*) repeats.

§Long allele: more than 7 (*TTTA*) repeats.

‖Unpublished data.

(eight patients), 19/19 (eight patients), 16/18 (seven patients), 17/17 (seven patients), 15/19 (five patients), 19/20 (four patients), 16/16 (three patients), and others (one patient for each). The repeat number of the *CYP19* (TTTA) repeat polymorphism ranged from 7 to 13 and seven genotypes were observed. The most frequent genotype was 7/7 (46 patients), followed by 7/11 (41 patients), 7/12 (12 patients), 11/11 (five patients), 11/12 (four patients), 7/13 (two patients), and 12/12 (one patient). The number of the *CYP11* (TTTTA) repeat polymorphism ranged from 3 to 9 and 9 genotypes were observed. The genotype frequency was as follows: 6/6 (39 patients), 4/6 (32 patients), 4/4 (19 patients), 6/8 (13 patients), 8/8 (three patients), 3/4 (two patients), and others (one patient for each). The repeat number of the *AR* (CAG) repeat polymorphism ranged from 18 to 37 and 18 genotypes were observed. The genotype frequency was as follows: 25 repeats (22 patients), 24 repeats (17 patients), 26 repeats (14 patients), 23 repeats (12 patients), 28 repeats (eight patients), 27 repeats (seven patients), 22 repeats (seven patients), 21 repeats (six patients), 19 repeats (four patients), 30 repeats (four patients), 20 repeats (two patients), 32 repeats (two patients), and others (one patient for each).

Of 13 polymorphisms, two polymorphisms, the *IGF-I* (CA) repeat polymorphism and the *CYP19* (TTTA) repeat polymorphism, were associated with survival. Patients with at least one copy of the long allele for the *IGF-I* polymorphism showed worse survival compared with those without the long allele ($P = .016$; Table 3, Fig 1). The rates of 10-year cancer-specific survival were 16.8% and 28.8%, and the median survival time was 41 months and 61 months for patients with and without the long allele of the *IGF-I* polymorphism, respectively. Similarly, the survival of patients with at least one copy of the long allele for the *CYP19* polymorphism were worse than without the long allele ($P = .025$; Table 1, Fig 2). The rates of 10-year cancer-specific survival were 16.7% and 29.7%, and the median survival times were 41 months and 61 months for patients with and without the long allele of the *CYP19* polymorphism, respectively.

Patients were classified into four groups according to the presence of the long allele of each polymorphism. Patients with long alleles of both the *IGF-I* and *CYP19* polymorphisms showed the worst pattern in Kaplan-Meier plots. The *P* values of all three possible pair-wise

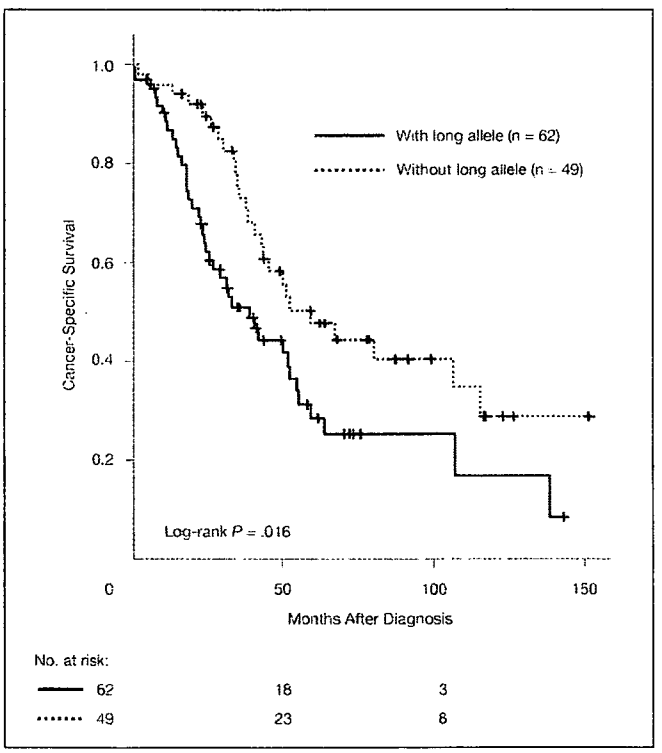

Fig 1. Kaplan-Meier curve according to the *IGF-I* polymorphism. Long allele is more than 18 (CA) repeats.

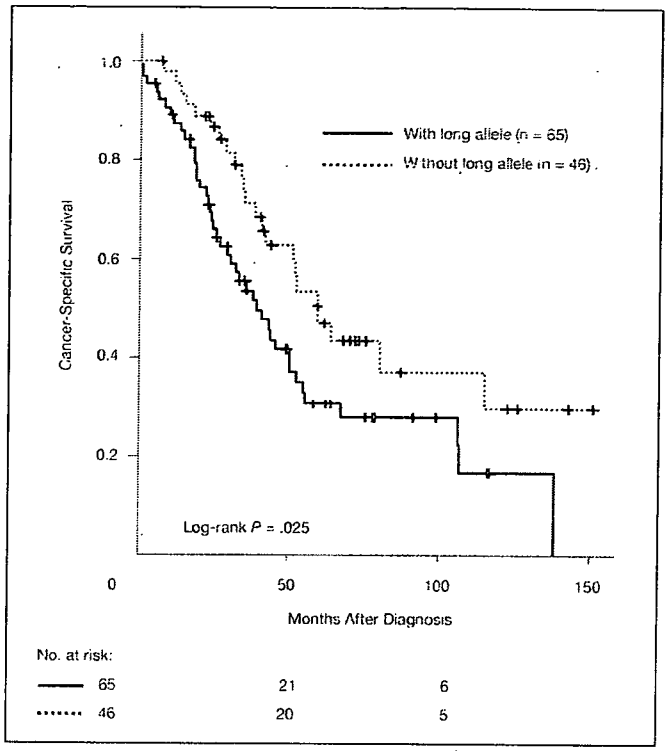

Fig 2. Kaplan-Meier curve according to the *CYP19* polymorphism. Long allele is more than 7 (TTTA) repeats.

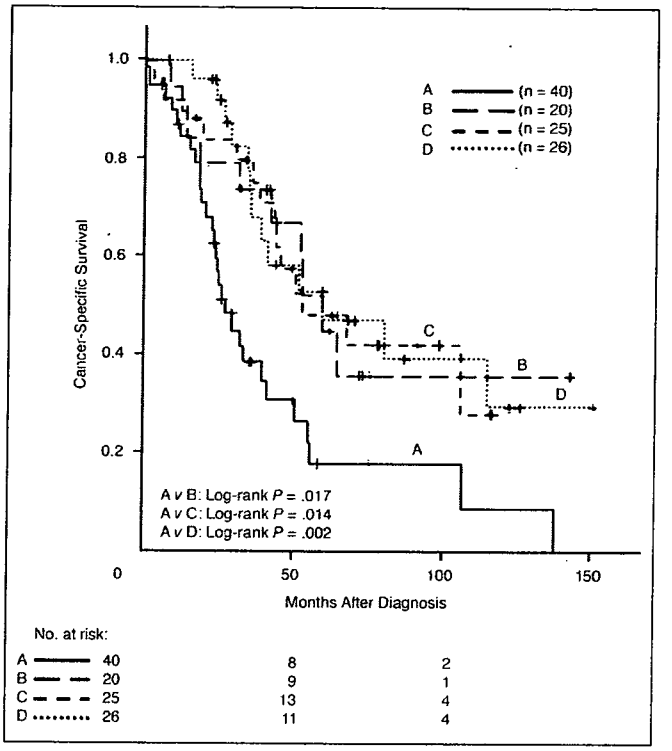

Fig 3. Kaplan-Meier curve according to combination of the two polymorphisms. (A) Patients with the *IGF-1* long allele and the *CYP19* long allele. (B) Patients with the *IGF-1* long allele and no *CYP19* long allele. (C) Patients with the *CYP19* long allele and no *IGF-1* long allele. (D) Patients with no *IGF-1* long allele and no *CYP19* long allele. *IGF-I* long allele is more than 18 (CA) repeats. *CYP19* long allele is more than 7 (TTTA) repeats.

logrank tests controlled by the worst pattern were less than .05 (Fig 3). There were no significant differences in survival between three groups except for the worst pattern. The rate of 10-year cancer-specific survival for patients with long alleles of both polymorphisms and other combinations was 8.8% and 29.4%, respectively, and the median survival times were 28 months and 61 months, respectively. The remaining 11 polymorphisms were not associated with patient survival.

Using five prognostic factors significantly determined by logrank test of dichotomized groups on serum markers as well as gene polymorphisms, the Cox proportional hazard regression analyses were performed against two models, A and B (Table 4). In model A, the presence of at least one copy of either the long allele of the *IGF-I* or *CYP19* polymorphism was also an independent risk factor predicting survival with HRs of 2.012 (95% CI, 1.120 to 3.623; $P = .019$) or 1.976 (95% CI, 1.086 to 3.595; $P = .026$) compared with no long allele, respectively. Furthermore, in model B, the presence of one or more copies of the long allele of both the *IGF-I* and *CYP19* polymorphisms was a stronger predictor for survival (HR, 2.570; 95% CI, 1.436 to 4.597; $P = .001$).

DISCUSSION

In metastatic prostate cancer, resistance to hormone therapy and the androgen-independent growth of cancer cells are considered key factors of patient survival because hormone therapy is the standard and main treatment in most of these patients.[6,34] In this study, we evaluated the polymorphisms of 13 genes previously reported to be associated with susceptibility to or progression of prostate cancer and found that the polymorphisms of *IGF-I* and *CYP19* as well as conventional biochemical markers (ie, pretreatment HGB, ALP, and PSA) were independent predictors of survival.

IGF-I regulates cell proliferation, differentiation, apoptosis and transformation through the IGF-I receptor with both paracrine and autocrine mechanisms, is required for development of the prostate gland and is involved in prostate cancer.[21] IGF-I also activates the AR directly in the absence of androgens, suggesting that IGF-I increases prostate cancer cell growth in both an androgen-dependent and androgen-independent manner.[35] A prospective case-controlled study demonstrated that men with high levels of serum IGF-I were at an increased risk of developing clinically evident prostate cancer within the next 5 to 10 years.[36] Furthermore, a strong association between the plasma level of IGF-I and extraprostatic and distant metastatic prostate cancer was recently indicated in a relatively large case-controlled study.[37] The *IGF-I* contains a polymorphic region composed of (CA) repeats approximately 1 kilo basepair upstream from the transcription initiation site, which is suggested to influence the gene transcription level.[38] Although the exact correlation between the polymorphism and the circulating IGF-I level has not been fully determined, several studies suggest that serum IGF-I levels increase with the number of 19-alleles.[39-41] A recent large cohort study demonstrated that the serum IGF-I level was significantly higher in carriers of the 192-bp allele, which corresponds to the 19 (CA) repeats allele, than noncarriers of the allele.[41] The same group further reported that the significantly higher serum IGF-I level was observed in carriers of

Table 4. Cox Proportional Hazard Regression Analysis of Predicting Factors for Cancer-Specific Survival of Metastatic Prostate Cancer Patients

Variables	Cancer-Specific Survival		
	HR	95% CI	*P*
Model A			
PSA, ng/ml			
> 260	1.923	1.043 to 3.544	.036
≤ 260			
HGB, g/dl			
Abnormal	2.833	1.488 to 5.405	.002
Healthy			
ALP, IU			
Abnormal	1.962	1.091 to 3.529	.024
Healthy			
IGF-I			
With	2.012	1.120 to 3.623	.019
Without long allele*			
CYP19			
With	1.976	1.086 to 3.595	.026
Without long allele†			
Model B			
PSA, ng/ml			
> 260	1.765	0.966 to 3.225	.064
≤ 260			
HGB, g/dl			
Abnormal	2.725	1.439 to 5.155	.002
Healthy			
ALP, IU			
Abnormal	2.072	1.156 to 3.712	.014
Healthy			
Combination of *IGF-I* and *CYP19*			
With long alleles of both two polymorphisms	2.570	1.436 to 4.597	.001
Others			

Abbreviations: HR, hazard ratio; PSA, prostate-specific antigen; HGB, hemoglobin; ALP, alkaline phosphatase; *IGF-I*, insulin-like growth factor; *CYP*, cytochrome.
*Long allele: more than 18 (*CA*) repeats.
†Long allele: more than 7 (*TTTA*) repeats.

192-bp or 194-bp which is identical to the long allele in this study.[42] Thus, the cut off point employed in this study is considered to be appropriate. Meanwhile, some conflicting results in regard to the association between the *IGF-I* polymorphism and serum IGF-I level.[43,44] Additional studies are needed to clarify the effect of the polymorphism on not only the serum IGF-I level, but also on the tissue IGF-I levels in the prostate.

It is known that the 19 (CA) repeat allele of the *IGF-I* polymorphism predicted a susceptibility to several types of cancer.[45] Recently, we demonstrated that the 19 (CA) repeat allele carriers had a significantly increased risk for prostate cancer or BPH compared with noncarriers of the allele.[29] Although no association between the aggressiveness of prostate cancer and the *IGF-I* polymorphism were observed in our previous case-control study,[29] this study suggested that the 19 or more (CA) repeat allele is associated with the aggressive phenotype or resistance to hormone therapies in advanced prostate cancer. In an animal study, transgenic adenocarcinoma of a mouse prostate (TRAMP) model showed an increased expression level of *IGF-I* mRNA in prostate tissue with cancer progression and metastasis.[46] Since IGF-I stimulates the growth of both metastatic and androgen-independent prostate cancer cells, these results indicate that a higher level of circulating and/or intra tumor IGF-I as influenced by the *IGF-I* polymorphism might have a significant impact on the survival of metastatic prostate cancer patients. If the results are confirmed in larger studies, modification of the IGF-I level or IGF-I signaling may be a promising therapeutic target for the treatment of advanced prostate cancer.

Aromatase encoded by *CYP19* is a key enzyme that converts testosterone into estrogen in males, and is suggested to play an important role in the development of benign prostate hyperplasia and prostate cancer.[47] The *CYP19* has a tetranucleotide (TTTA) repeat polymorphism in intron 4 and the polymorphism is reportedly associated with a risk of breast cancer, prostate cancer, and postmenopausal bone metabolism.[16,48,49] The biologic function of the *CYP19* polymorphism in enzyme activity or the expression level of aromatase has not been clarified. Because the polymorphism is located in the midportion of the intron, it is not likely to be associated with regulation of the transcript, splicing, or alteration of enzyme activity, but it may influence mRNA stability or in linkage disequilibrium with unidentified functional polymorphisms. Recently, Haiman et al[48] demonstrated that the presence of at least one allele more than 7 (TTTA) repeats of the *CYP19* polymorphism was associated with a lower level of plasma androstendion, a higher level of estrogens, and a higher estrone to androstendione ratio. Therefore, we used the 7 (TTTA) repeats as a cut off in this study, and other studies also used a similar cut off.[16,50] Although we did not measure pretreatment testosterone levels, our results are in line with previous reports which indicated that a lower pretreatment testosterone level predicted a lower response to hormone therapy and poor survival for patients with metastatic prostate cancer.[51] Advanced prostate cancers are adapted for a lower androgen environment and may achieve hormone-independent cell growth earlier in the clinical course. Those cancers are suggested to resist various hormone therapies.[8] However, as the circulating testosterone level has a circadian variation and is influenced by aging, it seems difficult to prove an association between the testosterone level and the polymorphisms.[52] A recent study observed no association between the *CYP19* polymorphism and susceptibility or aggressiveness of prostate cancer, suggesting that the racial or environmental difference also influence the significance of the polymorphisms in a development or progression of the cancer.[50] In Japanese population, the prognosis of metastatic prostate cancer is possibly affected by the *CYP19* polymorphism affecting the testosterone metabolism.

Androgen ablation therapy has been the standard treatment for metastatic prostate cancer since Huggins reported for the first time in 1941.[53] However, once cancer cells achieve resistance to hormone therapy, the mean survival is only approximately 12 months. Recently, two studies demonstrated a moderate survival benefit treated with chemotherapy using a taxane analog in hormone-refractory advanced prostate cancer.[54,55] However, the timing and target of this chemotherapy remain a controversial issue. Such chemotherapy at an earlier stage after diagnosis may further improve the prognosis of patients with poor survival, which may be defined by genetic polymorphisms as well as conventional risk factors. These results may provide a basic concept that this therapeutic modality, including the administration of early chemotherapy, may be modified according to the genetic polymorphism. Furthermore, the modification of the activity of the CYP19 enzyme or the IGF-I signaling

pathway may be a promising therapeutic target for the treatment of metastatic prostate cancer.

Interestingly, although recent programs on large-scale SNPs instituted by public and private initiatives are expected to reveal disease-associated genes,[56,57] the significant results with polymorphisms obtained in this study were from both simple dinucleotide repeat polymorphisms. Although we do not know whether there are any other significant SNPs in the vicinity of or linked with dinucleotide repeat polymorphisms, our results may underscore the importance of simple repeat polymorphisms in the genesis and progression of common diseases.

In conclusion, to our knowledge, this is the first study demonstrating the possible association of two polymorphisms, the *CYP19* (CA) repeat polymorphism and the *IGF-I* (TTTA) repeat polymorphism, with the survival of prostate cancer patients with distant metastasis. It is also suggested that intrinsic factors of cancer hosts as well as the cancer status and characteristics affect the prognosis of prostate cancer. Understanding the impact of genetic polymorphisms on the prognosis of prostate cancer could lead to the diversification and individualization of prostate cancer treatments, to follow-up strategies, and to novel therapeutic modalities.

REFERENCES

1. Quinn M, Babb P: Patterns and trends in prostate cancer incidence, survival, prevalence and mortality. Part II: Individual countries. BJU Int 90: 174-184, 2002
2. Reiner WG, Scott WW, Eggleston JC, et al: Long-term survival after hormonal therapy for stage D prostatic cancer. J Urol 122:183-184, 1979
3. Chodak GW, Vogelzang NJ, Caplan RJ, et al: Independent prognostic factors in patients with metastatic (stage D2) prostate cancer. The Zoladex Study Group. JAMA 265:618-621, 1991
4. Ernst DS, Hanson J, Venner PM: Analysis of prognostic factors in men with metastatic prostate cancer. Uro-Oncology Group of Northern Alberta. J Urol 146:372-376, 1991
5. Akashi T, Furuya Y, Ohta S, et al: Tissue factor expression and prognosis in patients with metastatic prostate cancer. Urology 62:1078-1082, 2003
6. Reynard JM, Peters TJ, Gillatt D: Prostate-specific antigen and prognosis in patients with metastatic prostate cancer–A multivariable analysis of prostate cancer mortality. Br J Urol 75:507-515, 1995
7. Matzkin H, Soloway MS, Schellhammer PF, et al: Prognostic factors in stage D2 prostate cancer treated with a pure nonsteroidal antiandrogen. Cancer 72:1286-1290, 1993
8. Jorgensen T, Kanagasingam Y, Kaalhus O, et al: Prognostic factors in patients with metastatic (stage D2) prostate cancer: Experience from the Scandinavian Prostatic Cancer Group Study-2. J Urol 158:164-170, 1997
9. Morote J, de Torres I, Caceres C, et al: Prognostic value of immunohistochemical expression of the c-erbB-2 oncoprotein in metastatic prostate cancer. Int J Cancer 84:421-425, 1999
10. Habuchi T, Suzuki T, Sasaki R, et al: Association of vitamin D receptor gene polymorphism with prostate cancer and benign prostatic hyperplasia in a Japanese population. Cancer Res 60:305-308, 2000
11. Stanford JL, Just JJ, Gibbs M, et al: Polymorphic repeats in the androgen receptor gene: Molecular markers of prostate cancer risk. Cancer Res 57:1194-1198, 1997
12. Li Z, Habuchi T, Mitsumori K, et al: Association of V89L SRD5A2 polymorphism with prostate cancer development in a Japanese population. J Urol 169:2378-2381, 2003
13. Kumazawa T, Tsuchiya N, Wang L, et al: Microsatellite polymorphism of steroid hormone synthesis gene CYP11A1 is associated with advanced prostate cancer. Int J Cancer 110:140-144, 2004
14. Habuchi T, Liqing Z, Suzuki T, et al: Increased risk of prostate cancer and benign prostatic hyperplasia associated with a CYP17 gene polymorphism with a gene dosage effect. Cancer Res 60:5710-5713, 2000
15. Lunn RM, Bell DA, Mohler JL, et al: Prostate cancer risk and polymorphism in 17 hydroxylase (CYP17) and steroid reductase (SRD5A2). Carcinogenesis 20:1727-1731, 1999
16. Suzuki K, Nakazato H, Matsui H, et al: Association of the genetic polymorphism of the CYP19 intron 4[TTTA]n repeat with familial prostate cancer risk in a Japanese population. Anticancer Res 23: 4941-4946, 2003
17. Blobe GC, Schiemann WP, Lodish HF: Role of transforming growth factor beta in human disease. N Engl J Med 342:1350-1358, 2000
18. Lukas J, Parry D, Aagaard L, et al: Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature 375:503-506, 1995
19. Herbst RS, Langer CJ: Epidermal growth factor receptors as a target for cancer treatment: The emerging role of IMC-C225 in the treatment of lung and head and neck cancers. Semin Oncol 29:27-36, 2002
20. Di Fiore PP, Pierce JH, Kraus MH, et al: ErbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237:178-182, 1987
21. Jones JI, Clemmons DR: Insulin-like growth factors and their binding proteins: Biological actions. Endocr Rev 16:3-34, 1995
22. Yu H, Rohan T: Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst 92:1472-1489, 2000
23. Cohen P, Graves HC, Peehl DM, et al: Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J Clin Endocrinol Metab 75:1046-1053, 1992
24. Pathology TJUAatJSo: General rule for Clinical and Pathological Studies on Prostate Cancer. Tokyo, Japan, Kanahara-Shuppan Co, 2001, pp 25-69
25. Gleason DF: Histologic grading of prostate cancer: A perspective. Hum Pathol 23:273-279, 1992
26. Morrison NA, Qi JC, Tokita A, et al: Prediction of bone density from vitamin D receptor alleles. Nature 367:284-287, 1994
27. Li Z, Habuchi T, Tsuchiya N, et al: Increased risk of prostate cancer and benign prostatic hyperplasia associated with transforming growth factor-beta 1 gene polymorphism at codon10. Carcinogenesis 25: 237-240, 2004
28. Wang L, Habuchi T, Takahashi T, et al: Cyclin D1 gene polymorphism is associated with an increased risk of urinary bladder cancer. Carcinogenesis 23:257-264, 2002
29. Tsuchiya N, Wang L, Horikawa Y, et al: CA repeat polymorphism in the insulin-like growth factor-I gene is associated with increased risk of prostate cancer and benign prostatic hyperplasia. Int J Oncol 26:225-231, 2005
30. Wang L, Habuchi T, Tsuchiya N, et al: Insulin-like growth factor-binding protein-3 gene -202 A/C polymorphism is correlated with advanced disease status in prostate cancer. Cancer Res 63:4407-4411, 2003
31. Wang LZ, Sato K, Tsuchiya N, et al: Polymorphisms in prostate-specific antigen (PSA) gene, risk of prostate cancer, and serum PSA levels in Japanese population. Cancer Lett 202:53-59, 2003
32. Shahbazi M, Pravica V, Nasreen N, et al: Association between functional polymorphism in EGF gene and malignant melanoma. Lancet 359: 397-401, 2002
33. Wang L, Habuchi T, Takahashi T, et al: No association between HER-2 gene polymorphism at codon 655 and a risk of bladder cancer. Int J Cancer 97:787-790, 2002
34. Collette L, de Reijke TM, Schroder FH: Prostate specific antigen: A prognostic marker of survival in good prognosis metastatic prostate cancer? (EORTC 30892). Eur Urol 44:182-189, 2003
35. Culig Z, Hobisch A, Cronauer MV, et al: Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 54:5474-5478, 1994
36. Chan JM, Stampfer MJ, Giovannucci E, et al: Plasma insulin-like growth factor-I and prostate cancer risk: A prospective study. Science 279:563-566, 1998
37. Chan JM, Stampfer MJ, Ma J, et al: Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. J Natl Cancer Inst 94:1099-1106, 2002
38. Rotwein P, Pollock KM, Didier DK, et al: Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. J Biol Chem 261:4828-4832, 1986
39. Vaessen N, Heutink P, Janssen JA, et al: A polymorphism in the gene for IGF-I: Functional properties and risk for type 2 diabetes and myocardial infarction. Diabetes 50:637-642, 2001
40. Missmer SA, Haiman CA, Hunter DJ, et al: A sequence repeat in the insulin-like growth factor-1 gene and risk of breast cancer. Int J Cancer 100: 332-336, 2002
41. Schut AF, Janssen JA, Deinum J, et al: Polymorphism in the promoter region of the insulin-like growth factor I gene is related to carotid intima-media thickness and aortic pulse wave velocity in subjects with hypertension. Stroke 34:1623-1627, 2003
42. Rietveld I, Janssen JA, van Rossum EF, et al: A polymorphic CA repeat in the IGF-I gene is associated with gender-specific differences in body height, but has no effect on the secular trend in body height. Clin Endocrinol (Oxf) 61:195-203, 2004

43. Friedrichsen DM, Hawley S, Shu J, et al: IGF-I and IGFBP-3 polymorphisms and risk of prostate cancer. Prostate 65:44-51, 2005

44. Rosen CJ, Kurland ES, Vereault D, et al: Association between serum insulin growth factor-I (IGF-I) and a simple sequence repeat in IGF-I gene: Implications for genetic studies of bone mineral density. J Clin Endocrinol Metab 83:2286-2290, 1998

45. Yu H, Li BD, Smith M, et al: Polymorphic CA repeats in the IGF-I gene and breast cancer. Breast Cancer Res Treat 70:117-122, 2001

46. Kaplan PJ, Mohan S, Cohen P, et al: The insulin-like growth factor axis and prostate cancer: Lessons from the transgenic adenocarcinoma of mouse prostate (TRAMP) model. Cancer Res 59: 2203-2209, 1999

47. Ellem SJ, Schmitt JF, Pedersen JS, et al: Local aromatase expression in human prostate is altered in malignancy. J Clin Endocrinol Metab 89: 2434-2441, 2004

48. Haiman CA, Hankinson SE, Spiegelman D, et al: A tetranucleotide repeat polymorphism in CYP19 and breast cancer risk. Int J Cancer 87:204-210, 2000

49. Somner J, McLellan S, Cheung J, et al: Polymorphisms in the P450 c17 (17-hydroxylase/17,20-Lyase) and P450 c19 (aromatase) genes: Association with serum sex steroid concentrations and bone mineral density in postmenopausal women. J Clin Endocrinol Metab 89:344-351, 2004

50. Li L, Cicek MS, Casey G, et al: No association between a tetranucleotide repeat polymorphism of CYP19 and prostate cancer. Cancer Epidemiol Biomarkers Prev 13:2280-2281, 2004

51. Imamoto T, Suzuki H, Akakura K, et al: Pretreatment serum level of testosterone as a prognostic factor in Japanese men with hormonally treated stage D2 prostate cancer. Endocr J 48:573-578, 2001

52. Winters SJ: Current status of testosterone replacement therapy in men. Arch Fam Med 8:257-263, 1999

53. Huggins C, Hodges VC: Studies on prostate cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res 1:293-297, 1941

54. Petrylak DP, Scher HI, Li Z, et al: Prognostic factors for survival of patients with bidimensionally measurable metastatic hormone-refractory prostatic cancer treated with single-agent chemotherapy. Cancer 70:2870-2878, 1992

55. Tannock IF, de Wit R, Berry WR, et al: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 351:1502-1512, 2004

56. Marnellos G: High-throughput SNP analysis for genetic association studies. Curr Opin Drug Discov Devel 6:317-321, 2003

57. Houlston RS, Peto J: The search for low-penetrance cancer susceptibility alleles. Oncogene 23:6471-6476, 2004

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Author Contributions

Conception and design: Norihiko Tsuchiya, Tomonori Habuchi
Provision of study materials or patients: Hiroyoshi Suzuki, Takehiko Segawa, Masaki Shimbo, Toshiyuki Kamoto, Tomohiko Ichikawa, Osamu Ogawa
Collection and assembly of data: Norihiko Tsuchiya, Lizhong Wang, Hiroyoshi Suzuki, Takehiko Segawa, Hisami Fukuda, Shintaro Narita, Masaki Shimbo, Toshiyuki Kamoto, Kenji Mitsumori, Tomohiko Ichikawa, Osamu Ogawa
Data analysis and interpretation: Norihiko Tsuchiya, Lizhong Wang, Shintaro Narita, Kenji Mitsumori, Akira Nakamura, Tomonori Habuchi
Manuscript writing: Norihiko Tsuchiya, Tomonori Habuchi
Final approval of manuscript: Norihiko Tsuchiya, Lizhong Wang, Hiroyoshi Suzuki, Takehiko Segawa, Hisami Fukuda, Shintaro Narita, Masaki Shimbo, Toshiyuki Kamoto, Kenji Mitsumori, Tomohiko Ichikawa, Osamu Ogawa, Akira Nakamura, Tomonori Habuchi

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Requirement of Androgen-Dependent Activation of Protein Kinase Cζ for Androgen-Dependent Cell Proliferation in LNCaP Cells and Its Roles in Transition to Androgen-Independent Cells

Takahiro Inoue, Toru Yoshida, Yosuke Shimizu, Takashi Kobayashi, Toshinari Yamasaki, Yoshinobu Toda, Takehiko Segawa, Toshiyuki Kamoto, Eijiro Nakamura, and Osamu Ogawa

Department of Urology (T.I., T.Yo., Y.Sh., T.Ko., T.Ya., T.Se., T.Ka., E.N., O.O.), Kyoto University, Graduate School of Medicine, and Anatomical Center of Kyoto University (Y.T.), Graduate School of Medicine, 54 Kawaharacho, Shogoin Sakyo-ku Kyoto 606-8507, Japan

A cell line that we designed, AILNCaP, proliferated in androgen-depleted medium after emerging from long-term androgen-depleted cultures of an androgen-sensitive prostate cancer cell line, LNCaP. Using this cell line as a model of progression to androgen independence, we demonstrated that the activity of the mammalian target of rapamycin/p70 S6 kinase transduction pathway is down-regulated after androgen depletion in LNCaP, whereas its activation is related to transition of this cell line to androgen-independent proliferation. Kinase activity of protein kinase Cζ is regulated by androgen stimulation in LNCaP cells, whereas it is activated constitutively in AILNCaP cells under androgen-depleted conditions. Treatment with a protein kinase Cζ pseudosubstrate inhibitor reduced p70 S6 kinase activity and cell proliferation in both cell lines. We identified that both protein kinase Cζ and p70 S6 kinase were associated in LNCaP cells and this association was enhanced by the androgen stimulation. We examined the expression of phospho-protein kinase Cζ and phospho-p70 S6 kinase in hormone-naive prostate cancer specimens and found that the expression of both kinases was correlated with each other in those specimens. Significant correlation was observed between the expression of both kinases and Ki67 expression. Most of the prostate cancer cells that survived after prior hormonal treatment also expressed both kinases. This is the first report that shows the significance of this pathway for both androgen-dependent and -independent cell proliferation in prostate cancer. Our data suggest that protein kinase Cζ/mammalian target of rapamycin/S6 kinase pathway plays an important role for the transition of androgen-dependent to androgen-independent prostate cancer cells. (*Molecular Endocrinology* 20: 3053–3069, 2006)

SINCE DEMONSTRATION BY Huggins and Hodges in the early 1940s that growth and survival of the prostate gland depends upon androgens (1), the mainstay of treatment for advanced prostate cancer has been androgen ablation. However, the vast majority of patients ultimately relapse after a period of initial response to this therapy, developing androgen-independent prostate cancer. Molecular mechanisms involved in prostate-cell proliferation in response to androgen, as well as emergence of androgen-independent prostate cancer, are under active investigation (2–14).

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Abbreviations: aPKC, Atypical PKC; AR, androgen receptor; CSFBS, charcoal-stripped fetal bovine serum; HEK, human embryonic kidney; mTOR, mammalian target of rapamycin; PDK-1, 3-phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PSA, prostate-specific antigen; TMA, tissue microarray.

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The LNCaP cell line was established from supraclavicular lymph node metastasis of human prostate cancer (15). Regardless of its expression of a promiscuous gain-of-function mutant androgen receptor (AR) in LNCaP cells, androgens regulate their growth and expression of prostate-specific genes, such as the prostate-specific antigen (16). Due to this androgen-sensitive characteristic, the LNCaP cell line has become a widely used model for *in vitro* prostate cancer research. Although acute androgen ablation results in growth arrest without inducing apoptosis in the cell lines, long-term androgen ablation transforms a subpopulation to androgen-independent clones (17). Thus, a number of androgen-independent LNCaP sublines have been developed in different laboratories (3, 17–20).

During the past decade, many reports have demonstrated that TOR (target of rapamycin) participates importantly in controlling cell growth, proliferation, and metabolism (21, 22). Regulation by TOR extends to a wide array of cellular functions including translation, transcription, mRNA turnover, protein stability, actin cytoskeletal organization, and autophagy (21–23).

The best-characterized downstream targets of mammalian target of rapamycin (mTOR) in mammalian cells include two signaling pathways that regulate translation: the 70-kDa ribosomal protein S6 kinase (S6K) pathway, and the eukaryotic translation initiation factor 4E-binding protein (4EBP1) pathway (22). Both 4E-BP and S6K pathways contribute to regulation of cell-cycle progression by TOR (22, 24). S6K is the well-known ribosomal protein S6 kinase in mammalian cells (22, 24). Microinjection of neutralizing anti-S6K antibodies can block mitogen-induced progression from the G1 to the S phase of the cell cycle (25). Phosphorylation of S6K contributes to an increase in kinase activity, which increases degrees of phosphorylation in ribosomal S6 polypeptides (26). S6 phosphorylation stimulates translation of mRNAs with 5′-terminal oligopyrimidine tracts (26). These terminal oligopyrimidine-containing mRNAs often code for components of the protein synthesis apparatus (26). Hence, S6K enhances the translational capacity of cells.

Multiple additional effectors for S6K include Cdc42 (27), Rac (27), 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (28), and atypical protein kinase C (aPKC) (29, 30). In coexpression experiments, a kinase-inactive mutant of the aPKC isoform PKCζ antagonized activation of S6K by epidermal growth factor, PDK-1, and activated Cdc42 and phosphatidylinositol 3-kinase (PI3K) (29). This suggested that PKCζ participates in activation of multimetric PI3K-S6K signaling complexes. PKCζ has been identified as a downstream target of PI3K (31, 32). Differing from conventional and novel classes of PKCs, PKCζ does not require diacylglycerol or calcium for activation (33–35). Additionally, PKCζ has been suggested to be involved in several growth-related processes (36), and PDK-1 has been suggested as an activator of PKCζ (31, 32).

In this study, we established a prostate cancer cell line, AILNCaP, that proliferated under androgen-depleted conditions after emerging from long-term, androgen-depleted culture of an androgen-sensitive prostate cancer cell line, LNCaP. We used this model, which mimics the situation in patients subjected to androgen ablation therapy who eventually became refractory to treatment, to investigate the role of intracellular signaling pathways in progression to androgen independence in prostate cancer.

We demonstrated that the mTOR/S6K signaling pathway is activated in an androgen-dependent manner in LNCaP cells but is activated constitutively in AILNCaP cells under androgen-depleted conditions. We also found that phosphorylation of PKCζ is regulated by androgen stimulation in LNCaP cells while being active in AILNCaP cells even under androgen-depleted conditions. Using a myristoylated PKCζ pseudosubstrate inhibitory peptide, which is specific for atypical PKC, we demonstrated that PKCζ is required for S6K activation and cell proliferation in both LNCaP and AILNCaP cells.

RESULTS

Characterization of Androgen-Independent Cell Lines AILNCaP

As previously described (37, 38), short-term androgen deprivation in LNCaP cells results in cell-cycle arrest in G_1 rather than apoptosis. Here, instead of cell-cycle arrest, AILNCaP cells could proliferate even though they were cultured in androgen-deprivation medium, representing androgen-independent cell proliferation (Fig. 1A). To identify the mechanisms that may be responsible for androgen-independent proliferation of these AILNCaP cells, we first examined whether mutations of the AR gene in addition to T877A spontaneously occurred in AILNCaP. We did not find any AR mutations except for T877A, which also was present in the parental LNCaP cells (data not shown). Next we used Western blotting to compare AR expression between these cell lines, and it was revealed that AR expression in AILNCaP cells increased 2.2-fold over that in LNCaP cells (Fig. 1B). In fact, AILNCaP cells expressed prostate-specific antigen (PSA) in response to R1881 stimulation, but needs much higher dose of R1881 stimulation for PSA expression both in mRNA and protein levels than LNCaP cells did (Fig. 1C). We then examined whether low-dose androgen increases the cell proliferation of AILNCaP cells. Our results showed that low-dose androgen stimulation such as 0.001 nM of R1881 did not induce up-regulation of S phase in AILNCaP cells and the proportion of cells in S phase was most abundant under androgen depleted conditions (Fig. 1D). On the other hand, LNCaP cells entered S phase with 0.1 nM of synthetic androgen (R1881) stimulation after 96-h culture in charcoal-stripped fetal bovine serum (CSFBS) (Fig. 1D). To further examine the role of an extremely low concentration of androgen retained in CSFBS in the proliferation of AILNCaP cells, those cells were cultured in 1 μM of bicalutimide, which could inhibit cell proliferation of LNCaP cells under normal serum. Our results demonstrate that AILNCaP cells showed no attenuation in cell proliferation at 1 μM of bicalutamide treatment (Fig. 1E). Hence, AILNCAP cells do not scavenge residual androgen from the CSFBS medium. These results indicate that hypersensitivity to androgen may not cause the androgen-independent cell proliferation of AILNCaP cells.

Androgen Deprivation Reduced Expression of Phosphorylated S6K as Well as the Amount of S6K Expression, Whereas They Are Constitutively Activated in AILNCaP under Androgen-Depleted Conditions

To investigate the mechanisms of how LNCaP cells becomes androgen-independent in cell proliferation, we first examined the differences of Erk and p38MAPK pathways in both cells (LNCaP and AILNCaP). It is well characterized that activation of Ras/ERK/MAPK cas-

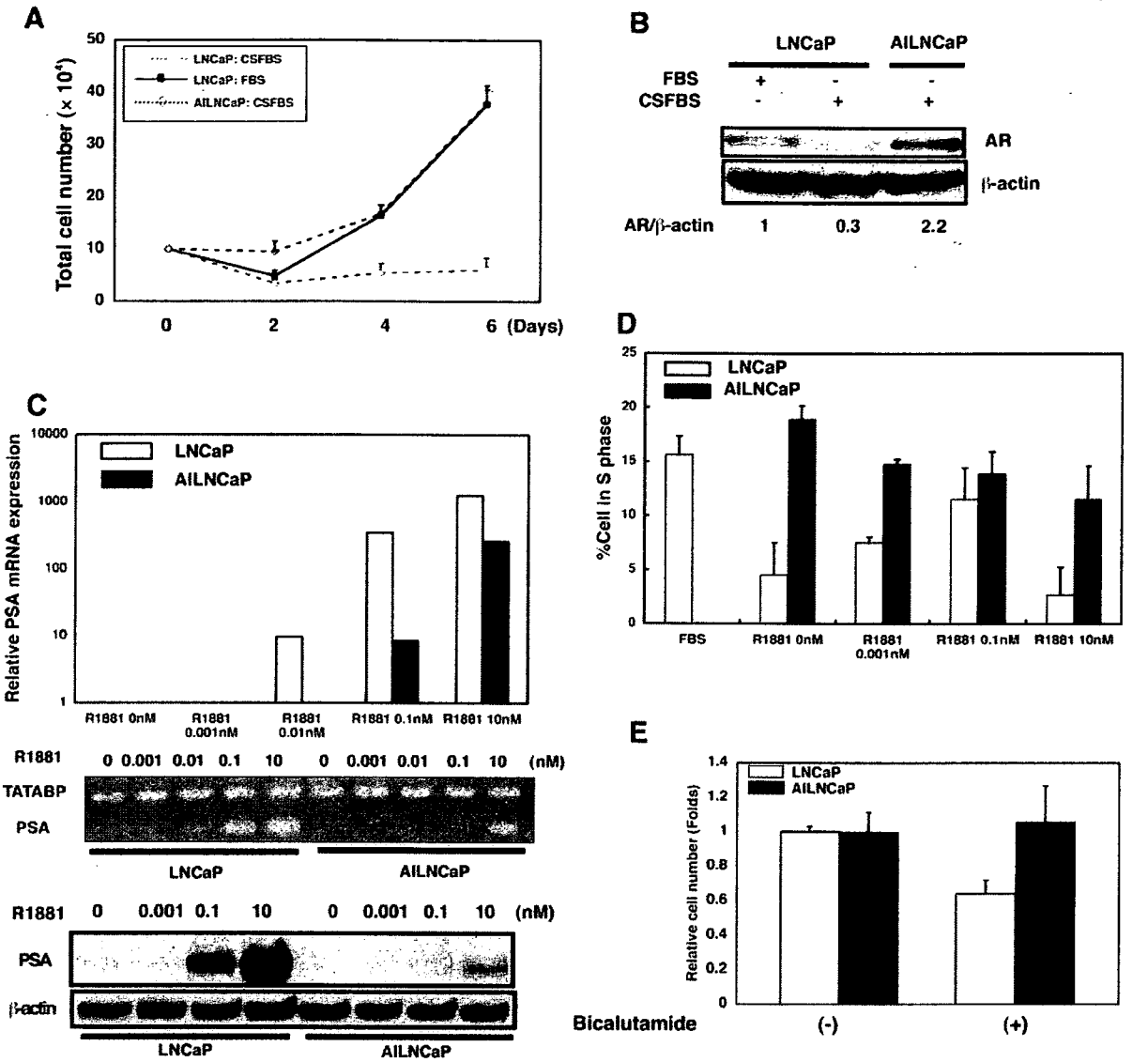

Fig. 1. Effect of Androgen Deprivation and Stimulation in LNCaP Cells and AILNCaP Cells

A, LNCaP cells were cultured in phenol red-free RPMI +10% CSFBS for 96 h and then 1 × 10^5 cells were seeded into 12-well plates (d 0). After 24 h, they were grown in 10% normal serum (FBS) (*filled squares*) or 10% CSFBS (*open squares*) for the indicated days. Cells were counted in a hemocytometer in quadruplicate. AILNCaP cells growing in phenol red-free RPMI + 10% CSFBS (*open diamonds*) also were analyzed. B, After 24 h in RPMI + 10% FBS, LNCaP cells were deprived of androgen in phenol red-free RPMI + 10% CSFBS for 96 h and total cell lysates were analyzed. AILNCaP cells in growth phase cultured in phenol red-free RPMI + 10% CSFBS also were analyzed, as were LNCaP cells in growth phase in RPMI + 10% FBS. Total cell extracts were analyzed for AR by Western blotting. The *number under each lane* of β-actin expression indicates the relative intensity of each AR expression normalized to β-actin expression. C, Dose-dependent expression analysis of PSA. After 24 h in RPMI + 10% FBS, LNCaP cells were depleted of androgen by culture in phenol red-free RPMI + 10% CSFBS for 96 h; then the indicated dose of synthetic androgen (R1881) was provided for 24 h. After 48 h in phenol red-free RPMI + 10% CSFBS, AILNCaP cells were treated with the indicated dose of synthetic androgen (R1881) for 24 h. Total cell extracts or RNA were analyzed for PSA by quantitative (*upper panel*) and conventional RTPCR (*middle panel*) or by Western blotting (*lower panel*). D, Percentages of LNCaP cells and AILNCaP cells in S phase determined by flow cytometry. LNCaP cells were cultured in phenol red-free RPMI + 10% CSFBS for 96 h and then the indicated dose of synthetic androgen (R1881) with 10% CSFBS or 10% FBS without R1881 was provided for 84 h. AILNCaP cells were cultured in phenol red-free RPMI +10% CSFBS and stimulated with indicated concentration of R1881 for 84 h before trypsinization and fixation. Values represent the mean ± SD derived from three independent experiments. All experiments were repeated at least three times. E, LNCaP cells were cultured in RPMI +10% FBS and then 1 × 10^5 cells were seeded into 12-well plates. AILNCaP cells were cultured in phenol red-free RPMI + 10% CSFBS and 1 × 10^5 cells seeded into 12-well plates also were analyzed. After 24 h, they were grown in RPMI +10% FBS (LNCaP) or phenol red-free RPMI +10% CSFBS (AILNCAP) with or without 1 μM of bicalutamide for 120 h. Cells were counted in a hemocytometer in quadruplicate. Data in panels represent the mean cell numbers relative to those without bicalutamide treatment ± SD (*error bars*).

cade plays significant roles in transition of prostate cancer to androgen-independent growth (39–41). Phosphorylated form of p38MAPK showed no significant difference between LNCaP and AILNCaP cells in the androgen-depleted conditions; however, phosphorylated Erk increased by1.5-fold in AILNCaP in comparison to LNCaP cells under the same culture conditions (Fig. 2A). To elucidate the role of this activation in transition of androgen-independent cell proliferation, we have examined the effect of PD98059 (MAPK kinase 1 inhibitor) on proliferation of AILNCaP cells. A 10-μM concentration of PD98059 could inhibit the expression of phosphorylated Erk significantly; however, this had a faint effect on the cell proliferation in AILNCaP cells (Fig. 2B). These results suggest that MAPK kinase signaling pathways are not predominantly responsible for androgen-independent cell proliferation in AILNCaP cells.

Next, we hypothesized that S6K inactivation could occur in LNCaP cells during androgen depletion because p70S6 kinase (S6K) has been reported to be an important regulator of cell proliferation (24, 25). Indeed, androgen deprivation reduced expression of both phosphorylated (Thr-389 and Ser-371) and total S6K expression in LNCaP cells, which resulted in reduction of S6K signaling. Additionally, the same trend was observed in the downstream target of S6K, S6 pathways (Fig. 2C). Moreover, androgen stimulation up-regulated the phosphorylation of S6K in LNCaP cells (Fig. 2D), which reflects the kinase acitivity of S6K. The kinase activity of S6K was reduced to 30% in LNCaP cells under androgen deprivation and it was partially restored by stimulation with synthetic androgen (Fig. 2E). Additionally, S6K kinase activity in AILNCaP cells under androgen-depleted conditions was up-regulated about by 2.6-fold over that in LNCaP cells under the same condition (Fig. 2E). Taken together, the kinase activity of S6K is partly regulated by androgen stimulation in LNCaP cells and it is activated constitutively in androgen-independent AILNCaP cells in the absence of androgen stimulation.

mTOR/S6K Signaling Pathway Is Required for Cell Proliferation in AILNCaP Cells

One main regulator of T389 phosphorylation that is crucial to activation of S6K (26, 42) is mTOR (43). We therefore treated cells with a specific inhibitor for mTOR, rapamycin, in the presence or absence of androgen to test whether androgen stimulation independently regulates S6K. This treatment abolished T389 phosphorylation of S6K in LNCaP cells regardless of androgen stimulation (Fig. 3A), suggesting that androgen acted upstream of mTOR. Moreover, treatment with rapamycin also reduced T389 and S371 phosphorylation of S6K in AILNCaP cells (Fig. 3B). Additionally, treatment with rapamycin partially inhibited the G1/S transition in LNCaP cells under normal serum and AILNCaP cells subjected to androgen deprivation. All these results suggest that activation of mTOR/S6K signaling pathway was also required for androgen-dependent cell proliferation in LNCaP cells and androgen-independent cell proliferation in AILNCaP cells (Fig. 3C).

No Significant Activation of AKT or TSC2 Is Observed in AILNCaP Cells Compared with Their Parental LNCaP Cells

Because earlier studies indicated that mTOR/S6K activation depends largely upon PI3K-dependent signaling (44, 45), we examined the effect of androgen deprivation upon activities of PI3K downstream targets (Akt and PDK-1) by Western blotting. The phosphorylated form of Akt and PDK-1 in LNCaP cells under androgen deprivation slightly increased by 1.3- and 2.0-fold, respectively, over those under normal serum, so neither was significantly responsible for S6K down-regulation in this condition (Fig. 4A).

We next examined TSC2 protein expression by Western blot analysis. TSC2 is a downstream target of the PI3K/Akt pathway, showing activity that negatively regulates the mTOR/S6K pathway (22, 23, 46, 47). Neither level of phosphorylation of TSC2 nor total TSC2 protein differed significantly between the two cell lines in androgen-depleted condition (Fig. 4B)

Based on these results, we investigated whether phosphorylation of S6K is PI3K sensitive, especially under androgen-depleted conditions in prostate cancer cells. Indeed, under androgen-depleted conditions, treatment with PI3K inhibitors, LY294002 or wortmannin, reduced phosphorylation of S6K (Fig. 4C). Interestingly, there was a difference in the level of S6K phosphorylation on threonine 389 with treatment of PI3K inhibitors, which was also reflected by the extent of S6K target protein S6 phosphorylation between LNCaP and AILNCaP cells (Fig. 4C). On the other hand, some discrepancies were evident between degree of attenuation of Akt phosphorylation and S6K phosphorylation by PI3K inhibitors in both cell lines (Fig. 4C). Considering that the phosphorylation status of Akt showed no significant difference between LNCaP cells and AILNCaP cells under androgen deprivation (Fig. 4C), other kinases downstream of PI3K as opposed to Akt might play major roles in mTOR/S6K activation in these cell lines.

Androgen Stimulation in LNCaP Cells Activates PKCζ, which Is Active in AILNCaP Cells without Androgen Stimulation

Recent studies suggested that PKC ζ is an upstream activator of S6K (29, 30) and that PKCζ/S6K signaling pathway regulates cell proliferation of prostate cancer in a murine prostate cancer model (48). So, we examined phosphorylation of the threonine residue (Thr410) of PKCζ in LNCaP cells and in AILNCaP cells. In LNCaP cells, androgen deprivation induced PKCζ dephosphorylation of Thr410 (Fig. 5A, *upper panel*). In contrast, phosphorylation at Thr410 could be seen in

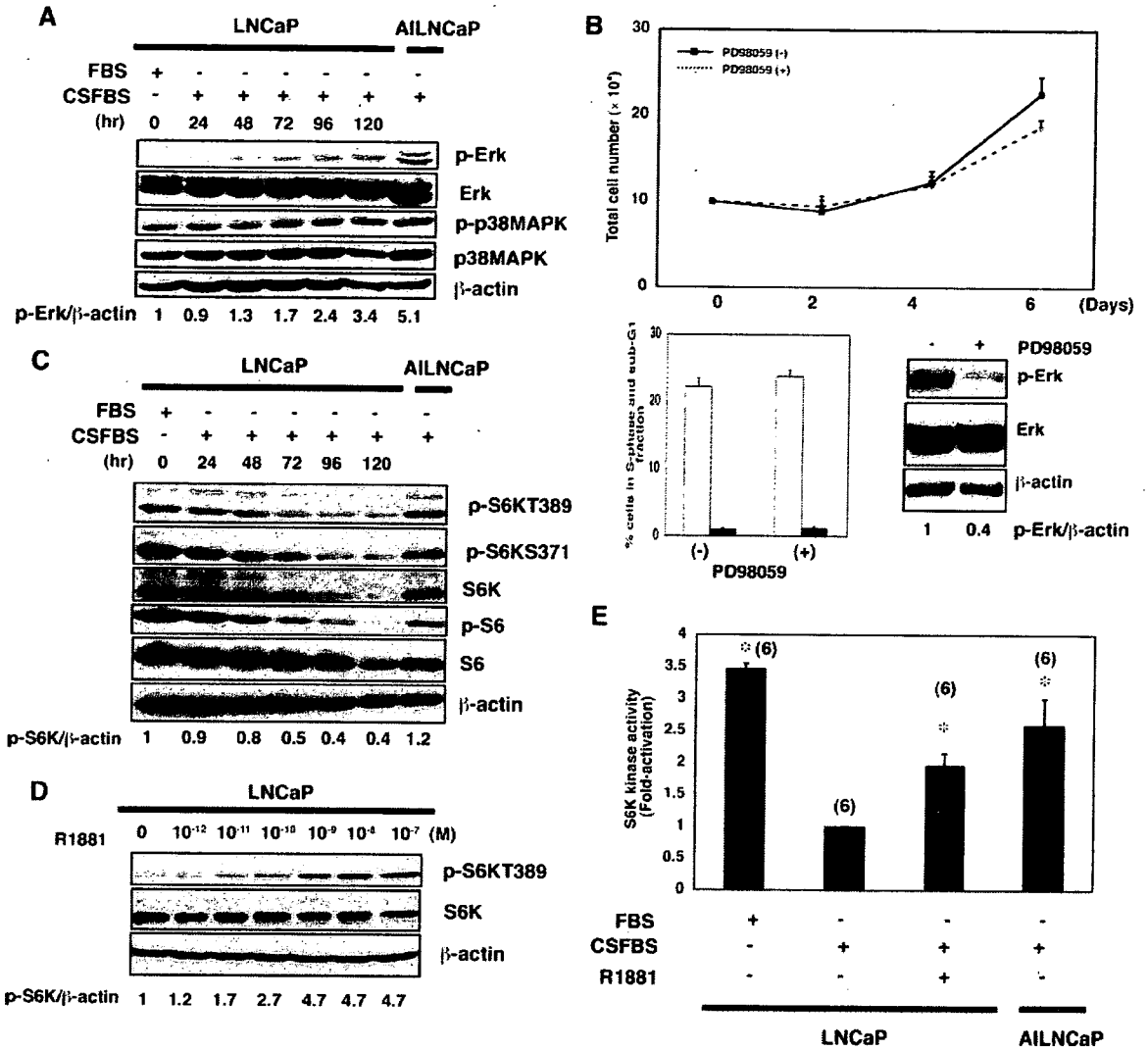

Fig. 2. The mTOR/S6K Signaling Pathway Is Regulated by Androgen Stimulation in LNCaP Cells and Is Activated in AILNCaP Cells without Androgen Stimulation

A and C, After 24 h culture in RPMI + 10% FBS, LNCaP cells were deprived of androgen in phenol red-free RPMI + 10% CSFBS for the time indicated, and total cell lysates were analyzed. AILNCaP cells in growth phase cultured in phenol red-free RPMI + 10% CSFBS also were analyzed, as were LNCaP cells in growth phase in RPMI + 10% FBS. Total cell extracts were analyzed for Erk phospho-T202/Y204 (p-Erk), p38MAPK phospho-T180/Y182 (p-p38MAPK), S6K phospho-T389 (p-S6KT389), S6K phospho-S371 (p-S6KS371), S6 phospho-S235/236 (p-S6), Erk, p38MAPK, S6K, and S6. The *number under each lane* of β-actin expression indicates the relative intensity of each phospho-Erk (A) or phospho-S6KT389 (C) expression normalized to β-actin expression. B, *Upper panel*, AILNCaP cells cultured in phenol red-free RPMI + 10% CSFBS were seeded 1×10^5 cells per well into 12-well plates (d 0). After 24 h, they were grown in phenol red-free RPMI + 10% CSFBS in the absence (*filled squares*) or presence of 10 μM PD98059 (*open diamonds*) for the indicated days. Cells were counted in a hemocytometer in quadruplicate. *Left lower panel*, Percentages of AILNCaP cells in S phase (*white bars*) and sub-G1 fraction (*black bars*) determined by flow cytometry. AILNCaP cells were cultured in phenol red-free RPMI + 10% CSFBS in the absence (−) or presence (+) of 10 μM PD98059 for72 h before trypsinization and fixation. Values represent the mean ± SD derived from three independent experiments. *Right lower panel*, After 48 h in phenol red-free RPMI + 10% CSFBS, AILNCaP cells were treated with 10 μM of PD98059 for 2 h. Total cell extracts were analyzed for Erk phospho-T202/Y204 (p-Erk) and Erk. D, After 24 h in RPMI + 10% FBS, LNCaP cells were deprived of androgen in phenol red-free RPMI + 10% CSFBS for 96 h; then the indicated final concentration of synthetic androgen (R1881) was added for 24 h. Total cell extracts were analyzed for S6K phospho-T389 (p-S6KT389) and for S6K. The *number under each lane* of β-actin expression indicates the relative intensity of each phospho-S6KT389 expression normalized to β-actin expression. E, S6K activity assays were carried out after growth of LNCaP cells in RPMI + 10% FBS as well as AILNCaP cells in phenol red-free RPMI + 10% CSFBS. LNCaP cells deprived of androgen in phenol red-free RPMI +10% CSFBS for 96 h and then stimulated with 10 nM of synthetic androgen (CSFBS+, R1881+), or sham-stimulated (CSFBS+, R1881−), for 24 h were also analyzed. S6K activity results are expressed as mean activities relative to those in androgen-depleted LNCaP cells after 120 h (CSFBS+, R1881−), ± SD (*error bars*) of (n) determinations. *Asterisks* indicate $P < 0.01$ for comparisons of S6K fold activities of LNCaP cells under CSFBS (CSFBS+, R1881−) values *vs.* others indicated. All experiments were repeated at least three times.

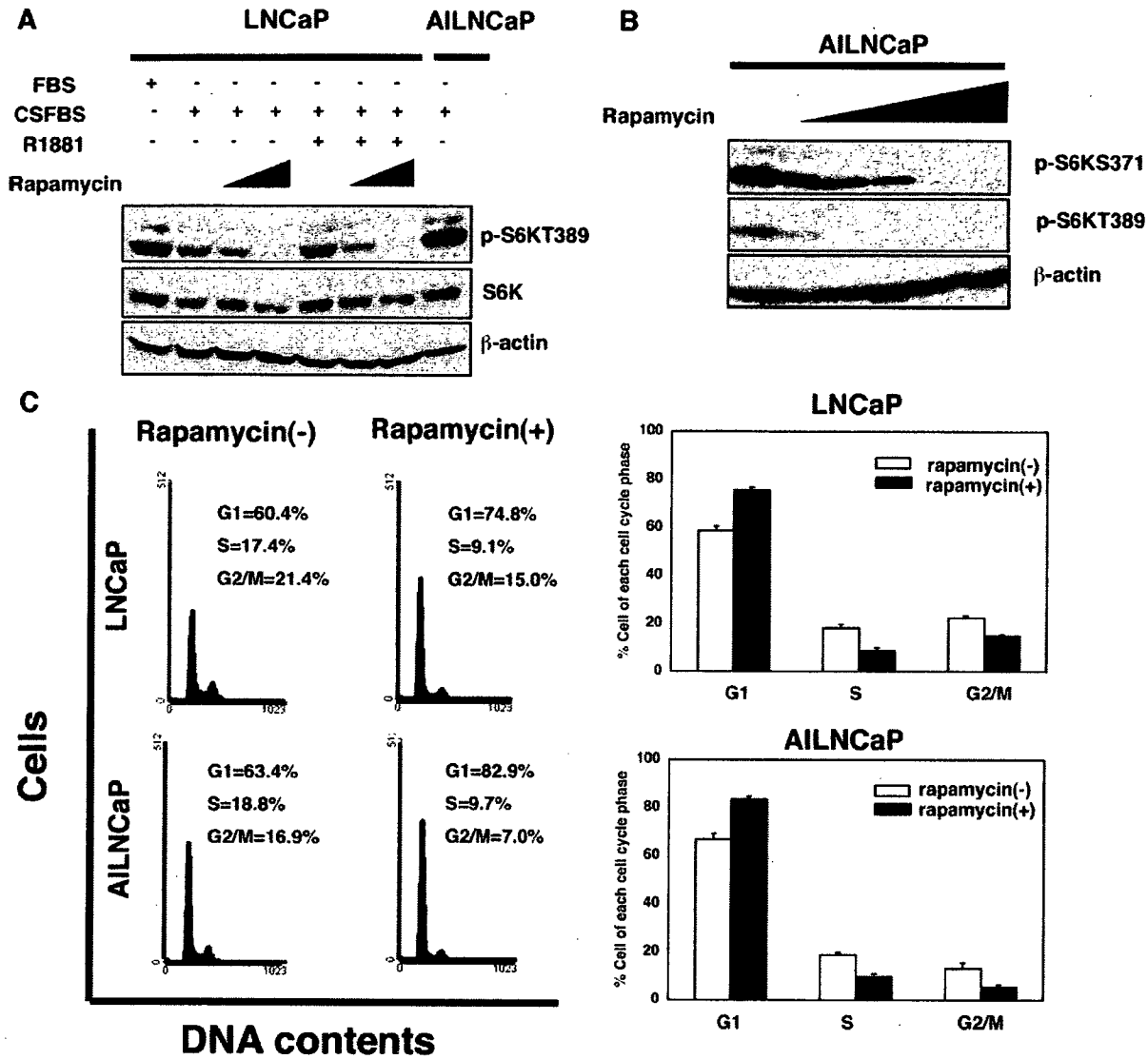

Fig. 3. Action of mTOR/S6K Is Essential for Cell Proliferation in Prostate Cancer Cells

A, After 24 h in RPMI + 10% FBS, LNCaP cells were cultured in phenol red-free RPMI + 10% CSFBS with or without 0.1 nM synthetic androgen R1881 for 48 h. LNCaP cells then were treated with 0.1 nM or 1 nM rapamycin for 24 h in the presence or absence of 0.1 nM R1881. Total cell extracts were analyzed for S6K phospho-T389 (p-S6KT389) and for S6K. B, After 48 h in phenol red-free RPMI + 10% CSFBS, AILNCaP cells were treated with 0.1, 0.5, 1, 5, or 10 nM rapamycin for 24 h. Total cell extracts were analyzed for S6K phospho-T389 (p-S6KT389) and S6K phospho-S371 (p-S6KS371). C, Cell-cycle distribution was determined by flow cytometry. After 24 h in RPMI + 10% FBS (LNCaP) or in phenol red-free RPMI + 10% CSFBS (AILNCaP), LNCaP cells and AILNCaP cells were cultured with or without rapamycin (1 nM) for 72 h. *Left*, Representative histograms of each condition. *Right*, Data in a panel represent the mean ± SD (*error bars*) of three independent experiments. These results are representative of three independent experiments.

AILNCaP cells even under androgen-deprived states (Fig. 5A). Furthermore, androgen stimulation up-regulated the degree of phosphorylation of PKCζ in LNCaP cells (Fig. 5A). In fact, AILNCaP cells retained higher PKCζ kinase activity than that in LNCaP cells under androgen-depleted conditions (Fig. 5B). Additionally, PKCζ kinase activity was partially restored by stimulation with synthetic androgen in LNCaP cells (Fig. 5B).

Previously, it was shown that exogenously expressed wild-type PKCζ was associated with S6K in human embryonic kidney (HEK) 293 cells (29, 30). So, we next addressed whether this was also observed in prostate cancer cell lines. We found that S6K coimmunoprecipitated with exogenously expressed wild-type PKCζ in LNCaP cells and PC3 cells as well as HEK 293 cells (Fig. 5C, *upper panel*). Furthermore, this complex formation was enhanced by androgen stimulation in LNCaP cells (Fig. 5C, *lower panel*). To verify the contribution of AR to PKCζ phosphorylation together with S6K phosphorylation in prostate cancer

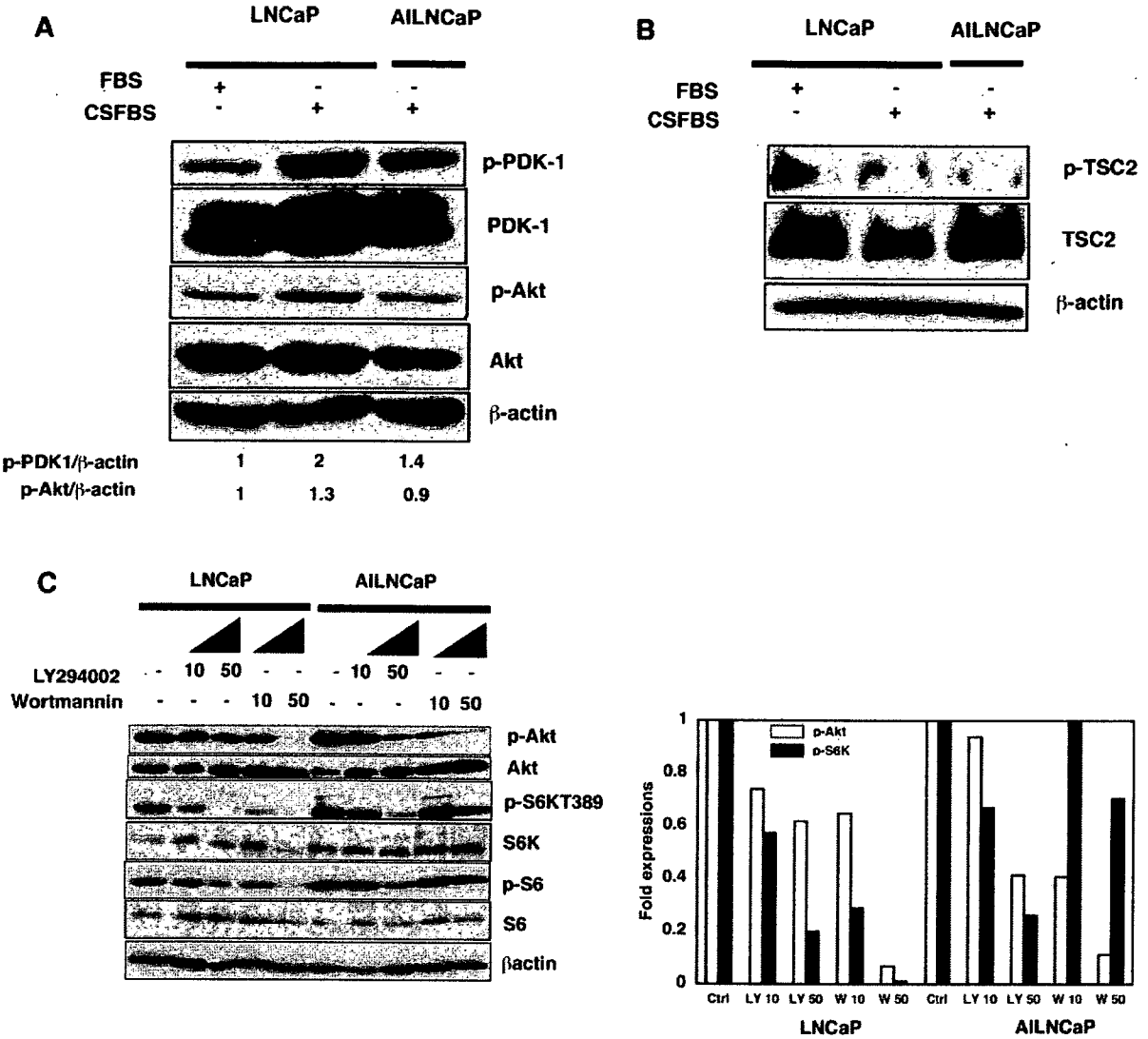

Fig. 4. Activity and Expression of PDK-1/Akt and TSC2 Cannot Explain Progression to Androgen Independence in LNCaP Cells

A, After 24 h in RPMI + 10% FBS, LNCaP cells were deprived of androgen in phenol red-free RPMI + 10% CSFBS for 96 h and total cell lysates were analyzed. AILNCaP cells in growth phase cultured in phenol red-free RPMI + 10% CSFBS also were analyzed, as were LNCaP cells in growth phase in RPMI + 10% FBS. Total cell extracts were analyzed for PDK-1 phospho-S241 (p-PDK-1), Akt phospho-S473 (p-Akt), PDK-1, and Akt. The *number under each lane* of β-actin expression indicates the relative intensity of each phospho-PDK-1 and phospho-Akt expression normalized to β-actin expression. B, Expression and phosphorylation of TSC2 were examined after culture under conditions described for panel A. Total cell extracts were analyzed for TSC2 phospho-T1426 (p-TSC2) and TSC2. C, After 48 h in phenol red-free RPMI + 10% CSFBS, both LNCaP cells and AILNCaP cells were treated for 30 min with or without 10 and 50 μM LY294002 or 10 and 50 nM wortmannin followed by culturing in fresh phenol red-free RPMI + 10% CSFBS for 120 min before harvesting. *Left*, Total cell extracts were analyzed for Akt phospho-S473 (p-Akt), S6K phospho-T389 (p-S6KT389), S6 phospho-S235/236 (p-S6), Akt, S6K, and S6. *Right*, A densitometric analysis of phospho-Akt and phospho-S6KT389 levels normalized to β-actin expression in each condition (Ctrl: no stimulation, LY10: 10 μM LY294002, LY50: 50 μM LY294002, W10: 10 nM wortmannin, W50: 50 nM wortmannin). These results are representative of three independent experiments.

cells, we used bicalutamide, a cognate AR antagonist, to block AR. As shown in Fig. 5D, in the presence of bicalutamide the action of androgen upon PKCζ and S6K phosphorylation in LNCaP cells was attenuated. This result was compatible with that observed during androgen depletion (Fig. 5A), suggesting that androgen might participate in this signaling activation through its cognate receptor. Together, these results suggest that androgen stimulation results in the activation of PKCζ kinase activity in LNCaP cells.

PKCζ Activity Is Required for the Cell Proliferation of LNCaP and AILNCaP Cells

To elucidate whether PKCζ activity is required for phosphorylation of S6K, treatment with a myristoy-

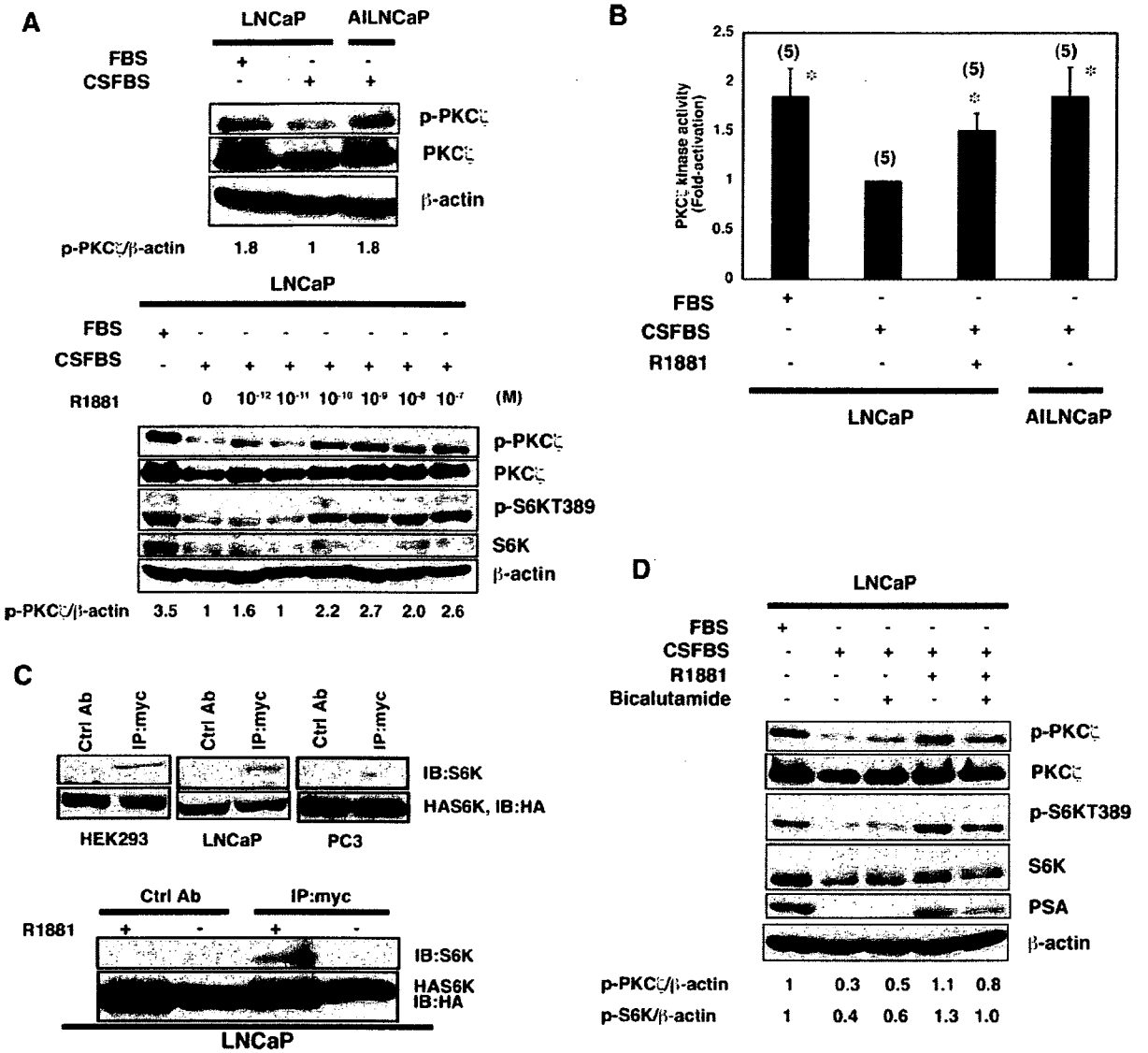

Fig. 5. Activity of PKCζ Is Regulated by Androgen Stimulation in LNCaP Cells and Is Activated in AILNCaP Cells without Androgen Stimulation

A, *Upper panel*, Expression and phosphorylation of PKCζ under the conditions described for Fig. 4A were determined by immunoblotting. Total cell extracts were analyzed for PKCζ phospho-T410 (p-PKCζ) and for PKC. The *number under each lane* of β-actin expression indicates the relative intensity of each phospho-PKCζ expression normalized to β-actin expression. *Lower panel*, After 24 h in RPMI + 10% FBS, LNCaP cells were deprived of androgen in phenol red-free RPMI + 10% CSFBS for 96 h; then the indicated final concentration of synthetic androgen (R1881) was added for 24 h. Total cell extracts were analyzed for PKCζ phospho-T410 (p-PKCζ), S6K phospho-S389 (p-S6KT389), PKCζ, and S6K. The *number under each lane* of β-actin expression indicates the relative intensity of each phospho-PKCζ expression normalized to β-actin expression. B, PKCζ activity assays were carried out after growth of LNCaP cells in RPMI + 10% FBS as well as AILNCaP cells in phenol red-free RPMI + 10% CSFBS. LNCaP cells deprived of androgen in phenol red-free RPMI +10% CSFBS for 96 h and then stimulated with 10 nM of synthetic androgen (CSFBS+, R1881+), or sham-stimulated (CSFBS+, R1881−), for 24 h were also analyzed. PKCζ activity results are expressed as mean activities relative to those in androgen-depleted LNCaP cells after 120 h (CSFBS+, R1881−), ± SD (*error bars*) (n = 5). *Asterisks* indicate $P < 0.01$ for comparisons of PKCζ fold activities of LNCaP cell under CSFBS (CSFBS+, R1881−) values *vs.* others indicated. C, *Upper panel*, HEK 293 cells were cotransfected with HA-S6K (1 μg) and PKCζ-myc (5 μg) per 6-cm dish. The cells were starved in serum-free DMEM for 24 h and then stimulated with EGF (100 ng/ml) for 30 min before harvesting. LNCaP cells were cotransfected with HA-S6K (6 μg) and PKCζ-myc (18 μg) per 10-cm dish. The cells were culture in DMEM supplemented with 10% FBS for 48 h before harvesting. PC3 cells were cotransfected with HA-S6K (2 μg) and PKCζ-myc (6 μg) per 6-cm dish. The cells were starved in serum-free DMEM for 24 h and then stimulated with EGF (100 ng/ml) for 30 min before harvesting. *Lower panel*, LNCaP cells were cotransfected with HA-S6K (6 μg) and PKCζ-myc (18 μg) per 10-cm dish. LNCaP cells were cultured with phenol red-free RPMI +10% CSFBS with 10 nM of synthetic androgen (R1881+), or without androgen (R1881−), for 48 h before harvesting. PKCζ-myc was immunoprecipitated (IP) with an anti-myc antibody. For control

lated PKCζ pseudosubstrate peptide, which is a proven specific inhibitor, was performed in LNCaP cells during androgen stimulation and in AILNCaP cells under androgen deprivation. Inhibition of PKCζ activity induced a decrease in the expression of both phosphorylated (Thr-389) and total S6K. The same trend was observed in downstream target S6, and the abundance of cyclin D1 signal was reduced in both cell lines (Fig. 6A). Additionally, cell cycle analysis revealed that 20 μM myristoylated PKCζ pseudosubstrate peptide not only inhibited the G1/S transition but also induced sub-G1 fraction in both cells, which strongly suggested the existence of apoptotic cells (Fig. 6B). This finding was confirmed by morphological analyses showing that around 10% of the cells presented the nuclei that were condensed or fragmented (Fig. 6C).

Next, we analyzed the requirement of PKCζ activity for S6K phosphorylation in AR-negative prostate cancer cells, such as PC3 cells and DU145 cells, by the same methods described above. Although 20 μM of myristoylated PKCζ showed little effect on the abundance of phosphorylated form of S6K, treatment with 50 μM of the inhibitor significantly reduced the above signal (Fig. 7A). Under this condition, the inhibitor induced apoptotic cell death (Fig. 7B) and inhibited cell proliferation (data not shown). So, activation of PKCζ/p70 S6 kinase pathway is required for cell proliferation in both AR-positive and -negative prostate cancer cell lines.

Phospho-PKCζ Expression Is Related to Phospho-S6K Expression in Human Prostate Cancer Specimens

We have previously described a tissue microarray (TMA) of prostate carcinoma from Japanese patients with comprehensive clinical data and evaluated the significance of prognostic markers such as Ki67, p53, and AR (49). This TMA consisted of 67 patients (184 spots) with hormone-naive prostate cancer (no prior hormone therapy) and 12 patients (29 spots) with hormone-treated prostate cancer, all of whom were treated by radical prostatectomy for localized disease. Using this TMA, we evaluated the relationship between phospho-PKCζ and phospho-S6K expressions by immunohistochemistry. The expression of AR and Ki67 were also examined in the same specimen.

In the hormone-naive prostate cancer specimens, 75% of spots (138 of 184 spots) were positively stained with both phospho-PKCζ and phospho-S6K antibodies (Table 1). Case 1 and case 2 show the representative specimens with positively or negatively staining of both antibodies (Fig. 8). Spearman's rank order correlation analysis revealed the statistical significance of the modest correlations between the expression of phospho-PKCζ and that of phospho-S6K in these specimens ($P < 0.01$, $\gamma = 0.663$) (Table 1). Furthermore, significant elevation of Ki67 labeling indexes was observed in specimens that were positively stained by both antibodies (total of 138 spots) in comparison with the rest of specimens (total of 46 spots) ($P < 0.001$). These results indicate that activation of PKCζ/S6 kinase pathway is associated with cell proliferation of hormone-naive prostate cancer cells *in vivo*. There was no correlation with Gleason grade and the expression of both kinases.

We analyzed viable cancer cells morphologically in the hormone-treated prostate cancer specimens. Most of the cells might be in a transition state in the continuum between hormone-naive prostate cancer and hormone-refractory cancer cells (50). We found 86% of the spots (25 of 29 spots) were positive for both phospho-PKCζ and phospho-S6K antibodies (Table 1 and Fig. 8). Although the number of hormone-treated prostate cancer specimen is too small for evaluation of PKCζ/S6K pathway, these results implied the possibility that activation of this pathway may be associated with transition of prostate cancer cells to androgen-independent growth or survival of prostate cancer cells in androgen-depleted condition *in vivo*.

DISCUSSION

Growing evidence suggests that AR plays an essential role in transition of androgen-independent cell proliferation in prostate cancer (3). Hence, we first examined the protein levels of AR in both AILNCaP cells and LNCaP cells. In accordance with previous reports (17–20), we found that AR protein level was higher in AILNCaP cells than that in LNCaP cells under androgen-deprived condition. However, in our AILNCaP cells, the level of PSA mRNA induced by androgen stimulation was lower than that in LNCaP cells (17–20), which was in contrast to the previous report (3). Additionally, bicalutamide did not have any significant effect on the growth of AILNCaP cells, but it could significantly reduce cell proliferation of LNCaP cells.

experiments, the cell lysates were incubated with protein G-Sepharose conjugated with control mouse IgG (Ctrl Ab). Coimmunoprecipitating HA-S6K was detected using a S6K-specific polyclonal antibody (Santa Cruz). Total cell lysates were analyzed for HA-S6K using HA antibody (Covance). All experiments were independently repeated two to three times. D, After 24 h in RPMI + 10% FBS, LNCaP cells were deprived of androgen in phenol red-free RPMI + 10% CSFBS for 96 h. With or without pretreatment with 10 μM bicalutamide for 24 h, LNCaP cells were treated with or without 10 nM synthetic androgen (R1881) for another 24 h in phenol red-free RPMI + 10% CSFBS. Total cell extracts were analyzed for PKCζ phospho-T410 (p-PKCζ), S6K phospho-S389 (p-S6KT389), PKCζ, S6K, and PSA. The *number under each lane* of β-actin expression indicates the relative intensity of each phospho-PKCζ expression and phospho-S6KT389 normalized to β-actin expression. These results are representative of three independent experiments.

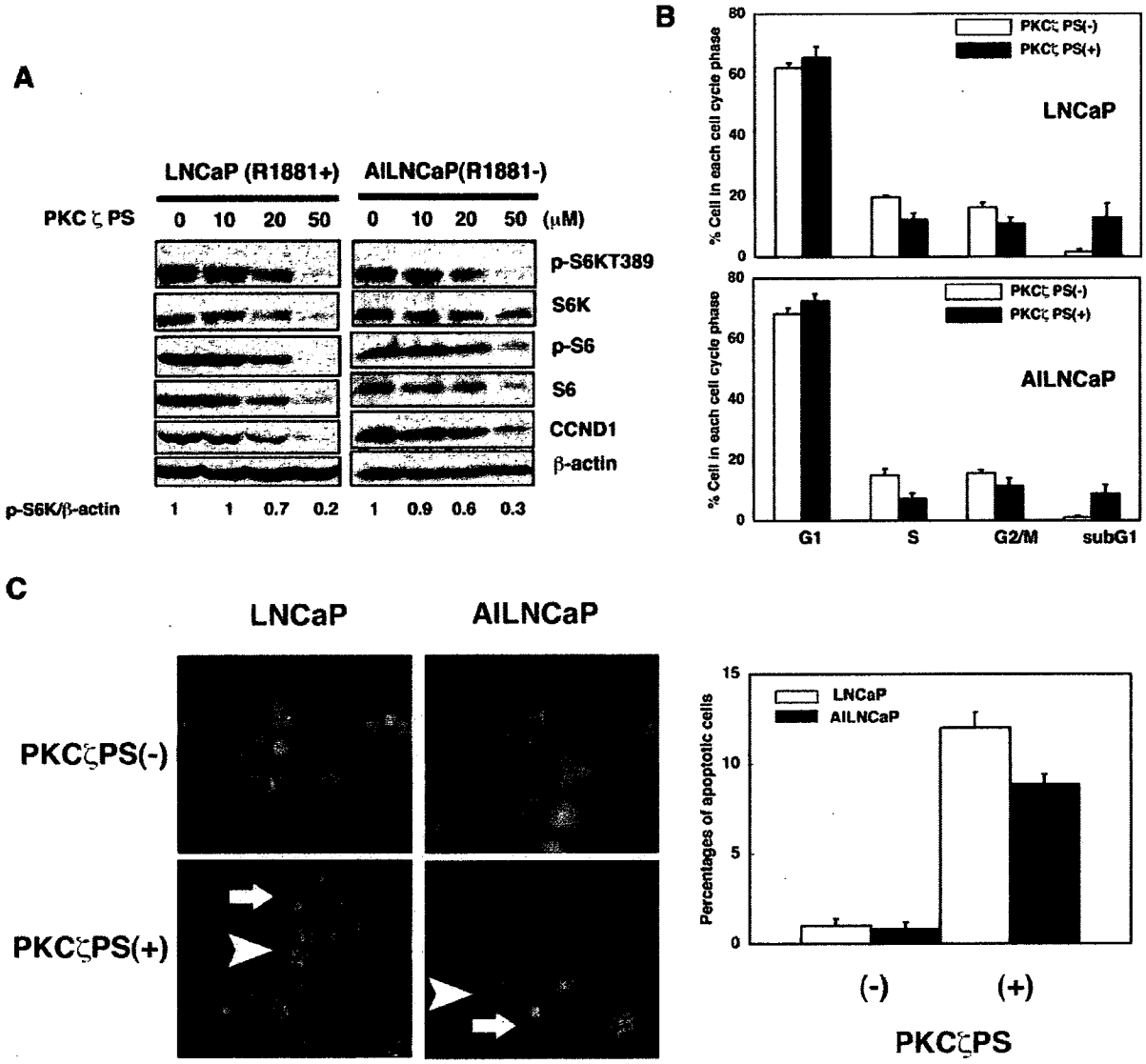

Fig. 6. Activity of the mTOR/S6K Signaling Pathway in LNCaP Cells Is Regulated by Activity of PKCζ, which Is Required for Androgen-Independent Cell Proliferation

A, After 24 h in phenol red-free RPMI + 10% CSFBS, LNCaP cells were cultured for another 24 h with R1881 (10 nM) stimulation. Thereafter, LNCaP cells were treated with 10, 20, or 50 μM myristoylated PKCζ pseudosubstrate inhibitor (PKCζ PS) for 120 to 150 min. AILNCaP cells in phenol red-free RPMI + 10% CSFBS without R1881 also were treated with 10, 20, and 50 μM myristoylated PKCζ pseudosubstrate inhibitor (PKCζ PS) for 120–150 min. Total cell extracts were analyzed for S6K phospho-T389 (p-S6KT389), S6 phospho-S235/236 (p-S6), S6K, S6, and cyclin D1 (CCND1). The *number under each lane* of β-actin expression indicates the relative intensity of each phospho-S6KT389 expression normalized to β-actin expression. B, Cell-cycle distribution was determined by flow cytometry. After 24 h in RPMI + 10% FBS, LNCaP were treated with (+) or without (−) 20 μM myristoylated PKCζ pseudosubstrate inhibitor (PKCζ PS) for24 h. After 48 h in phenol red-free RPMI + 10% CSFBS, AILNCaP were treated with (+) or without (−) 20 μM myristoylated PKCζ pseudosubstrate inhibitor (PKCζPS) for24 h. Data in panels represent the mean ± SD (*error bars*) of three independent experiments. C, Apoptosis was detected morphologically by using Hoechst 33342. LNCaP cells and AILNCaP cells treated with 20 μM myristoylated PKCζ pseudosubstrate inhibitor for 24 h were collected. The cells were treated with 10% formalin neutral buffer solution, followed by rinsing with phosphate-buffer saline, and Hoechst 33342 was added at a final concentration of 0.167 μg/μl and incubated for 20 min at room temperature in the dark. Cells aliquots were placed on slides and a fluorescent microscope was used to count 200 fluorescent cells per condition. Nuclear fragmentation and chromatin condensation were scored as dead. Representative morphology after treatment with myristoylated PKCζ pseudosubstrate inhibitor was represented. *Left upper panels*, There was no increase in chromatin condensation in LNCaP cells and AILNCaP cells treated with vehicles [PKCζPS (−)]. *Left lower panels*, *White arrows* indicate cells with chromatin condensation and *white arrowheads* indicate nuclear fragmentation [PKCζPS (+)]. *Right panel*, Graph represents three independent experiments in which 200 fluorescent cells were counted and scored for chromatin condensation and nuclear fragmentation.