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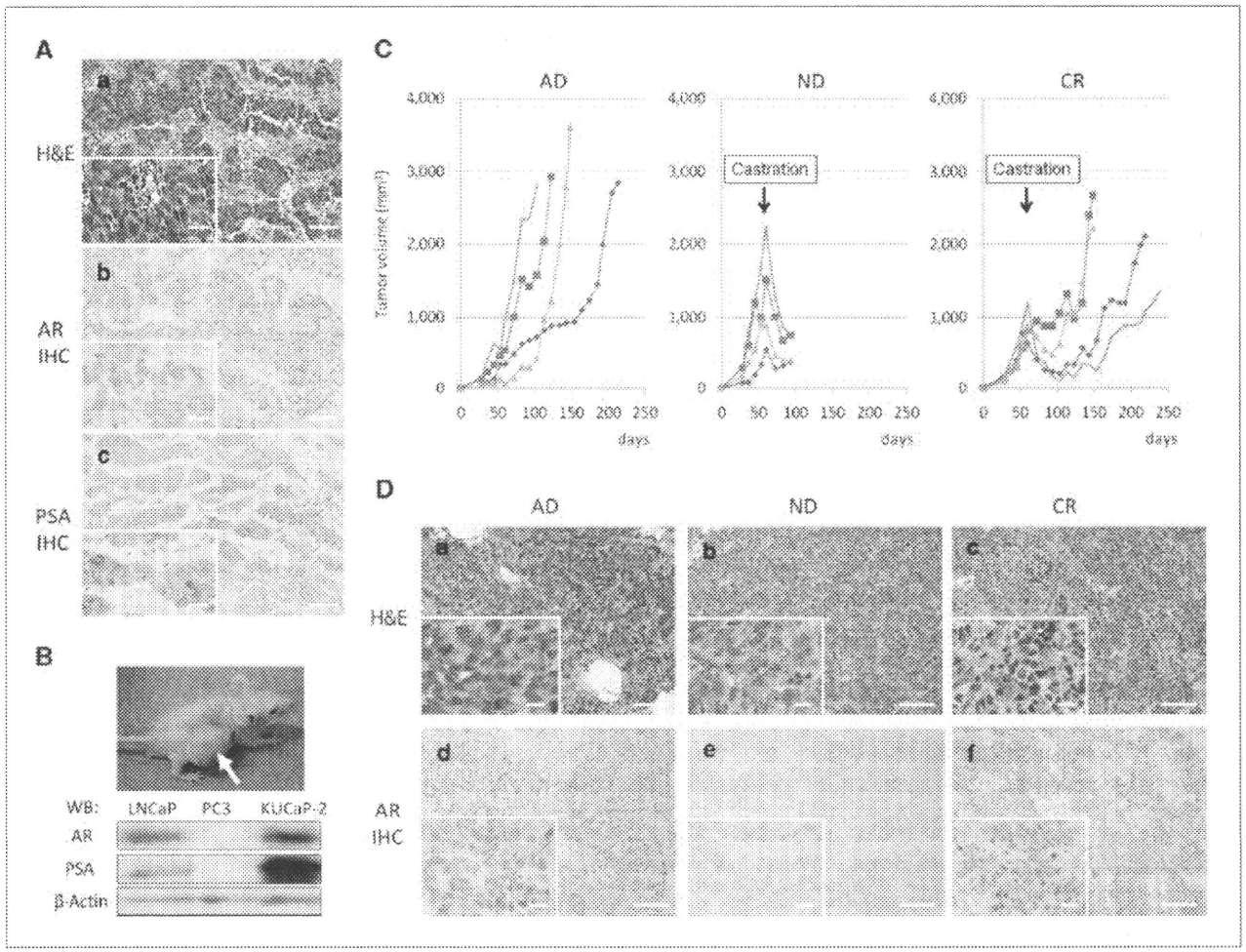

Figure 1. KUCaP-2, a novel established prostate cancer xenograft expressing AR and PSA, regresses after castration of mice and develops castration resistance with nuclear expression of AR. A, H&E staining (a), AR immunohistochemistry (IHC; b), and PSA immunohistochemistry (c) of tumor tissues used for the establishment of KUCaP-2. Scale bars, 50 μm, inset 5 μm. B, KUCaP-2 tumor (arrow, top) expressing AR and PSA detected with Western blotting (WB; LNCaP and PC3 presented as a positive and negative control; bottom). C, the sequential changes in xenograft tumor volume of KUCaP-2 before the tumor extraction at the AD, ND, and CR stages (n = 4 each). D, H&E staining (a–c), AR immunohistochemistry (d–f) at AD, ND, and CR stages of tumors. Scale bars, 50 μm, inset 5 μm.

a luciferase reporter assay in LNCaP cells. EP4 signaling promoted AR activation without androgen to ~50% of the level achieved with androgen stimulation and did not induce additional AR activation in the presence of androgen. Then, we examined whether the increase in cAMP levels and the acceleration of PKA activity was associated with AR activation. PSA expression in LNCaP cells without androgen was induced by both forskolin and dbcAMP but was inhibited by H-89 (Fig. 3C). These results indicate that the signal activation of EP4-cAMP-PKA-AR axis is associated with the castration resistance of LNCaP cells.

The castration of mice decelerated xenograft tumor growth in LNCaP-mock cells but not in LNCaP-EP4 cells (Fig. 3D). The serum PSA values of castrated mice bearing LNCaP-EP4 xenografts were significantly higher than those of mice bearing LNCaP-mock xenografts (median PSA at sacrifice: 4.0 and 32.5 ng/mL in LNCaP-mock and LNCaP-EP4 cells, respectively, $P < 0.05$). These results show that EP4 overexpression induces castration-resistant progression of LNCaP cells *in vivo*.

EP4 antagonist suppressed castration-resistant progression of LNCaP-EP4 and KUCaP-2 tumors. ONO-AE3-208 is an EP4-specific antagonist (5). The Ki values of ONO-AE3-208 for the prostanoid receptors are 1.3, 30, 790, and 2,400 nmol/L for EP4, EP3, FP, and TP, respectively, and >10,000 nmol/L for the other prostanoid receptors (15). To examine the EP4 antagonistic effect of ONO-AE3-208 on LNCaP-EP4 cells, intracellular cAMP concentrations were examined under a variety of ONO-AE3-208 concentrations in androgen-depleted conditions, indicating that 10 to 100 nmol/L of ONO-AE3-208 is sufficient to antagonize overexpressed EP4. This concentration of ONO-AE3-208 reached the Ki of EP3 and could also antagonize EP3. The EP3 signal inhibits adenylate cyclase, and thus the antagonism of EP3 increases intracellular cAMP concentrations (15). However, the suppression level of cAMP was proportional to the ONO-AE3-208 concentrations, suggesting

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that antagonistic effect against EP3 might be slight. The PSA expression of LNCaP-EP4 cells without androgen was also suppressed by the same concentrations of ONO-AE3-208 (Fig. 4A).

We then examined the *in vivo* antitumor effect of ONO-AE3-208. I.p. injection of ONO-AE3-208 (10 mg/kg; once a day) suppressed the castration-resistant growth of LNCaP-EP4 xenograft tumors (Fig. 4B). The serum PSA values of LNCaP-EP4 xenograft mice were also significantly decreased (median PSA at sacrifice: 5.7 and 3.7 ng/mL in controls and AE3-208, respectively, $P < 0.05$). The mean body weight of mice in the control and AE3-208 groups were almost the same, and no mice died during the treatment, indicating that ONO-AE3-208 was well tolerated at the concentrations used. The same dose of ONO-AE3-208 also suppressed the castration-resistant growth of KUCaP-2 tumors (Fig. 4C). The PSA production of KUCaP-2 tumors was significantly decreased (median PSA at sacrifice: 17.4 and 9.4 ng/mL in controls and AE3-208, respectively, $P < 0.05$). There were no significant differences in EP4 expression between the tumors of the control and AE3-208 groups (data not shown), indicating that ONO-AE3-208 antagonized EP4 without suppressing the receptor expression. In summary, EP4 antagonism with ONO-AE3-208 might be an effective and tolerable treatment modality for CRPC, in which EP4 overexpression induced castration-resistant progression (Fig. 4D).

Discussion

As CRPC is a heterogeneous group of diseases (16), many experimental models are required to elucidate the mechanisms for castration resistance. However, limited tissue availability for molecular studies and few available human prostate cancer cell lines with both AR- and androgen-dependent states have restricted prostate cancer research. Xenografts are models in which human tissue is transplanted into an immunodeficient mouse. In this way, human prostate cancer can be propagated *in vivo* for long periods to allow the study of tumor progression under different experimental hormonal conditions and to support the testing of novel therapies. Before 1993, only one prostate cancer xenograft, LNCaP

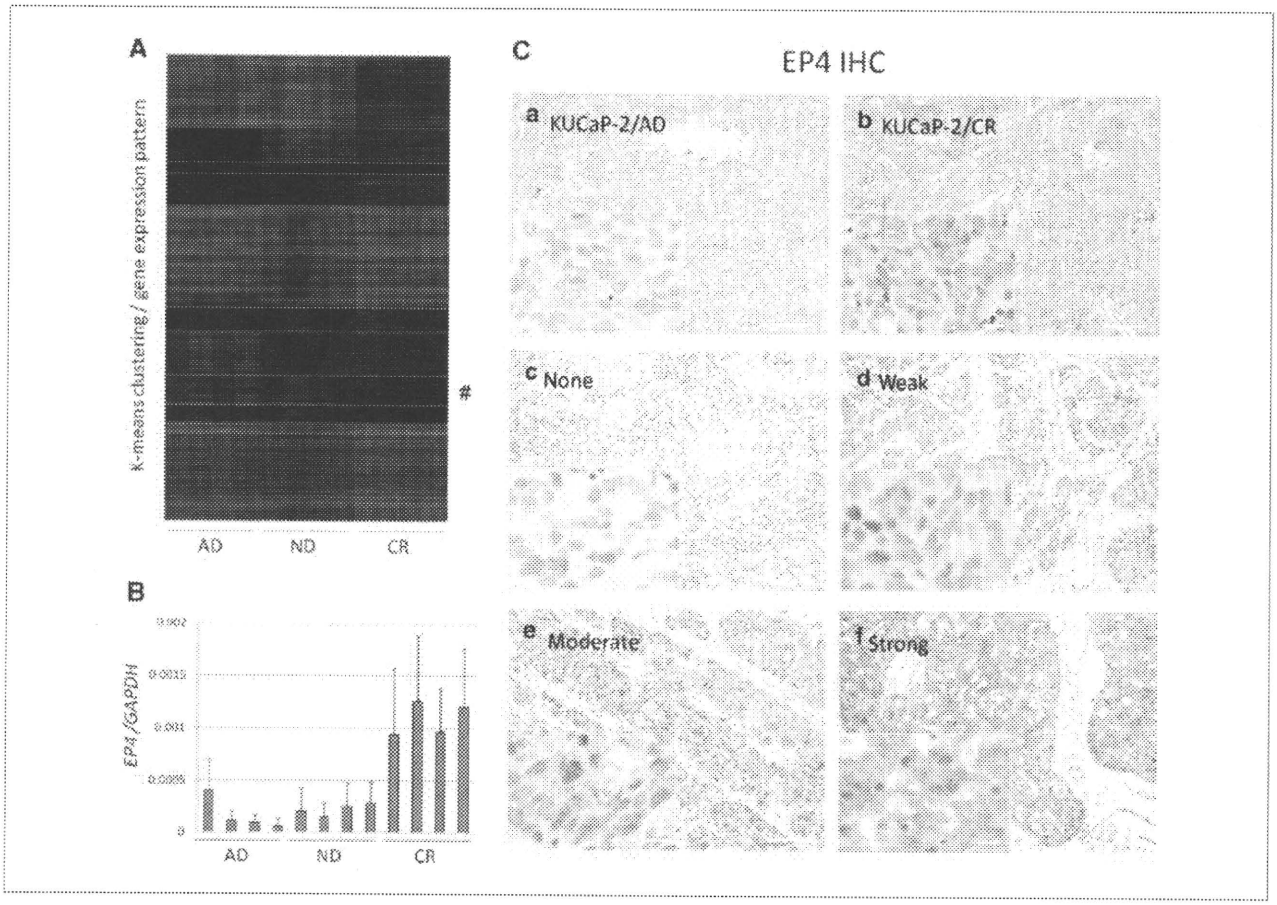

Figure 2. EP4 expression was upregulated during the castration-resistant progression of KUCaP-2. A, the *k*-means clustering of the DNA microarray data for AD, ND, and CR stage tumors of KUCaP-2. The *EP4* is in the cluster indicated with #. B, expression levels of *EP4* validated by real-time PCR analysis. C, the EP4 immunohistochemistry of AD stage (a) and CR stage (b) tumors of KUCaP-2. The staining intensity of EP4 in clinical samples of prostate cancer patients graded as none (c), weak (d), moderate (e) and strong (f). Scale bars, 50 μm, inset 5 μm.

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Table 1. Significantly upregulated genes at CR compared with AD and ND stages (10 highest CR/ND ratio)

Gene name (symbol)	CR/AD		CR/ND	
	Ratio	*P*	Ratio	*P*
Prostaglandin E receptor EP4 subtype (EP4)	15.7	0.01048	15.7	0.02903
Lumican (LUM)	23.6	0.00110	14.2	0.01095
Natriuretic peptide receptor C (NPRC)	2.3	0.19044	8.1	0.04921
N-methyl-D-aspartate 3A (GRIN3A)	9.1	0.00006	7.4	0.00029
Neuroligin 1 (NLGN1)	7.9	0.01844	6.1	0.04429
Coiled-coil domain containing 68 (CCDC68)	7.4	0.00013	4.4	0.00460
Tissue factor pathway inhibitor (TFPI)	4.1	0.04105	4.2	0.04678
Secretoglobin, family 1D, member 2 (LIPB)	8.2	0.01950	3.8	0.06641
Nudix-type motif 11 (UNDT11)	4.0	0.00444	3.7	0.01913
Eukaryotic translation initiation factor 1A Y-linked (EIF1AY)	2.6	0.07360	3.3	0.04354

(17), had been reported to be androgen dependent. LNCaP tumors shrink slightly after castration, usually with less than a 10% reduction in volume, and regrow less than 5 weeks after castration. Thereafter, several androgen-dependent xenografts have also been established. The LAPC-4 (18), LuCaP-23 (19), and PC346P (20) xenograft models reportedly show a response to castration similar to that of LNCaP. The CWR22 (21) and LAPC-9 (22) models showed recurrent growth after androgen ablation after 3 to 6 months, which was similar to our established xenograft, KUCaP-2. Similar to these models that mimic the clinical behavior of prostate cancer, KUCaP-2 may provide an excellent system to study the mechanisms associated with the castration-resistant progression of prostate cancer and help us develop novel treatment modalities against CRPC.

Most androgen-dependent xenografts were derived from patients with CRPC, as seen in KUCaP-2, because of the difficulty to obtain enough samples from patients with HNPC. It was suggested that prostate cancer contain a heterogeneous mixture of cells that vary in their dependence on androgen for growth and survival, and that treatment with androgen ablation therapy provides selective pressure and alters the relative concentration of these cells, thereby leading to the outgrowth of CRPC (22). These tumors presumably contain a mixture of growth-arrested, androgen-responsive tumor cells in addition to androgen-independent cells at the time of implantation into mice. In the androgenic environment of the intact male mouse, the androgen-responsive cells would gain a growth advantage and eventually develop into androgen-dependent xenografts.

The castration-resistant KUCaP-2 tumors expressed AR in their nuclei and produced PSA, suggesting that AR was activated with significantly low circulating androgen and is associated with the castration-resistant progression. Recent findings suggest that AR is an important transcription factor that mediates survival and proliferation signaling not only in HNPC but also in CRPC (23, 24). The androgen-independent activation of AR is mediated by several pathways (25, 26). The acquisition of mutations in *AR* is likely to be an important pathway (3, 27). However, KUCaP-2 harbors wild-type AR and progresses to castration resistance without AR mutation. Another possible pathway is its hypersensitivity to low levels

Table 2. Patient characteristics and EP4 staining grade in HNPC and CRPC

	HNPC	CRPC	*P*
Number	27	31	
Median serum PSA (ng/mL)	7.9 (3.8–31.3)	15.5 (0.5–949)	0.0066*
Median Gleason sum	7 (3–9)	9 (6–10)	0.0001*
EP4 staining grade†			0.0001‡
None	10 (37.0%)	5 (16.1%)	
Weak	17 (63.0%)	9 (29.1%)	
Moderate	0 (0%)	10 (32.2%)	
Strong	0 (0%)	7 (22.6%)	

*Mann-Whitney *U* test.
†The grading was determined on the intensity of staining for at least 20% of the cancer cells.
‡χ^2 test between HNPC and CRPC.

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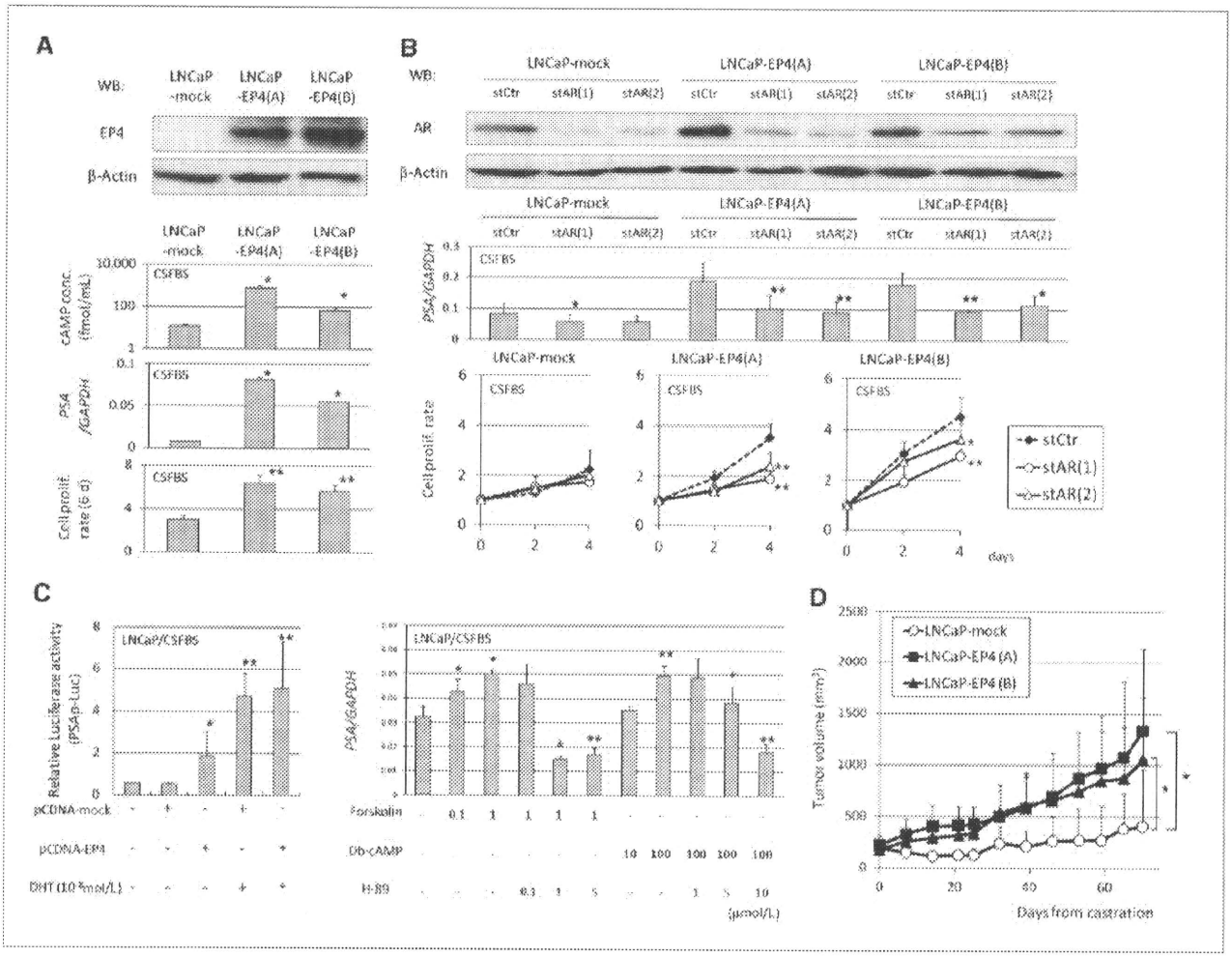

Figure 3. Overexpression of EP4 with cAMP-PKA signal activation promoted the castration-resistant progression of LNCaP cells through AR activation. A, EP4 protein expression detected with Western blotting in LNCaP-EP4(A) and LNCaP-EP4(B) compared with LNCaP-mock (top), intracellular cAMP concentrations, *PSA* expression, and cell proliferation ratio versus day 0 in androgen-depleted conditions (CSFBS) for 6 d (bottom). *, $P < 0.05$; **, $P < 0.005$ versus LNCaP-mock. B, AR expression attenuated using a stealth RNAi system detected with Western blotting (top), *PSA* expression levels on day 4 (middle), and cell proliferation ratio on days 2 and 4 versus day 0 (bottom) in CSFBS. *, $P < 0.05$; **, $P < 0.005$ versus LNCaP-mock. C, relative luciferase activities of PSAp-Luc in LNCaP cells with transfection of pcDNA3.1-mock and pcDNA3.1-EP4 in CSFBS and 5α-dihydrotestosterone (DHT) stimulation, respectively. *, $P < 0.05$; **, $P < 0.005$ versus control (left). PSA expression of LNCaP cells in CSFBS for 6 d under the administration of forskolin, dbcAMP, and H-89. *, $P < 0.05$; **, $P < 0.005$ versus preadministration (right). D, the sequential changes in xenograft tumor volume after the castration of mice (1×10^7 cells, $n = 5$ each). *, $P < 0.05$.

of androgens, induced by increased expression of the AR itself (10). In KUCaP-2, the *AR* mRNA expression slightly increased from the AD to ND and CR stages. However, the AR protein expression levels decreased at the ND stage and recovered at the CR stage to the same level as the AD stage both in the AR immunohistochemistry (Fig. 1D) and Western blotting (data not shown) analysis. One possible explanation for this discrepancy is that the AR protein might be degraded without androgen at ND stage and stabilized at the CR stage (28, 29). These results indicated that the upregulation of *AR* might merely be an adaptation of tumor cells to the condition of low androgen stimulation and not an essential indicator of castration-resistant progression. EP4 upregulation was observed during castration-resistant progression in KUCaP-2. EP4 expression was higher in clinical CRPC than in HNPC. The xenograft of EP4-overexpressing LNCaP cells developed castration resistance through AR activation. These results revealed that EP4 upregulation might lead to AR activation, resulting in the castration-resistant progression of prostate cancer. It was reported that the activation of a membrane-localized G protein-coupled receptor induced nuclear partition and activation of AR through the accumulation of intracellular cAMP and PKA activation (30). As EP4 is a G protein-coupled receptor, our data showing that EP4-cAMP-PKA axis activates AR are consistent to the report.

EP4 is one of the prostaglandin E2 (PGE2) receptors. PGE2, the product of cyclooxygenase-2 (COX-2) conversion of plasma membrane phospholipids, is the most common prostanoid and is associated with inflammatory disease (14) and cancer (31, 32). It was suggested that inflammation plays a

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role in prostate carcinogenesis (33, 34) and the regular consumption of non-steroidal anti-inflammatory drugs (NSAID) may reduce the risk of prostate cancer (35–37). Therefore, NSAIDs, including COX-2 inhibitors, have been tested in the treatment (38) and prevention (39) of prostate cancer. However, these approaches have met with limited success (40) and, sometimes, severe cardiovascular side effects (41), probably because COX-2 produces multiple products with pleiotropic effects in addition to PGE2. Therefore, targeting downstream signaling pathways of PGE2 may represent an attractive new strategy. There are four subtypes of PGE2 receptors, EP1 to EP4. The intracellular signaling differs among the receptor subtypes; EP1 is coupled to calcium mobilization, EP3 inhibits adenylate cyclase, and EP2 and EP4 stimulate adenylate cyclase in various types of cells (42). The effects of PGE2 are dependent on the ligand concentration and the target cell receptor expression (32). Experimental studies have suggested that increased EP2 and EP4 expression is important during colorectal and prostate cancer progression (43, 44). In KUCaP-2, EP2 expression did not increase significantly during castration-resistant progression (data not shown), indicating that EP4 might be more strongly associated with castration resistance than EP2 in this model. To examine the association of PGE2 and cancer progression, the serum PGE2 concentrations of mice bearing KUCaP-2 were examined by PGE2 Express EIA kit (500141; Cayman Chemical). Unfortunately, reproducible results could not be obtained, probably because of the instability of PGE2.

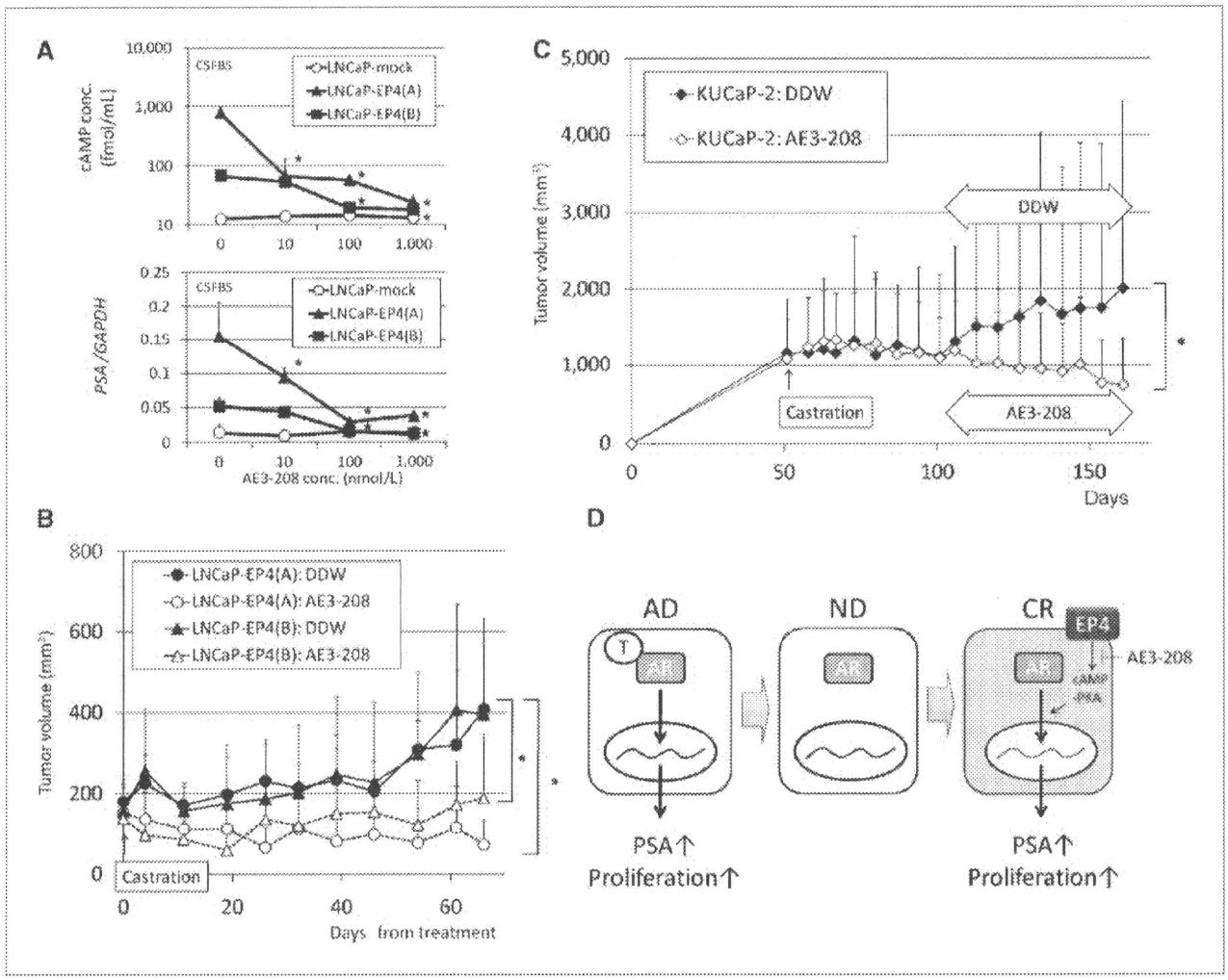

Figure 4. Castration-resistant progression of LNCaP-EP4 and KUCaP-2 was suppressed by ONO-AE3-208 treatment. A, the cAMP concentration (top) and *PSA* expression (bottom) of LNCaP-mock, LNCaP-EP4(A), and LNCaP-EP4(B) under the *in vitro* administration of ONO-AE3-208 in CSFBS. *, $P < 0.05$ versus LNCaP-mock. B, the sequential changes in LNCaP-EP4(A) and LNCaP-EP4(B) xenograft tumors treated with i.p. injection of 200 μL/d distilled water (DDW) and 10 mg/kg/d ONO-AE3-208 (AE3-208) started soon after the castration of mice and continued for 70 d (0.5×10^7 cells, $n = 5$ each). *, $P < 0.05$. C, the sequential changes in KUCaP-2 tumors treated with the same volume of DDW and AE3-208 solution started 50 d after castration and continued for 60 d ($n = 5$ each). *, $P < 0.05$. D, schematic representation of the relationship between EP4 overexpression and castration resistance. After castration, the upregulated EP4 induces activation of the AR without androgen and promotes castration-resistant cell proliferation and PSA production, which is suppressed by ONO-AE3-208 administration. T, androgen.

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Therefore, it might be difficult to examine the serum PGE2 concentrations in clinical samples. The secreted PGE2 concentrations in LNCaP-EP4 cells were higher than in LNCaP-mock cells [20.3 ± 15.4, 48.7 ± 4.9, and 44.7 ± 11.5 pg/mL in LNCaP-mock, LNCaP-EP4(A), and LNCaP-EP4(B), respectively]. However, the administration of PGE2 into LNCaP-EP4 could not induce cell proliferation and PSA production. To elucidate the association of PGE2 and cancer progression needs further examinations.

The cell proliferation of LNCaP-EP4 was significantly higher than that of LNCaP-mock under androgen-depleted medium but not under normal medium (data not shown), indicating that EP4 overexpression enhanced androgen-independent but not androgen-dependent proliferation of LNCaP cells *in vitro*. However, the *in vivo* tumor growth of LNCaP-EP4 was significantly higher than that of LNCaP-mock in intact mice (193 ± 76 and 121 ± 46 mm^3 on day 30, respectively, $P = 0.003$). Moreover, the xenograft tumor take-up rate of LNCaP-EP4 was higher than that of LNCaP-mock (~100% and 60%, respectively). It was reported that PGE2 regulated angiogenesis in PC3 cells through EP2 and EP4 (44). Therefore, it was suggested that EP4 overexpression might increase cell proliferation of LNCaP cells *in vivo* through angiogenesis. The PSA expression of LNCaP-EP4 was significantly higher than that of LNCaP-mock under androgen-depleted medium. Although EP4 expression was higher in LNCaP-EP4(B) than in LNCaP-EP4(A), the intracellular cAMP concentration and PSA expression levels were higher in LNCaP-EP4(A) than in LNCaP-EP4(B). To investigate the reasons for the discrepancy, we performed transient transfection analyses with several different amounts of pcDNA3.1-EP4. The PSA expression levels were correlated with EP4 expression levels in LNCaP cells transiently transfected with the EP4 expression vector, suggesting that the reasons for this discrepancy might be clonal variations of LNCaP-EP4 (data not shown). The expression of TMPRSS2, one of other AR-regulated genes (45), was also increased with EP4 overexpression and decreased by the attenuation of AR (data not shown). These results indicated that EP4 may increase PSA expression partly in an AR-dependent manner; however, we do not exclude possibilities that EP4 increases PSA and TMPRSS2 expressions through an AR-independent manner. Analysis of these mechanisms needs further investigations.

It was suggested that the EP4-cAMP-PKA axis can activate the β-catenin/TCF signaling pathway, leading to cancer progression (46), and that the EP4-specific antagonist, ONO-AE3-208, inhibits the progression of EP4-expressing colorectal cancer (47, 48). The present study is the first report showing that ONO-AE3-208 reduces the castration-resistant progression of prostate cancer cells induced by EP4 overexpression. ONO-AE3-208 did not suppress the proliferation of KUCaP-2 in intact mice (data not shown), suggesting that EP4 antagonism might have no antitumor effect against HNPC. DU145 and PC3 are AR-negative prostate cancer cells with high EP4 expression. The *in vitro* administration of 100 nmol/L ONO-AE3-208 decreased intracellular cAMP concentrations of these cells. However, it did not suppress their cell proliferation *in vitro* and *in vivo* (data not shown). It was suggested that the EP4-cAMP-PKA axis might not be associated with their cell proliferation.

In conclusion, we found that EP4 overexpression is one of the mechanisms responsible for progression to CRPC using a novel xenograft model KUCaP-2. The administration of EP4 antagonist *in vivo* suppressed the castration-resistant progression of KUCaP-2, indicating that EP4 may be a potential target for the treatment of CRPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Research

Correction: Online Publication Dates for *Cancer Research* April 15, 2010 Articles

The following articles in the April 15, 2010 issue of *Cancer Research* were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:

Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, Feral C, Papayannopoulou T, Lowy A, Blair SL, Cheresh D, Ginsberg M, Varner JA. Integrin $\alpha4\beta1$ signaling is required for lymphangiogenesis and tumor metastasis. Cancer Res 2010;70:3042–51. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3761.

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Clinical Significance of Polymorphism and Expression of Chromogranin A and Endothelin-1 in Prostate Cancer

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Abbreviations and Acronyms

BPH = benign prostatic hyperplasia
BPHcont = nonprostate cancer specimen BPH region
BPHpca = prostate cancer specimen BPH region
CHGA = chromogranin A
ET = endothelin
IHC = immunohistochemistry
LD = linkage disequilibrium
PCapca = prostate cancer region
PCR = polymerase chain reaction
PSA = prostate specific antigen
RRP = radical retropubic prostatectomy

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Purpose: We investigated the clinical significance of chromogranin A and endothelin-1 polymorphism and expression in prostate cancer.
Materials and Methods: We analyzed 2 *CHGA* polymorphisms by polymerase chain reaction-restriction fragment length polymorphism in DNA samples of 435 patients with prostate cancer and 316 age matched male controls. Chromogranin A and endothelin-1 expression was evaluated by immunohistochemistry in prostate specimens of 114 men with prostate cancer who underwent radical retropubic prostatectomy and in 27 with bladder cancer who underwent radical cystectomy and served as controls.
Results: For the *CHGA* Glu264Asp polymorphism men with the *GG* genotype were at 2.05 times higher risk for prostate cancer than men with the *CC* genotype ($p = 0.014$). In men with prostate cancer higher chromogranin A immunohistochemistry grade was associated with higher stage and higher Gleason score ($p = 0.011$ and 0.044, respectively). Multivariate analysis showed that chromogranin A immunohistochemistry grade was an independent variable for predicting biochemical failure after radical prostatectomy ($p = 0.023$). Higher endothelin-1 expression was observed in prostate cancers ($p = 0.011$), especially those with a higher Gleason score ($p = 0.042$). There was no significant relationship between chromogranin A polymorphisms, and chromogranin A and endothelin-1 expression.
Conclusions: Polymorphism and expression of chromogranin A and endothelin-1 have clinical significance in prostate cancer. Chromogranin A expression was an independent predictor of biochemical failure after prostatectomy in patients with localized prostate cancer.

Key Words: prostate; prostatic neoplasms; polymorphism, genetic; chromogranin A; endothelin-1

NEUROENDOCRINE cells have an important role in normal prostates and BPH as well as in primary and metastatic prostate cancer.[1,2] Of the biogenic amines and neuropeptides secreted by neuroendocrine cells CHGA is a candidate marker for diagnosing and predicting the prognosis of prostate cancer. Patients with prostate cancer have significantly higher serum CHGA than those with BPH and controls.[2] A group reported that CHGA protein expression determined by IHC is a useful prognostic marker of biochemical failure after radical prostatectomy.[3] To date only 1 group

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has performed IHC analysis and found higher CHGA expression in benign epithelial cells adjacent to prostate cancer lesions than in the BPH region.[4] On the other hand, CHGA polymorphisms can influence CHGA expression, which eventually affects baseline blood pressure,[5] but the relationship between CHGA polymorphisms and prostate cancer remains unclear.

ETs, which are endogenous small peptides secreted by endothelium, exert paracrine and autocrine effects through cell surface receptors and influence cellular processes, such as angiogenesis, cellular proliferation, and tissue repair and development.[6–8] Plasma ET-1 levels in patients with hormone refractory, metastatic prostate cancer are higher than in patients with organ confined prostate cancer or controls.[9] Another IHC study showed ET-1 over expression in cases of advanced prostate cancer and high grade prostatic intraepithelial neoplasia.[10] Recently CHGA and ET-1 interaction was reported in a group of twins as well as in vitro experiments.[11] The study showed that polymorphisms in the CHGA promoter region are associated with serum ET-1 and CHGA stimulated ET-1 secretion in endothelial cells in a dose dependent manner. To our knowledge the association between CHGA and ET-1 in prostate cancer has not been assessed.

We analyzed 7 polymorphisms in the promoter region and the Glu264Asp polymorphism in exon 6 of *CHGA* in a Japanese population to evaluate the relationship to prostate cancer risk and clinical characteristics. We evaluated CHGA and ET-1 protein expression to determine whether they are related to localized prostate cancer pathological features and treatment outcomes. Also, we assessed the relationships among the *CHGA* genotypes, CHGA protein expression and ET-1 protein expression.

MATERIALS AND METHODS

Subjects

A total of 751 men, including 435 with prostate cancer and 316 controls, were enrolled in this study. All patients with prostate cancer were diagnosed at Akita University Medical Center, Kyoto University affiliated hospital and related community hospitals. They were pathologically diagnosed using specimens obtained from transrectal needle biopsy or transurethral prostate resection due to lower urinary tract symptoms. Prostate cancer clinical or pathological stage at diagnosis was determined by reviewing the medical records based on the TNM system. Prostate cancer was classified as stage A—T1a-bN0M0, stage B—T1c-2N0M0, stage C—T3-4N0M0 and stage D—T1-4N1M0-1 or T1-4N0-1M1 by the modified Whitmore-Jewett system. Controls were native Japanese men older than 60 years who had undergone health inspection at a community hospital.

IHC was done in prostate specimens from 114 men with stage T2-4 prostate cancer who underwent RRP and in BPH specimens from 27 who underwent radical cystectomy for bladder cancer. Since endocrine therapy may affect the number of neuroendocrine cells, patients with prostate cancer treated with endocrine therapy before RRP were excluded from analysis.[12] Clinical information was reviewed in the medical records. DNA and prostate specimens were collected after obtaining informed consent with approval from the institutional ethics committee.

CHGA Polymorphism Genotyping

We selected 7 polymorphisms in the *CHGA* promoter region for LD analysis. DNA direct sequencing was done in 200 samples to analyze the genotypes of those polymorphisms. The Appendix lists PCR primer sequences. Genotype data were imported into Haploview, version 3.32 (Daly Laboratory, Board Institute, Cambridge, Massachusetts) to test LD among polymorphisms in the *CHGA* promoter region. D' greater than 0.8 was considered a strong LD.

Finally, we analyzed 2 *CHGA* polymorphisms, including rs9658635 in the promoter region and Glu264Asp in exon 6, using certain primers (table 1). After confirming successful PCR amplification each product was digested at 37C overnight with 5 U *Bcc* I or *BfuC* I restriction enzymes (New England Biolabs, Beverly, Massachusetts). For the rs9658635 polymorphism restriction fragments were 114 and 21 bp for the *T* allele, and 135 bp for the *C* allele. For the Glu264Asp polymorphism restriction fragments were 129 and 106 bp for the *G* allele, and 235 bp for the *C* allele. To avoid genotyping errors caused by incomplete digestion or other technical failures we repeated the experiment at least twice for all samples and compared the genotype with the DNA sequencing results in 100 randomly selected samples.

Table 1. *PCR primers*

	Reference Single Nucleotide Polymorphism	
	rs9658635	rs9658655
Polymorphism	T-415C	Glu264Asp
Primers	Forward-5' CCTAGATATTGGAGAGAGCCATGAGTGA 3'	Forward-5' AGGGTGGCAGGCAAAGAG 3'
	Reverse-5' CCATGTGTACTGAGGTCCCTGGCAG 3'	Reverse-5' AAGGTGGAATGAGGTTATGG 3'
Length (bp)	135	235
Enzyme	*BccI*	*BfuCI*
Fragments (bp)	21 + 114	106 + 129

IHC Staining and Evaluation

We performed IHC staining for CHGA and ET-1 using a certain protocol. Briefly, deparaffinized, rehydrated sections were steamed for 20 minutes to enhance antigen retrieval. Immunohistochemical labeling with mouse anti-human CHGA antibody (DakoCytomation, Glostrup, Denmark) (×800) or ET-1 antibody (Alexis Biochemicals, Lausen, Switzerland) (×250) was done overnight at 4C. Slides were labeled with the anti-mouse EnVision™+ system labeled with horseradish peroxidase for 30 minutes. The liquid DAB+ Substrate-Chromogen System (DakoCytomation) was applied at room temperature for 30 minutes. Slides were counterstained with hematoxylin solution for nuclear staining. Specimens were examined by 2 independent researchers blinded to sample background data.

CHGA positive stained cells were counted in 10 high power visual fields at 200× magnification to determine which had the most positive cells (fig. 1, *A*). Since the number of CHGA positive cells in the BPH region was greatly different than that in the prostate cancer region, CHGA positive cells in 3 regions were counted, including BPHcont, BPHpca and PCapca. Counting was done 3 times per sample and the mean was used for statistics. The mean value of each sample was categorized as grade 1—less than 10, grade 2—10 to 29 and grade 3—30 or greater for the prostate cancer region. Since neuroendocrine cells are consistently found in the periurethral ducts and verumontanum,[13] those regions were excluded from counting.

Cytoplasmic ET-1 staining intensity was scored on a semiquantitative scale as 1—weak, 2—moderate and 3—strong (fig. 1, *B*). The percent of cytoplasmic ET-1 positive cells was divided into 4 groups, including 1—less than 25%, 2—25% to 50%, 3—50% to 75% and 4—greater than 75%. Total immunoreactivity grade was calculated by multiplying the 2 scores[14] and defined as grade 1—6 or less, grade 2—8 and grade 3—greater than 8.

Statistical Analysis

All data were entered into an Access® database and analyzed using Excel® 2007 and SPSS®, version 16.0J. We examined differences in mean age in the 3 groups using the independent t test. Hardy-Weinberg equilibrium analysis was done to compare observed and expected genotype frequency using the Pearson chi-square test. We used binary logistic regression to assess the association between prostate cancer risk and genotypes by calculating the OR and 95% CI. We hypothesized that the *C* allele of the rs9658635 polymorphism would be an inherent genetic risk factor for prostate cancer and prostate cancer progression. Statistical modeling was done independently on the relative risk of the *CC* or *CT* genotype against the *TT* genotype for rs9658635 using the logistic regression model adjusted by age. For Glu264Asp the *G* allele was hypothesized as an inherent genetic risk factor for prostate cancer and prostate cancer progression.

We used 1-way ANOVA to compare the number of CHGA IHC positive cells among the 3 groups and Kendall's τ-b rank correlation coefficients to examine the relationship between IHC grade and Gleason score or clinical stage. The biochemical failure-free interval was defined as the time from the date of RRP to the date when PSA increased to more than 0.4 ng/ml. We estimated relationships between polymorphisms or IHC grade and biochemical failure-free survival in stage T2-4 prostate cancer cases by the Kaplan-Meier method and evaluated them by the log rank test. The Cox multivariate proportional hazards model was used for multivariate analysis. We examined relationships between polymorphisms and IHC grades in patients with prostate cancer using Fisher's exact test. All statistical tests and p values were 2-tailed with results considered significant at $p < 0.05$.

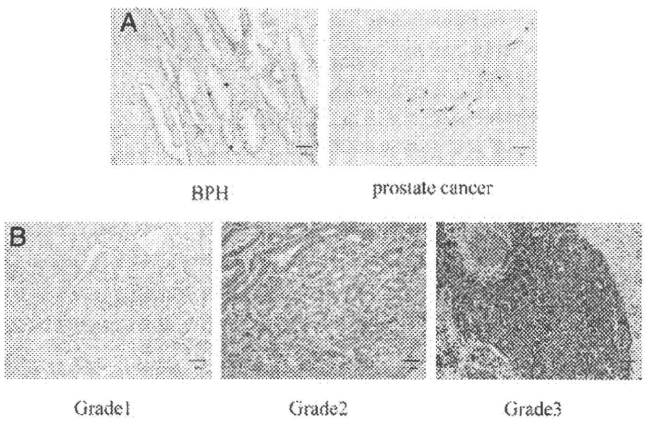

Figure 1. IHC in BPH and prostate cancer regions. *A*, CHGA cytoplasmic staining pattern. *B*, representative ET-1 IHC stains of different grades.

RESULTS

Characteristics

Mean age ± SD in patients with prostate cancer and male controls was 70.28 ± 7.43 and 69.46 ± 7.22 years, respectively ($p = 0.289$). Stage was A to C, D1 and D2 in 10, 191, 83, 25 and 126 patients with prostate cancer, respectively. In the prostate cancer group Gleason score was less than 7, 7, greater than 7 and unavailable in 14, 202, 164 and 55 patients, respectively.

CHGA Associations

Polymorphism genotypes vs prostate cancer risk and clinicopathological factors. Genotype distributions in all groups were consistent with Hardy-Weinberg equilibrium. Since more than 90% of D′ values in the 7 polymorphisms in the *CHGA* promoter region equaled 1 (fig. 2), the rs9658635 polymorphism, which was reported to be associated with CHGA expression,[5] was chosen as a representative polymorphism for further analysis. Statistical analysis of genotype frequency showed no relationship between the rs9658635 polymorphism and the prostate cancer risk ($p > 0.05$, table 2). For the Glu264Asp polymorphism we found a significantly increased prostate cancer risk in men with the *GG*

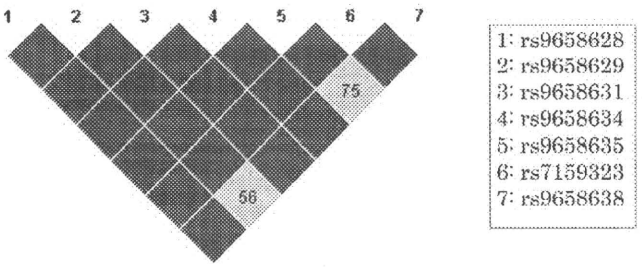

Figure 2. There were strong LDs among 7 CHGA promoter region polymorphisms. Red diamonds indicate D' = 1. Gray diamonds indicate D' less than 1.

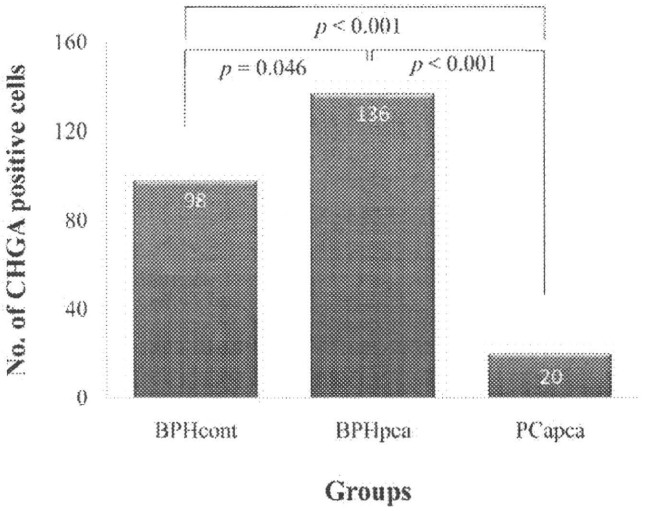

Figure 3. There were significant differences among mean number of CHGA IHC positive cells in BPHcont, BPHpca and PCapca.

genotype and the *GC* genotype than in those with the *CC* genotype (OR 2.05, 95% CI 1.16–3.63; p = 0.014 and OR 1.97, 95% CI 1.10–3.52; p = 0.023, respectively, table 2). There was no significant association of the rs9658635 or the Glu264Asp *CHGA* polymorphism with prostate cancer clinical stage or Gleason score (p >0.05).

IHC grade vs prostate cancer clinicopathological factors and prognosis. BPHcont, BPHpca and PCapca showed a mean ± SD of 97 ± 81, 136 ± 109 and 20 ± 48 CHGA IHC positive cells, respectively (p <0.001, fig. 3). Compared with BPHcont BPHpca had more and PCapca had fewer CHGA positive cells (p = 0.046 and <0.001, respectively). In patients with prostate cancer a higher CHGA IHC grade was more often found in those with pT3-4 than pT2 disease (p = 0.011). There was a significant association between CHGA IHC grade and Gleason score (p = 0.044). On univariate analysis a higher probability of biochemical failure after RRP was significantly associated with higher CHGA IHC grade (p = 0.001, fig. 4), higher Gleason score (p = 0.039), higher stage (p = 0.025) and higher PSA at diagnosis (p <0.001). On multivariate analysis CHGA IHC grade was an independent factor predicting possible biochemical failure after RRP (p = 0.023, table 3).

ET-1 IHC Grade

Prostate cancer showed a higher ET-1 IHC grade than BPH (chi-square 9.030, p = 0.011). Of patients with prostate cancer we noted a higher ET-1 IHC grade in those with a higher Gleason score (chi-square 4.149, p = 0.042, fig. 5). There was no statistically significant relationship between ET-1 IHC grade and clinical stage or biochemical failure after RRP (p = 0.661 and 0.230, respectively).

CHGA Polymorphism Genotypes vs CHGA and ET-1

Cross-tabulation results showed no significant association of the *CHGA* rs9658635 or the Glu264Asp polymorphism with CHGA or ET-1 IHC grade (table 4). We found no significant relationship between CHGA and ET-1 expression (p >0.05).

Table 2. *CHGA polymorphisms vs prostate cancer risk and clinicopathological factors*

	Prostate Ca vs Control		Clinical Stage D vs A + B + C		Gleason Score 8 or Greater vs Less Than 8	
Genotype Polymorphism	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
CHGA promoter rs9658635:						
TT	1		1		1	
CT	1.12 (0.81–1.55)	0.502	1.41 (0.87–2.31)	0.167	1.26 (0.79–1.98)	0.331
CC	0.83 (0.55–1.25)	0.363	1.24 (0.65–2.39)	0.512	1.03 (0.56–1.91)	0.914
CT + CC	1.02 (0.76–1.38)	0.892	1.37 (0.86–2.18)	0.187	1.19 (0.77–1.84)	0.424
TT + CT:CC	1.29 (0.89–1.86)	0.185	0.98 (0.55–1.76)	0.954	1.10 (0.64–1.91)	0.729
Exon 6 Glu264Asp:						
CC	1		1		1	
GC	1.97 (1.10–3.52)	0.023	1.80 (0.64–5.10)	0.268	2.98 (0.96–9.22)	0.059
GG	2.05 (1.16–3.63)	0.014	2.11 (0.76–5.87)	0.154	2.62 (0.86–8.04)	0.091
GC + CC	2.01 (1.15–3.51)	0.014	1.98 (0.72–5.43)	0.817	2.77 (0.91–8.37)	0.072
CC + GC:GG	0.86 (0.64–1.15)	0.314	0.80 (0.54–1.20)	0.279	1.02 (0.69–1.51)	0.904

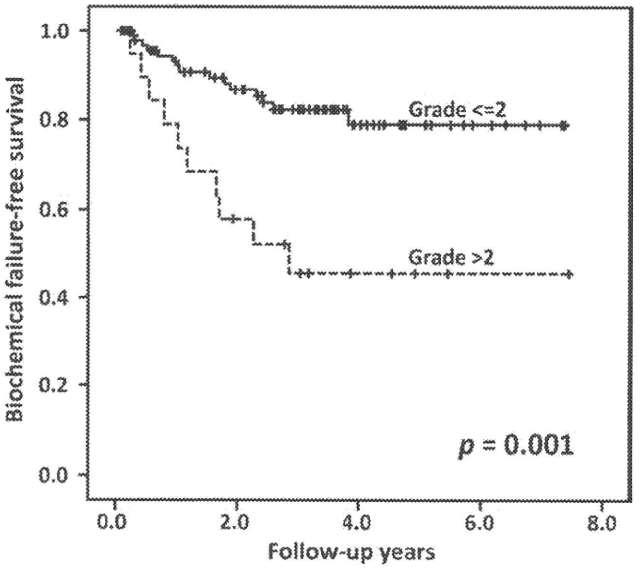

Figure 4. Higher CHGA IHC grade was associated with higher probability of biochemical failure after RRP.

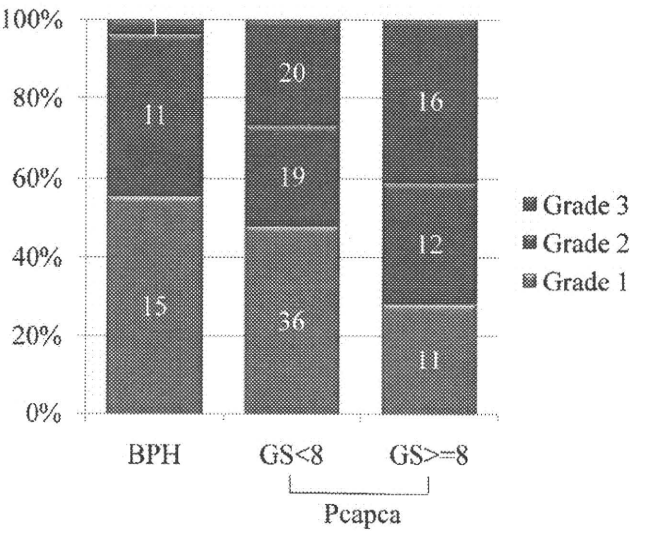

Figure 5. Compared with BPH prostate cancer showed higher ET-1 IHC grade. Higher ET-1 IHC grade was associated with higher Gleason score (*GS*) prostate cancer.

DISCUSSION

We first investigated the influence of CHGA polymorphisms on prostate cancer clinicopathological factors. Results revealed a significant association between the *G* allele of the *CHGA* Glu264Asp polymorphism and the risk of prostate cancer in a native Japanese population, suggesting that the *CHGA* Glu264Asp polymorphism may be a useful marker for estimating the prostate cancer risk. To our knowledge this is the first study to investigate whether *CHGA* gene variants influence prostate cancer. The *G* to *C* allele variant of the Glu264Asp polymorphism caused the 264 amino acid CHGA to change from glutamic to aspartic acid. Pancreastatin, an impairing glucose metabolism peptide of 52 amino acids, is located in this CHGA encoding region.[15] Pancreastatin inhibits the release of glucose stimulated insulin from pancreatic islet β cells.[16] Since insulin has an important role in prostate cancer pathogenesis,[17] it is reasonable that the *CHGA* Glu264Asp polymorphism affects prostate cancer carcinogenesis through functional alteration of pancreastatin by regulating insulin secretion. Also, the importance of the pancreastatin polymorphism was supported by the observation that the Gly297Ser polymorphism influences glucose uptake.[18] Pancreastatin is a useful prognostic indicator in patients with neuroendocrine tumors[19] but there was no association between the CHGA Glu264Asp polymorphism and prostate cancer prognosis.

The CHGA rs9658634 polymorphism is reportedly associated with serum CHGA and the ET-1 level.[5,11] However, the CHGA polymorphism showed no relationship with CHGA or ET-1 IHC expression in our study, which could have been due to several reasons. 1) The different methods of measuring CHGA expression may have led to different results. Since CHGA is secreted by other tissues as well as the prostate, serum CHGA represents total CHGA expression in the whole body. We used prostate surgical specimen IHC grade instead of the serum level to more specifically reflect prostate CHGA expression in the prostate. In support of our findings another research group found no correlation between serum CHGA and immunohistochemical results.[20] 2) The discrepancy may have been be due to our population. Only men older than 60 years with prostate cancer were enrolled in our study

Table 3. *Biochemical failure and clinical factor Cox proportional hazards model*

	Univariate		Multivariate	
Clinical Factors	HR (95% CI)	p Value	HR (95% CI)	p Value
CHGA IHC grade greater than 2 or not	3.585 (1.609–7.987)	0.002	2.713 (1.149–6.407)	0.023
Gleason score 8 or greater or not	2.237 (1.020–4.908)	0.044	1.512 (0.650–3.517)	0.337
T stage greater than 2 or not	2.422 (1.088–5.394)	0.030	0.969 (0.375–2.504)	0.948
PSA greater than 10 ng/ml or not	5.682 (1.950–16.557)	0.001	4.611 (1.483–14.336)	0.008

Table 4. *CHGA polymorphisms vs CHGA and ET-1 expression*

	CHGA IHC Grade				ET-1 IHC Grade			
	1	2	3	p Value	1	2	3	p Value
rs9658635:				0.964				0.497
TT	29	6	9		19	14	11	
CT	32	10	9		18	14	19	
CC	13	3	3		10	3	6	
Glu264Asp:				0.423				0.272
CC	4	0	0		1	2	1	
GC	27	11	10		17	17	14	
GG	43	8	11		29	12	21	

whereas other studies included subjects without cancer regardless of age or gender. This may result in the lack of a significant relationship between CHGA polymorphisms and expression in patients with prostate cancer.

However, our study shows that in patients with localized prostate cancer and no history of endocrine therapy a higher CHGA IHC grade was associated with worse tumor stage and higher Gleason score. A group also reported that IHC staining for CHGA is significantly associated with Gleason score,[20] although a contradictory result was reported.[21] Furthermore, the Cox multivariate regression model showed that CHGA IHC grade was an independent variable for predicting biochemical failure after RRP. In a study of lymph node positive cases of prostate cancer the investigators found that CHGA expression is associated with biochemical failure after RRP.[22] However, the history of endocrine therapy before RRP, which was associated with CHGA expression,[12] was not controlled in that study. Other studies in D2 prostate cancer cases showed that higher CHGA IHC grade is associated with a worse prognosis.[21,23] Taken together, CHGA IHC grade, which represents neuroendocrine differentiation, could predict the prognosis in patients with prostate cancer.

Compared with BPHcont, the number of CHGA IHC positive cells was higher in BPHpca and lower in PCapca (fig. 3). This agrees with the result that CHGA positive cells had more prominent expression in benign epithelial cells adjacent to prostate cancer lesions than in the prostate cancer region.[4] Also, there is a tendency toward a decreased number of neuroendocrine cells in untreated patients with prostate cancer compared with that in patients with BPH and male controls with a normal prostate.[24] Hence, higher serum CHGA in patients with prostate cancer may result from BPHpca, which has many more neuroendocrine cells than in patients with BPH. This indicates that to predict prostate cancer susceptibility more efficiently we should focus on the cancerous region and the adjacent noncancerous region.

Neuroendocrine cells in the BPH region are negative for α-methylacyl coenzyme A racemase while neuroendocrine cells in prostate cancer are positive for α-methylacyl coenzyme A racemase.[25] In vitro cells of the androgen dependent line LNCaP were induced to show neuroendocrine differentiation by androgen deprivation[26] or agents that increase intracellular cyclic adenosine monophosphate.[27] Results indicate that PCapca neuroendocrine cells, which have hormone insensitive characteristics, may differentiate from prostate cancer cells. Patients with the worst prostate cancer stages had a higher CHGA IHC grade, indicating that more prostate cancer cells had transformed into neuroendocrine cells with hormone insensitive characteristics and resulting in a worse prognosis. Whether our patients with hormone refractory prostate cancer had many more hormone insensitive neuroendocrine cells than our patients with localized prostate cancer should be explored in the future.

Serum ET-1 has no value for estimating prostate cancer prognosis.[28] We noted no association of ET-1 expression with clinical stage or biochemical failure after RRP. However, prostate cancer showed significantly higher ET-1 expression than the BPH region and higher ET-1 IHC grade was associated with a higher Gleason score.

CONCLUSIONS

A *CHGA* genetic variant may modify prostate cancer carcinogenesis and CHGA expression may be a useful biomarker to predict the higher malignant potential of localized prostate cancer and biochemical failure after RRP. Thus, results suggest that CHGA is involved in prostate cancer carcinogenesis and progression.

ACKNOWLEDGMENTS

Mrs. Mitobe provided technical assistance.

APPENDIX

DNA Sequencing Primers for CHGA Promoter Region

Fragment No. (polymorphisms)	Reference Single Nucleotide Polymorphism	Primers	
		PCR	DNA Sequencing
1:		Forward-5′ CAGGTTCTCATTTAGGGACA 3′ Reverse-5′ AAAGGTCAGTTTCCTGGTTG 3′	Forward-5′ TTTAGGGACAGGCGTGAGCACAGGT 3′ Reverse-5′ TCAGTTTCCTGGTTGGCTTCCCTT 3′
G-1106A	rs9658628		
A-1018T	rs9658629		
T-998G	rs9658631		
2:		Forward-5′ CATCAGTTACCTGTCAAGTGCGT 3′ Reverse-5′ CCCCGTGCTATTTTTCCTAAGT 3′	Forward-5′ TGTCAAGTGCGTTTCCTCTGT 3′ Reverse-5′ TTCCTAAGTGCCCTCTGCCT 3′
G-462A	rs9658634		
T-415C	rs9658635		
3:		Forward-5′ GCCCAGGGACACAAGGCAAAT 3′ Reverse-5′ TCGGCGTGCGTCCGTCTGTC 3′	Forward-5′ CACCTCTTGGAAACCAGATACC 3′ Reverse-5′ TGCGTCCGTCTGTCGGTCGATG 3′
C-89A	rs7159323		
C-57T	rs9658638		

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Statins Reduce the Androgen Sensitivity and Cell Proliferation by Decreasing the Androgen Receptor Protein in Prostate Cancer Cells

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BACKGROUND. Statins (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors) are cholesterol-lowering drugs that are widely used to prevent and treat atherosclerotic cardiovascular disease. Recent epidemiological studies suggest that statins reduce serum prostate-specific antigen (PSA) levels and decrease the risk of prostate cancer. In the present study, we determined the molecular mechanisms related to the regulation of PSA, androgen receptor (AR) and cell proliferation in prostate cancer cell lines by statins.
METHODS. Western blotting, quantitative real-time polymerase chain reaction, cytotoxicity analysis and a cell proliferation assay were used to resolve the regulatory role of statins (mevastatin and simvastatin) in three prostate cancer cell lines, RWPE-1, 22Rv1, and LNCaP.
RESULTS. Western blotting revealed that both mevastatin and simvastatin downregulated AR and PSA protein. However, these statins did not downregulate AR mRNA expression, while they decreased PSA mRNA. The protease inhibitor MG132 inhibited the downregulation of AR protein which suggested that statins decreased AR protein levels by increasing AR proteolysis. Furthermore, statins reduced cell proliferation in AR positive cells but not in AR negative cells, suggesting that statins regulate cell proliferation via AR expression. In addition, cell proliferation assay at various concentrations of dihydrotestosterone (DHT) showed that statins decreased androgen sensitivity in LNCaP cells.
CONCLUSIONS. Statins decreased AR protein by proteolysis but not mRNA transcription. The drop in AR levels resulted in a reduction in androgen sensitivity and a decrease in cell proliferation in AR positive prostate cancer cells. *Prostate* © 2010 Wiley-Liss, Inc.

KEY WORDS: **statin; prostate cancer; androgen receptor; prostate specific antigen**

INTRODUCTION

Statins, also known as the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, are drugs used for cholesterol reduction and are prescribed to treat hypercholesterolemia in 13 million patients in the United States [1]. Several studies in men with prostate cancer, who had a history of statin exposure, have shown risk reduction or improvement in progression free survival. Among patients in the Cancer Prevention Study II Nutrition cohort, the California Men's Health Study, the Veteran Affairs Medical Center Study in Oregon and the US Male Health Professional cohort, there were statistically significant

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reductions in the risk of developing advance prostate cancer among patients using cholesterol-lowering medications for 5 or more years [2–4]. Moreover, other reports have suggested that statins may decrease the levels of serum PSA, a prostate cancer biomarker, and thereby potentially impact on the risk of prostate cancer detection [5,6].

In the present study, we determined the molecular mechanisms related to the regulation of PSA, androgen receptor (AR) and cell proliferation in prostate cancer cell lines associated with the use of statins.

MATERIALS AND METHODS

Cell Culture

Human prostate cancer RWPE-1 (keratinocyte serum free medium), 22Rv1 (RPMI1640 medium), and LNCaP cells (RPMI1640 medium) were cultured in media which were purchased from Invitrogen (San Diego, CA), and contained 10% fetal bovine serum. LNCaP cells that had been propagated between 10 and 30 times were used. Castration-resistant derivatives of LNCaP cells, namely LNCaP-CxR cells (referred to as CxR cells) and hydrogen peroxide-resistant LNCaP cells, namely LNCaP-HPR50 cells (referred to as HPR50 cells) were established and maintained as described previously [7]. 22Rv1-AR-GFP cells, which were derived from 22Rv1 cells and stably expressed AR-Green Fluorescent Protein (GFP) protein, were established as described previously [8].Briefly, 22Rv1 cells were transfected with AR-GFP expressing plasmid that were kindly provided by Dr. Toshihiko Yanase (Fukuoka University, Fukuoka, Japan) [9]. They were cultured for 2 weeks in selection medium containing 500 μg/ml of geneticin (Nacalai Tesque, Kyoto, Japan). Protein expression in the clones that were obtained was verified using Western blotting and a fluorescence microscope (BIOZERO, Keyence, Tokyo, Japan). Isolated clones were maintained in the presence of 500 μg/ml of geneticin. The cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies and Reagents

Antibodies against AR (sc-815), PSA (sc-7316), and GFP (sc-8334) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody, mevastatin, and simvastatin were purchased from Sigma (St. Louis, MO). MG132 and cycloheximide were obtained from Calbiochem (Gibbstown, NJ) and Nacalai Tesque, respectively.

Western Blotting

Whole-cell extracts were prepared as previously described [7,10,11]. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA), based on Bradford's method. Whole-cell extracts (30 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ) using a semi-dry blotter. The blotted membranes were incubated for 1 hr at room temperature with a primary antibody. Membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody. The bound antibody was visualized using an ECL kit (GE Healthcare Bio-Science) and membranes were exposed

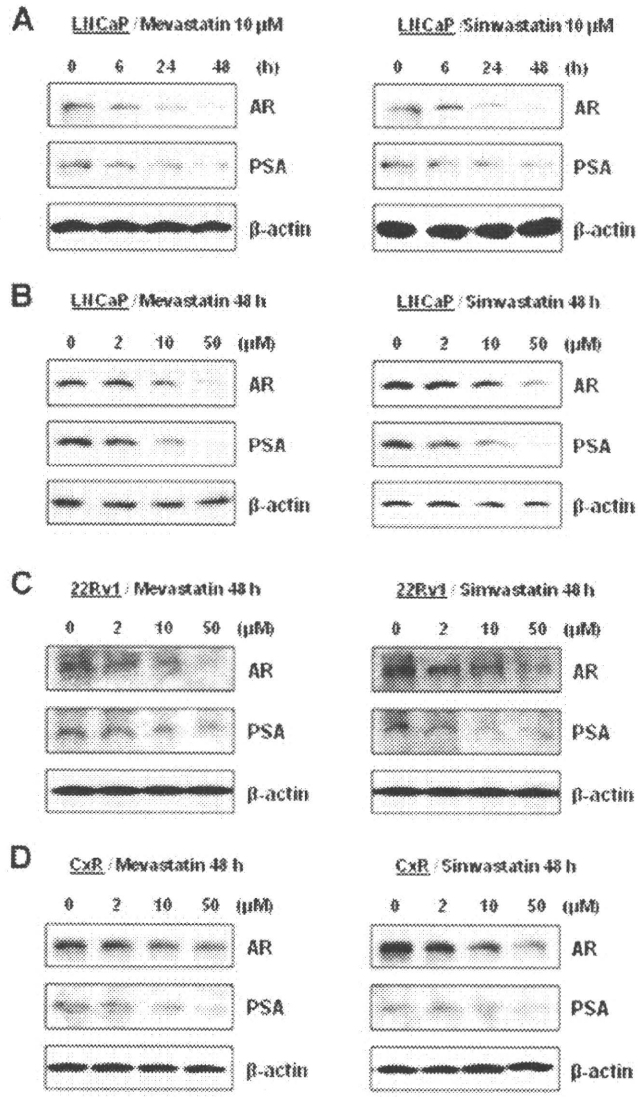

Fig. 1. Downregulation of AR and PSA protein by mevastatin and simvastatin. Mevastatin and simvastatin were added to media containing (**A–D**), LNCaP (A,B), 22Rv1 (C), and CxR (D) cells at the concentrations and for the durations indicated. The whole-cell extracts were subjected to SDS–PAGE. Western blotting was performed using the antibodies indicated.

to high performance chemiluminescence film (GE Healthcare Bio-Science).

RNA Isolation and RT-PCR

RNA isolation and RT-PCR was performed as described previously [7,10,11]. Briefly, total RNA was prepared from culture cells using RNeasy mini kits (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1.0 μg of total RNA using a Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis) according to the manufacturer's protocol.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed as described previously [7,10,11]. Briefly, the synthesized cDNA was diluted 1:2 and 2.0 μl of the diluted mixture was used. Quantitative real-time PCR with a TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) was performed using ABI 7900HT. The expression level of each target gene was corrected for the corresponding GAPDH expression level. The results are representative of at least three independent experiments.

Cytotoxicity Analysis

Cytotoxicity analysis was performed as previously described [7,8]. Briefly, RWPE-1, 22Rv1, LNCaP, CxR, and HPR50 cells (2.5×10^3) were seeded into 96-well plates. The following day the indicated concentrations of mevastatin, simvastatin, and dihydrotestosterone (DHT) were added. After 72 hr, the surviving cells were stained using the Alamar Blue assay (TREK Diagnostic Systems, Cleveland, OH) for 180 min at 37°C. Absorbance in each well was measured using a plate reader (ARVO™ MX; Perkin Elmer, Inc., Waltham, MA).

Statistical Analysis

The Mann–Whitney's *U*-test was used for statistical analysis, and significance was set at the 5% level.

RESULTS

First, using Western blotting we examined whether or not mevastatin and simvastatin downregulated AR and PSA protein expression. As shown in Figure 1, both AR and PSA expression were downregulated in a time and dose dependent manner in LNCaP (A and B), 22Rv1 (C) and CxR (D) cells after the addition of mevastatin and simvastatin. However, quantitative

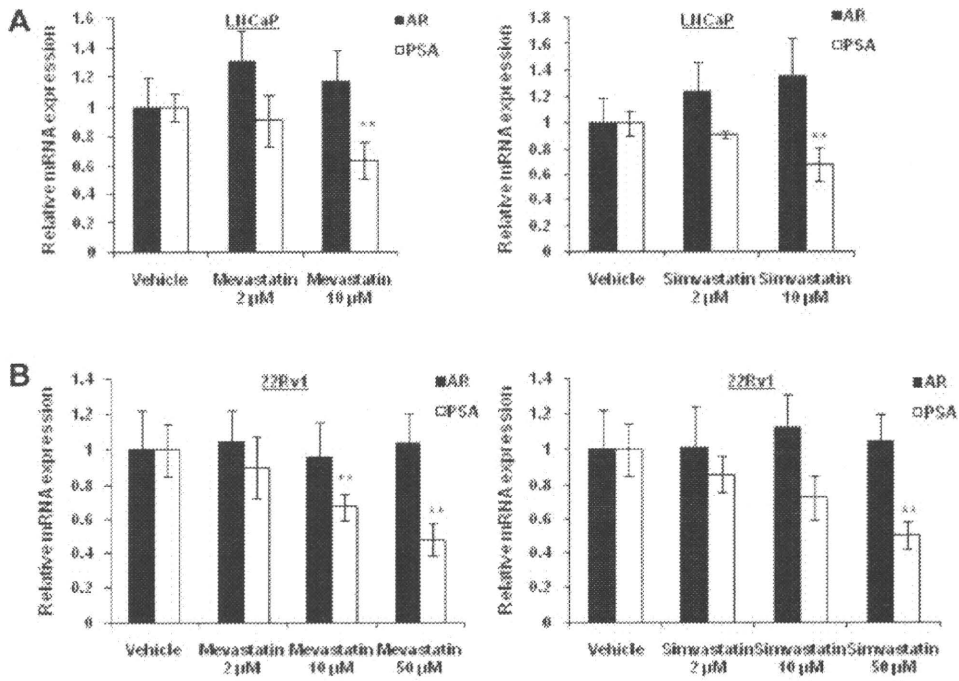

Fig. 2. Downregulation of PSA mRNA expression but not AR mRNA expression by mevastatin and simvastatin. LNCaP (**A**) and 22Rvl (**B**) cells were exposed to the concentration of mevastatin and simvastatin indicated for a period of 48 hr. Then, the extraction of total RNA and the synthesis of cDNA were performed. Quantitative real-time PCR was performed using the primers and probes for AR, PSA, and GAPDH. The transcript level of the target transcript was corrected with the corresponding GAPDH transcript level. All values represent at least three independent experiments. The AR and PSA transcript levels of cells applied to the vehicle were defined as I. Boxes: mean values; Bars ± SD, $^{**}P < 0.05$ (compared with that of vehicle).

real-time PCR of AR, PSA, and GAPDH indicated that while mRNA expression of PSA was downregulated by statins, AR was not downregulated. This finding indicated that AR expression was not transcriptionally regulated by mevastatin and simvastatin (Fig. 2). The downregulation of PSA can be explained by the fact that PSA was transcriptionally regulated by AR, as has been previously reported [10]. To confirm that statins regulate AR expression by regulating the protein level rather than the transcriptional level, the proteasome inhibitor MG132 was applied in our experiments. As shown in Figure 3, mevastatin and simvastatin promote AR protein degradation in LNCaP (A) and CxR (B) cells, but this was prevented by MG132. The protein level of PSA was also similarly regulated by the AR protein levels (Fig. 3A,B). Furthermore, the application of cycloheximide, a translational inhibitor, did not affect the down regulation of AR in the presence or absence of dihydrotestosterone (DHT), indicating that statins did not regulate AR expression at the translational level (Fig. 3C). Finally, exogenously introduced expression vector AR-GFP [9] was also downregulated by mevastatin, which again supports the data that statins downregulated AR by proteolysis (Fig. 3D).

Next, the inhibitory effect of statins on cell growth was examined as shown in Figure 4. Mevastatin and simvastatin suppressed cell growth in AR positive cells (LNCaP and 22Rv1) in a dose dependent manner, but this inhibitory effect was limited in AR negative RWPE-1 cells (Fig. 4A,B). Additionally, statins inhibited cell growth, not only in LNCaP cells but also in castration-resistant LNCaP derivative CxR cells [7] and hydrogen peroxide-resistant LNCaP derivative HPR50 [7]. These data possibly suggest that the inhibitory effect of statins is harbored in castration resistant prostate cancer cells.

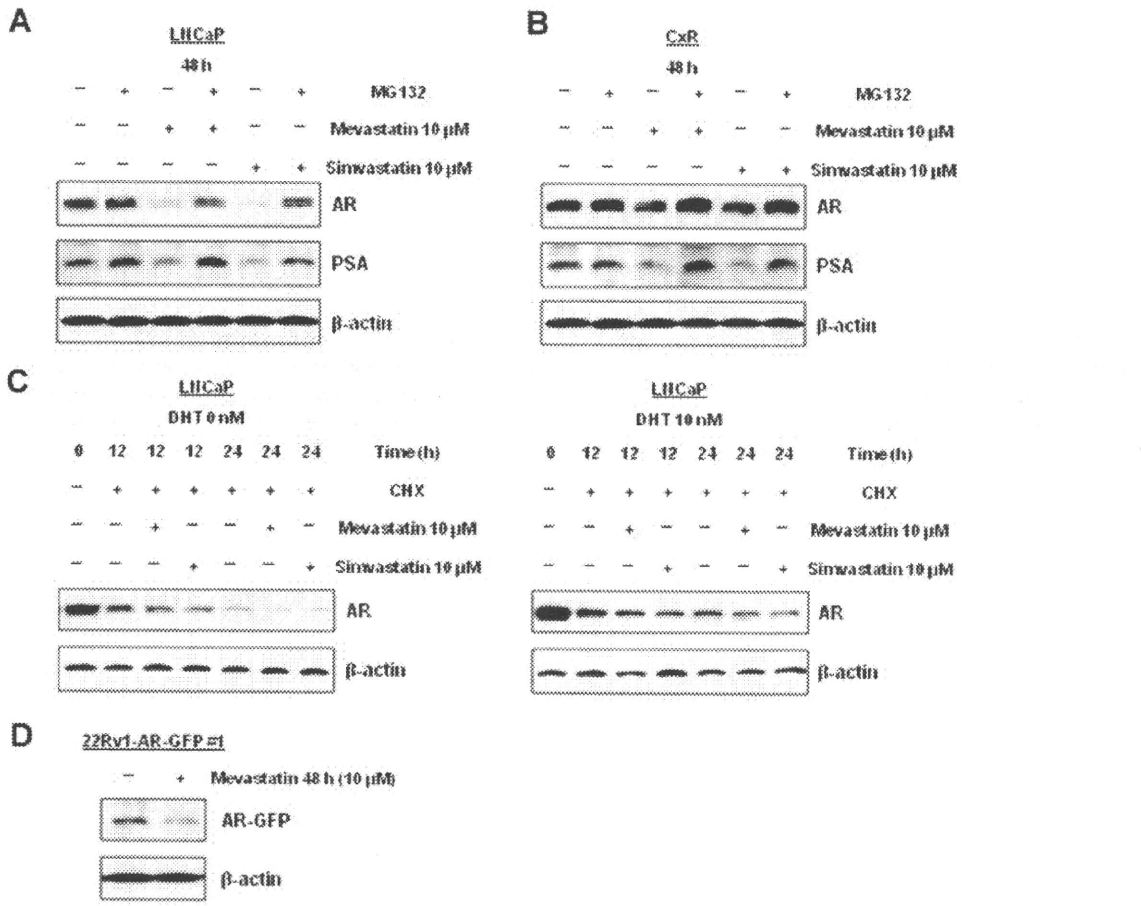

Fig. 3. Promotion of AR degradation by mevastatin and simvastatin. LNCaP (**A**) and CxR (**B**) cells were exposed to the concentrations of mevastatin and simvastatin indicated for a period of 48 hr, with or without 5 μM of MG132. The whole-cell extracts were subjected to SDS–PAGE. Western blotting was performed using the antibodies indicated. LNCaP cells (**C**) were exposed to 10 μM of mevastatin and simvastatin, with or without 1 μg/ml of cycloheximide for the durations indicated in the absence (**left panel**) or presence (**right panel**) of 10 nM of DHT. The whole-cell extracts were subjected to SDS–PAGE. Western blotting was performed using the antibodies indicated. 22Rv1-AR-GFP cells (**D**) were exposed to 10 μM of mevastatin for 48 hr. The whole-cell extracts were subjected to SDS–PAGE. Western blotting was performed using antibodies against GFP and β-actin.