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An Original Patient-Derived Xenograft of Prostate Cancer With Cyst Formation

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BACKGROUND. The high rate of failure of new agents in oncology clinical trials indicates a weak understanding of the complexity of human cancer. Recent understanding of the mechanisms underlying castration resistance in prostate cancer led to the development of new agents targeting the androgen receptor pathway; however, their effectiveness is limited. Hence, there is a need for experimental systems that are able to better reproduce the biological diversity of prostate cancer in preclinical settings. In this study, we established a unique patient-derived xenograft (PDX) model to identify biomarkers for treatment efficacy and resistance and better understand prostate cancer biology.

METHODS. A prostate cancer tissue sample from a Japanese patient was transplanted subcutaneously into male, severe combined immune-deficient (SCID) mice and this PDX mouse model was named KUCaP3. Sequential tumor volume changes were observed before and after castration. Androgen receptor (AR), prostate-specific antigen (PSA), and other molecular markers were examined immunohistochemically. Sequence analysis of *AR* was also performed to detect mutations. Proteomic analysis of cyst fluid and sera samples of KUCaP3 mice were analyzed by mass spectrometry (MS).

RESULTS. KUCaP3 cell line, derived from human tissue, was successfully and serially passaged in vivo with approximately 60% take rate. KUCaP3 exhibited cyst formation, showed androgen-dependent growth initially, and developed castration-resistant growth several months after castration of the mice. Immunohistochemical analysis showed that KUCaP3 was positive for AR, PSA, CK18, and α-methyl acyl-coenzyme A racemase, but negative for CK5/6 and ERG. The *AR* gene in KUCaP3 cells contained a substitution from CAT (histidine) to TAT (tyrosine) at the nucleotide positions corresponding to codon 875 (H875Y) in the ligand-binding domain. Chemiluminescent immunoassay revealed higher levels of PSA in cystic fluid and the serum of KUCaP3-bearing mice. MS analysis detected 23 proteins of human origin in cystic fluids of KUCaP3.

CONCLUSIONS. We developed KUCaP3, an androgen-dependent PDX model with cyst formation. Several proteins including PSA were detected in the cystic fluid and sera of tumor-bearing mice. This original PDX model has the potential to be used as a clinically relevant model to evaluate molecular markers for prostate cancer diagnosis and treatment. *Prostate 76:994–1003, 2016.* © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

Prostate cancer is one of the most frequently diagnosed forms of human cancer in the Western world. However, the mechanisms underlying occurrence, metastasis, and transition to castration-resistance are still unknown. Standard oncology research methodologies involve the use of animal models, but gene expression and function differ between human and animals. Immunodeficient mice harboring subcutaneous prostate cancer cell line xenografts have been commonly used as preclinical cancer models for mechanistic understanding and therapeutic studies [1]. However, these models fail to reproduce the diverse heterogeneity of clinical prostate cancer since most of cell lines are established after long-term in vitro culture and exhibit homogeneous characteristics. Furthermore, cell line xenografts rarely show specific architecture such as ductal structure, and therefore do not accurately mimic the original malignancy [2]. These limitations can be overcome using patient-derived tumor tissue xenografts (PDX), established by direct implantation of cancer tissues into immunodeficient mice.

Lawrence et al. [3] reported that castration-resistant prostate cancer (CRPC) PDX models can be established from transurethral resection of the prostate (TURP) specimens with moderate success. They suggested that three factors determined the take rate: quality of the tissue, abundance of cancer cells, and viability. They described a contemporary xenografting method that involves the placement of the xenograft beneath the renal capsule in host mice [4]. We previously reported two novel prostate cancer PDX models, namely KUCaP1 and KUCaP2 [5,6]. KUCaP1 tumors harbor the W742C mutant androgen receptor (AR), regress quickly after castration, and do not showed growth relapse in long-term follow-up. The anti-androgen drug, bicalutamide, promotes KUCaP1 tumor growth by virtue of the AR mutation [5]. This model can reproduce androgen withdrawal syndrome observed in clinical settings [7]. KUCaP2 tumors, on the other hand, harbor wild-type AR, regress rapidly after castration, but show growth 1–2 months later. Comparing the expression profiles of KUCaP2 tumors at each stage using DNA microarrays, we found that prostaglandin E receptor 4 (EP4) was highly expressed in castration-resistant KUCaP2 tumors, while an EP4 antagonist significantly suppressed castration-resistant KUCaP2 tumor growth. This model simulates CRPC in clinical settings and suggests that an EP4 antagonist is potentially an effective treatment modality for CRPC [6].

In this study, we successfully established an original PDX prostate cancer model, termed as KUCaP3. The tumors harbored an H875Y mutant AR, regressed rapidly after castration, and showed growth relapse after several months, mimicking clinical CRPC cases. Moreover, its characteristic feature was formation of a cystic tumor, containing high level of secreted prostate-specific antigen (PSA) in the cyst fluid. We immunohistochemically characterized this model using several biomarkers in addition to using a proteomic approach to analyze the cystic fluid and serum of the tumor-bearing mice.

MATERIALS AND METHODS

Patients and Tissue Samples

The clinical materials used to establish KUCaP3 were obtained through TUR of the prostate performed on a 74-year-old Japanese male patient who later died of CRPC. All tissue samples were acquired after obtaining informed consent from the patient according to protocols approved by the Institutional Review Board (IRB) of Kyoto University Hospital (IRB approved number G52: Research about an individualized treatment for urological cancer using gene profiling).

Animals

All experiments involving laboratory animals were performed in accordance with the Guideline for Animal Experiments of Kyoto University and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine. C.B-17/IcrCrj severe combined immune-deficient (SCID) mice (Charles River Japan, Yokohama, Japan) were used as the host animal of KUCaP3 xenograft.

Generation, Serial Transplantation, and Renal Capsular Grafting of KUCaP3

The primary prostate tumor tissue was harvested, minced into 20–30 mm^3 pieces, and subcutaneously transplanted on both sides of the back of 5-week-old SCID mice. Additionally, 50 μl Matrigel (Becton Dickinson, Franklin Lakes, NJ) was injected

around the implant. The first KUCaP3 xenograft was established in only one out of five mice, nearly a year after the inoculation. The xenograft tumors were extracted from this mouse and transplanted to another five mice, this time without Matrigel. For passage, xenograft tumors were subcutaneously transplanted into ten mice on one side of the back. Renal capsular grafting was performed as previously described [8].

Sequence Analysis

Genomic DNA from the xenograft tissue of the second generation was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and Sanger sequencing was performed for all exons of the human *AR* gene as previously reported [5]. RNA from the xenograft tissue obtained at the fifth and eighth generation was converted to cDNA from total RNA using the RiverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) and the sequences of *AR* exons 4–8 were analyzed by Sanger sequencing using the primers previously described [9].

Liquid Chromatography-Mass Spectrometry Analysis (LC-MS/MS)

The cyst fluid and sera of three KUCaP3 mice were collected and analyzed by LC-MS/MS approach. LC-MS/MS analysis was performed as previously reported [10]. We originally developed an abundant serum protein depletion device based on high-performance hollow-fiber membranes (HFM). All the samples were first treated with this device for low-molecular weight (LMW) proteins to be separated from high-molecular weight proteins, which often inhibit LMW proteins from being detected by mass-spectrometric approach. Secondly to the treatment, trypsin digestion, strong cation exchange chromatography, and C18 reverse-phased chromatography were performed before analysis by a quadrupole-TOF mass spectrometer. The acquired MS/MS spectra were compared with the Swiss-Prot mammalian database (NCBI) containing 64,691 entries for mammal proteins using Mascot software ver.2.2.4 (Matrix Science, MA). The search tolerance was set to 0.6 Da for precursor ions and 0.3 Da for product ions. One trypsin miscleavage per peptide was allowed, and certain modifications (oxidation of methionine, carbamidomethylation of cysteine) were considered. The proteins were judged as human origin, which were detected only as human proteins, or whose MASCOT scores were maximized if assigned as human proteins.

Chemiluminescent Immunoassay

PSA in the sera and cyst fluid of KUCaP3 was estimated by chemiluminescent immunoassay (CLIA) using Architect® PSA (Abbott, Abbott Park, IL).

Antibodies and Reagents

Anti-AR (C-19 sc-815) and anti-PSA (C-19 sc-7638) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX) and anti-ERG antibody (ab92513) was obtained from Abcam (Cambridge, UK). Anti-α-methylacyl-coenzyme A racemase (AMACR) monoclonal antibody (M3616), anti-cytokeratin (CK)-18 (M7010), anti-neuron-specific enolase (NSE) (M0873), and anti-CK5/6 (M7237) were purchased from Dako (Carpinteria, CA). Anti-chromogranin A (CGA) (NCL-CHROMO-430) antibody was obtained from Leica (Newcastle, UK).

Immunohistochemistry

Immunohistochemistry was performed by standard indirect immunoperoxidase procedures using appropriate dilutions (as shown) of each primary antibody (AR 1:100, PSA 1:100, ERG 1:1000, AMACR 1:150, CK18 1:30, NSE 1:5000, CK5/6 1:100, and CGA 1:300) as previously described [6].

RESULTS

KUCaP-3 Characterization

Tumor tissues used to establish KUCaP3 were obtained from a 74-year-old Japanese prostate cancer patient 13 years after his diagnosis. The patient was given complete androgen blockade therapy, three regimens of chemotherapy, and local radiation therapy. Since he had difficulty during urination indicating local progression, we performed transurethral resection (TUR) of the prostate. The tumor tissue was obtained at this TUR and was transplanted subcutaneously into male SCID mice after the patient provided informed consent. The patient then received lumbar laminectomy for disease progression and the metastatic bone tissue was also obtained. The treatment and PSA time course are shown in Figure 1.

One of the five tissues transplanted into mice exhibited initial growth at approximately 9 months after implantation. This xenograft was labeled as KUCaP3 and maintained by serial passage, with a take rate of approximately 60% at each passage. The KUCaP3 tumor showed specific morphological features; macroscopically, it formed a cystic tumor (Fig. 2A) and microscopically, it formed a papillary tumor containing stroma, which was not detected in the original TUR specimen

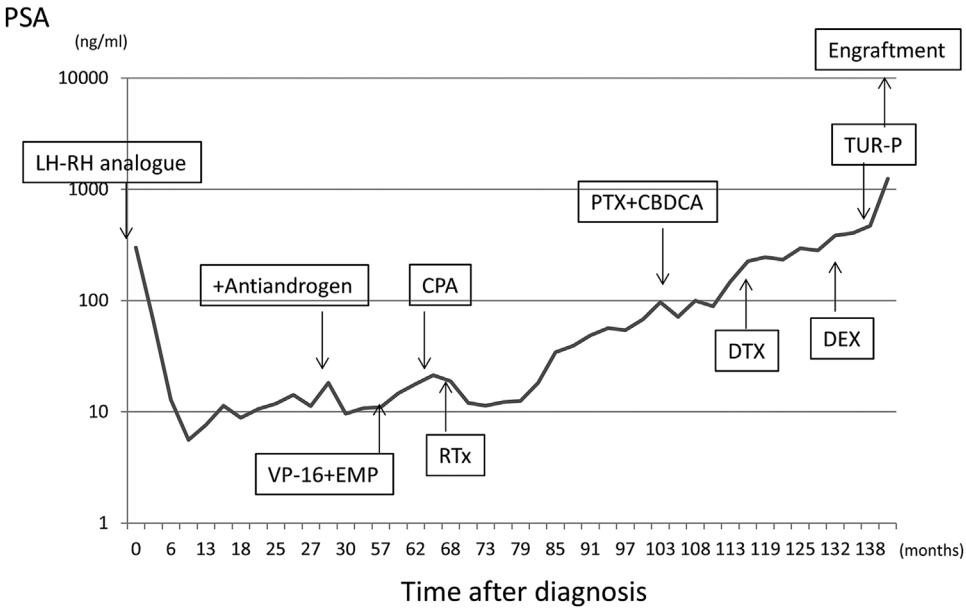

Fig. 1. Clinical course of the donor patient for KUCaP3. Prostate-specific antigen (PSA) time course and treatments administered to the patient whose prostate cancer tissue was used to establish KUCaP3. The tissues established as KUCaP3 obtained from transurethral resection of the prostate (TURP) are indicated. VP-16, etoposide; EMP, estramustine phosphate; CPA, cyproterone acetate; RTx, radiation; PTX, paclitaxel; CBDCA, carboplatin; DTX, docetaxel; DEX, dexamethasone.

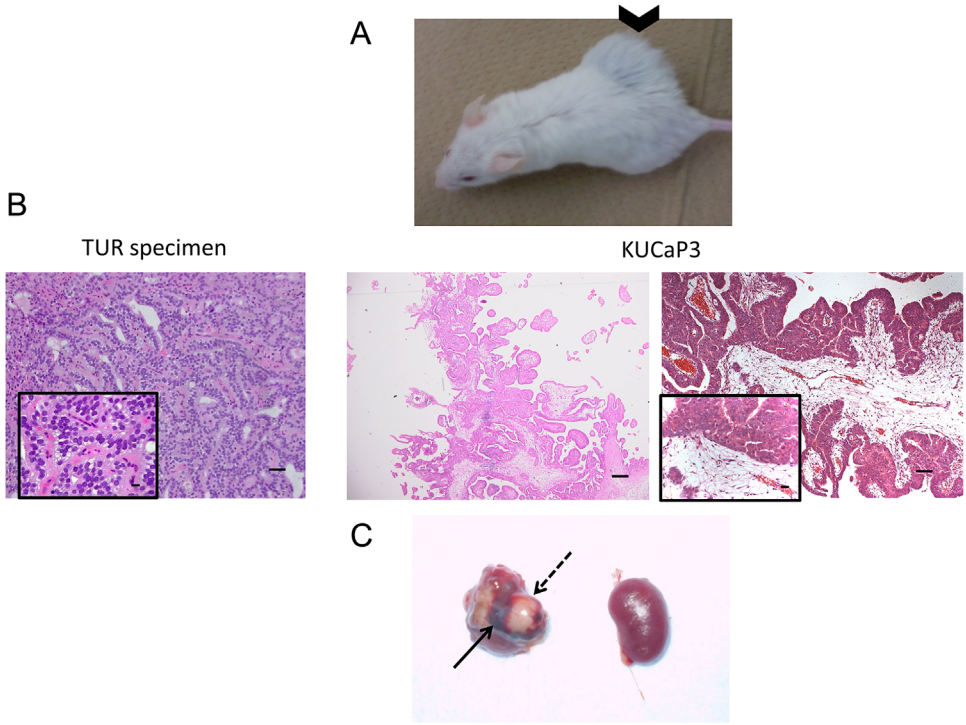

Fig. 2. Macroscopic and microscopic findings for KUCaP3. **A**: Severe combined immune-deficient mouse bearing subcutaneous tumor (arrowhead). **B**: Histopathology of a TUR specimen and a KUCaP3 tumor. Hematoxylin and eosin staining (20×, bar = 20 μm, 50×, bars = 50 μm, insert: 200×, bars = 5 μm). **C**: A KUCaP3 tumor engrafted and grown under the renal capsule showed cystic lesion (solid arrow) adjacent to solid lesion (dashed arrow).

(Fig. 2B). Cyst formation was observed in each KUCaP3 mouse implanted with the xenograft beneath the renal capsule (Fig. 2C) as well as when they acquired castration resistance as described below.

Next, we immunohistochemically analyzed several molecular markers using human specific antibodies to check the origin and character of KUCaP3. AR and PSA expression were positive as those observed in the original TUR specimen (Fig. 3). Figure 4 shows positive results for CK18 and AMACR but not for CK5/6 and ERG, implying that the cells were derived from human prostate cancer differentiated epithelial cells and not from basal cell origin. Immunohistochemically confirmed presence of neuroendocrine markers, NSE and CGA implies that the tumor cells were focally positive, indicating that KUCaP3 cells are heterogeneous, similar to the cellular characteristics of tumors in human CRPC, though this was not verified by hematoxylin-eosin staining. We then examined whether KUCaP3 growth requires androgen stimulation. We investigated the sequential changes in KUCaP3 tumor volume after castration (castration group: $n = 7$), and compared the changes to those observed in non-castrated mice (control group: $n = 6$). The tumor size at day 0 was $1{,}376 \pm 813\,mm^3$ in the control group and $1{,}095 \pm 807\,mm^3$ in the castration group. The KUCaP3 tumor regressed rapidly after castration. The tumor size at day 93 was $20{,}042 \pm 24{,}387\,mm^3$ in the control group and $427 \pm 465\,mm^3$ in the castration group (Fig. 5A and B). However, some tumors in the castration group showed heterogeneous characteristics; four out of seven tumors regrew several months after castration (Fig. 5C).

Sequence analysis of *AR* in KUCaP-3 tumors revealed a substitution mutation from CAT (histidine) to TAT (tyrosine) at the nucleotide positions corresponding to codon 875 (H875Y) in the ligand-binding domain. DNA sequence analysis of TURP specimens from the patient revealed that the wild-type and H875Y mutant alleles coexisted in the prostate tumors, but only wild-type *AR* was detected in the lumbar vertebrae metastatic tissue samples derived from laminectomy at the time of the patient's disease progression after the TURP. These results suggest that H875Y mutant AR was selected at transplantation (Fig. 6A) and this mutation was maintained even after serial transplantations until at least the 5th and 8th generations. The wild-type *AR* was partially detected in the 5th generation, suggesting that KUCaP3 tumors were basically heterogeneous, as seen in the immunohistochemical results (Fig. 6B).

Furthermore, we investigated PSA levels in the sera of KUCaP3 mice. CLIA showed $888.3 \pm 240.6\,ng/ml$ PSA in the sera of three KUCaP3 tumor-bearing mice, with $9{,}913.3 \pm 7{,}834.5\,ng/ml$ in the cyst fluid of the same mice. This result strongly indicates that the cyst fluid of KUCaP3 mice contains high levels of proteins secreted by the KUCaP3 tumor tissue.

Mass-Spectrometry of KUCaP3 Cystic Fluid and Sera in Tumor-Bearing Mice

We performed LC-MS/MS analysis of the cyst fluid and sera samples from three KUCaP-3 mice as previously reported [10]. A total of 346 proteins were

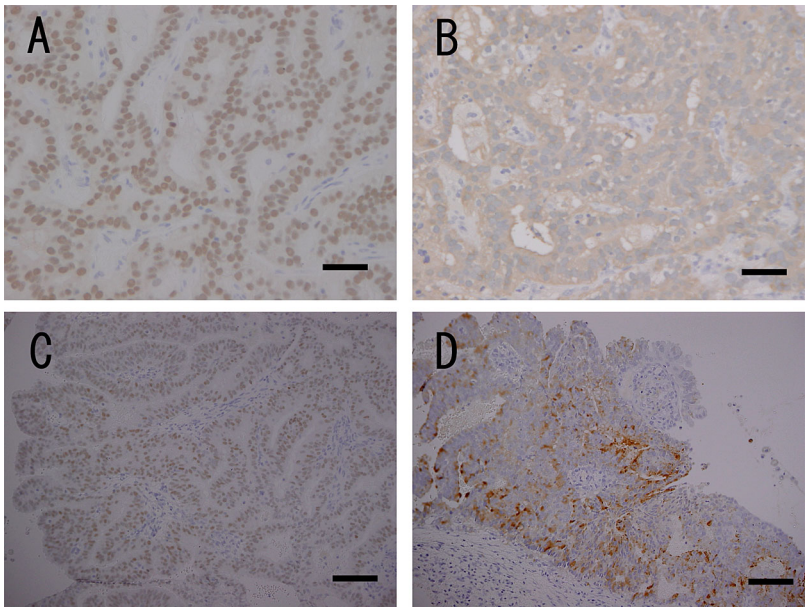

Fig. 3. Immunohistochemical assay for AR and PSA expression by KUCaP3. AR (**A**) and PSA (**B**) expression in the original TUR specimen and AR (**C**) and PSA (**D**) expression by KUCaP3 (200×, bars = 5 μm).

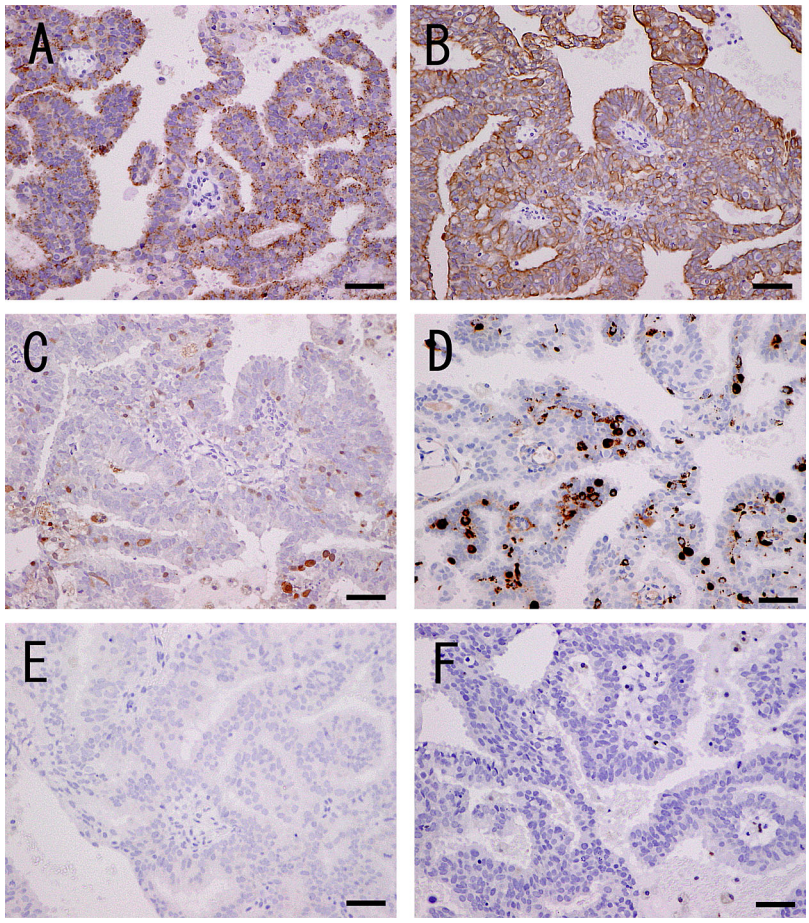

Fig. 4. Immunohistochemical assay for AMACR (**A**), CK18 (**B**), NSE (**C**), CGA (**D**), CK5/6 (**E**), and ERG (**F**) expression by KUCaP3 (200×, bars = 5 μm).

detected at least in one sample. Among these, 52 proteins were confirmed as of human origin and 23 proteins (including PSA KLK3) were detected in two or more samples (Table I contains a list of the proteins with their MASCOT scores).

DISCUSSION

Xenografts are experimental models in which human tissues are transplanted into an immunodeficient animal. These models are useful experimental resources wherein human cancer cells can be propagated in vivo for long periods to aid the study of tumor progression under various conditions and testing of novel therapies. PDX models established directly from human cancer tissues simulate various clinical cancer situations and characteristics more closely compared with xenografts of established cancer cell lines.

Human prostate cancer cell lines including LNCaP, PC3, and their sub-lines have been the draft horses of prostate cancer research field for decades and there was no doubt that the many and varied investigations using theses cell lines have been informative. The development of various potent, though expensive drugs in recent years has necessitated use of prognostic or predictive markers in routine clinical practice. However, no validated and reliable markers are currently available. Therefore, alternative preclinical models that provide more accurate translation of research investigations are essential to monitor and improve treatment outcomes. Prostate cancer is a heterogeneous disease and therefore, a diverse range of tumor specimens are required to understand the implications of any given treatment strategy. PDX models can help overcome these challenges and are therefore, gaining acceptance in basic and translation research.

KUCaP3, the third original PDX developed by our group, is unique in cyst formation that contains abundant prostate-specific proteins. Established from a CRPC specimen, it expresses AR and PSA strongly, and regresses rapidly after mouse castration. Some tumors showed growth after castration, showing

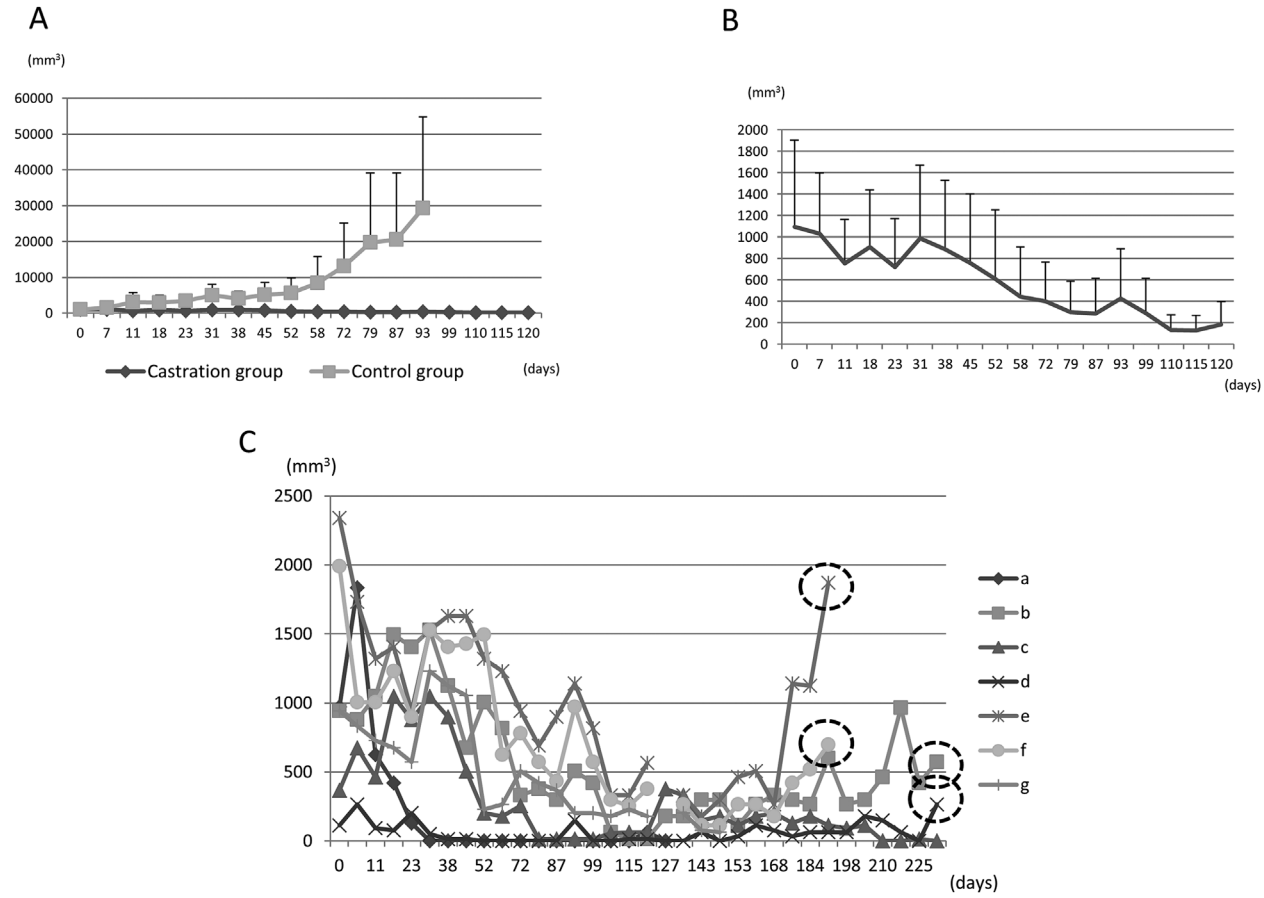

Fig. 5. KUCaP3 requires testicular androgen for growth. **A**: Sequential changes in KUCaP3 tumor volume after castration compared with those without castration. The error bars show standard deviation. **B**: After castration, KUCaP3 tumor volume was immediately reduced. **C**: KUCaP3 tumor showed growth relapse in four out of seven mice at 5–6 months after castration. The tumors labeled b, d, e, and f acquired castration resistance (indicated by dashed circle).

evidence of castration resistance. *AR* sequence analysis revealed that KUCaP3 harbors an H875Y mutation and this mutation was maintained after several passages. Morphologically, the glandular epithelium lining of the cystic wall of tumor tissues is unique to this PDX tumor. These features were maintained when KUCaP3 tumor tissues were transplanted beneath the renal capsules independent of the inoculation site. It is not clear why KUCaP3 retained cystic features and need to be investigated further. Based on these characteristics, KUCaP3 has potential as a useful model with three prominent features: (i) well-differentiated prostate cancer morphology; (ii) androgen-dependent growth, with the ability to acquire castration resistance; and (iii) secreted proteins in cystic fluid for investigation of molecular markers for prostate cancer.

A few prostate cancer PDX models are available that initially exhibit androgen-sensitive growth characteristics, but after castration of mice bearing established PDX tumors the models show variable responses including tumor shrinkage and tumor regression followed by growth relapse, thus mimicking clinical transition to CRPC. PC346P is a PDX model derived from TUR specimens of a Caucasian male diagnosed with advanced prostate cancer [11]. PC346P model shows response to castration followed by growth relapse as KUCaP3, however the duration for acquisition of growth relapse after tumor regression is shorter. CWR22 is an androgen-dependent PDX tumor, where the xenograft regresses after androgen withdrawal [12]. However, some CWR22 tumors relapse after 3–10 months, thus closely mimicking KUCaP3 tumors. KUCaP2 tumors established in our laboratory also express androgen-dependent tumor growth initially but subsequently acquire castration-resistant growth [6]. Similar to previously established PDX models, KUCaP3 allows the evaluation of androgen responsiveness in clinically relevant studies as well as the mechanisms of transition to castration-resistance.

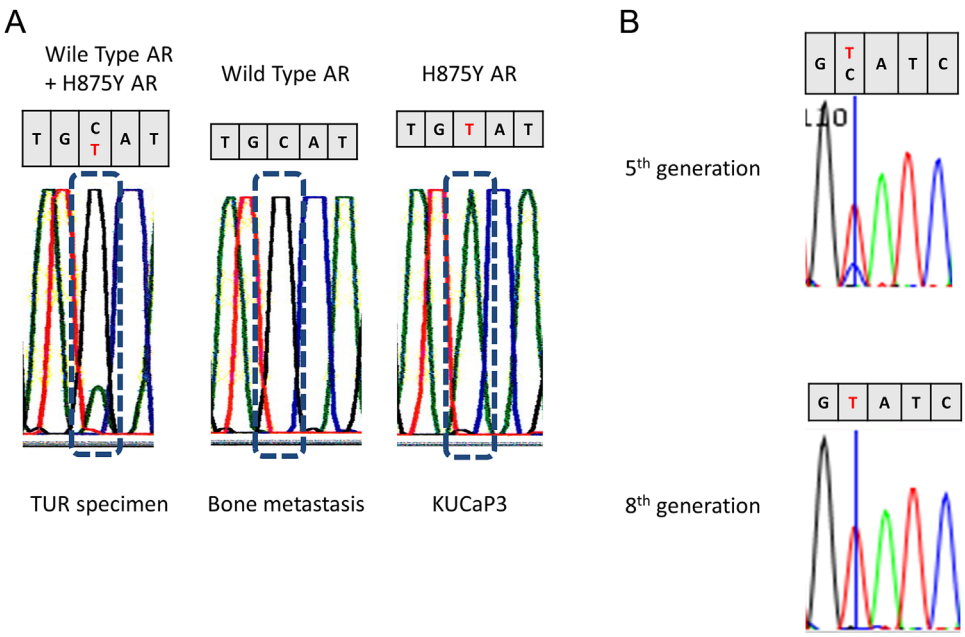

Fig. 6. *AR* of KUCaP3 harbors the H875Y point mutation in its ligand-binding domain. **A**: Left: Partial *AR* DNA sequences of TURP specimens. Middle: Partial *AR* DNA sequences of lumbar vertebrae metastasis specimens derived from patients' laminectomy. Right: Partial *AR* DNA sequences of KUCaP3 samples. Dashed square indicates the mutation site. **B**: Partial *AR* DNA sequences of 5th and 8th generation of KUCaP3 samples.

Lee et al. previously reported the analysis of secreted factors of prostate cancer epithelial cells, PCa-118b using PDX models [13]. However, they separated and selected epithelial cells from stromal cells in the tumor under special in vitro conditions and collected the condition medium for analysis. The KUCaP3 model developed in the present study is unique in that, molecules secreted by the cancer cells with murine stromal cells can be analyzed directly in vivo, thus proving more advantageous in the investigation of prostate cancer biology and molecular markers.

Using LC-MS/MS methods, established by Tanaka et al., we identified proteins expressed in the cyst fluid of KUCaP3 mice. PSA was consistently identified in all samples. Among the other proteins shown in Table I, some have been previously reported as associated with prostate cancer. For example, Testican-1, also known as Spock1, is upregulated in prostate cancer [14]. Cysteine-rich secretory protein 3 is upregulated in prostate cancer relative to its expression in normal prostate, a finding based on electronic profiling of expressed sequence tags [15]. Recent studies have reported an increase in the expression of midkine in various human neoplasias, while Konishi et al. [16] reported that this protein can serve as a diagnostic and prognostic marker of prostate cancer, based on immunohistochemistry. Identification of these proteins could enhance the validity of our analysis. Investigation of the biological and cancer-related significance of these detected proteins in prostate cancer is currently underway.

KUCaP3 harbors a mutant AR, H875Y, similar to that in CWR22, an androgen-dependent human prostate cancer cell xenograft [17]. *AR* mutations and amplifications are infrequently found in localized hormone-naïve prostate cancer, but *AR* aberrations are associated with progression to CRPC. The incidence of *AR* mutations in CRPC patients is reported as 10–30%, and it increases especially after treatment with androgen-signaling target agents such as bicalutamide, enzalutamide, and abiraterone [18]. Almost 160 *AR* mutations have been reported to date and a majority of these are single-base substitutions: 50% of them identified in prostate cancer specimens in the ligand-binding domain of AR [19]. The exact incidence of each *AR* mutation in CRPC remains unknown due to difficulty in direct acquisition of CRPC specimens to evaluate *AR* mutations. However, the analysis of circulating cell-free DNA (cfDNA) has been recently shown as a promising approach for characterization of the CRCP tumor genome. Azad et al. collected plasma of 62 metastatic CRPC patients ceasing abiraterone, enzalutamide, or other agents for disease progression and analyzed *AR* gene aberrations from cfDNA, including H875Y *AR* mutation in five patients. Since H875Y *AR* mutation was found in patients progressing on abiraterone or with prior abiraterone treatment, KUCaP3 may be an

TABLE I. Proteins Detected From the Crude Serum and Cystic Samples by LC-MS/MS

Swissprot ID	MASCOT score/sample 1	MASCOT score/sample 2	MASCOT score/sample 3	MASCOT score/sera	Protein
FINC_HUMAN	6,277	2,408	3,213	376	Fibronectin
NPC2_HUMAN	664	682	350	134	Epididymal secretory protein E1
KLK3_HUMAN	925	818	1,189	118	Prostate-specific antigen
TICN1_HUMAN	183	179	212	35	Testican-1
CRIS3_HUMAN	631	306	546		Cysteine-rich secretory protein 3
DSC2_HUMAN	166	364	139		Desmocollin-2
GDF15_HUMAN	567	267	277		Growth/differentiation factor 15
IBP2_HUMAN	52	244	124		Insulin-like growth factor-binding protein 2
KLK11_HUMAN	620	123	287		Kallikrein-11
MK_HUMAN	635	47	34		Midkine
PEBP1_HUMAN	610	323	583		Phosphatidylethanolamine-binding protein 1
REG1A_HUMAN	372	997	82		Lithostathine-1-alpha
SPIT1_HUMAN	251	154	204		Kunitz-type protease inhibitor 1
CMGA_HUMAN	791	298		1,167	Chromogranin-A
TMSL3_HUMAN		203	62	239	Thymosin beta-4-like protein 3
CALD1_HUMAN	58	51		43	Caldesmon
AGR2_HUMAN	250		283		Anterior gradient protein 2 homolog
ARMET_HUMAN		38	101		Protein ARMET
ATS1_HUMAN		35	40		A disintegrin and metalloproteinase with thrombospondin motifs 1
CH3L2_HUMAN	221	162			Chitinase-3-like protein 2
EHD1_HUMAN		175	217		EH domain-containing protein 1
KLK2_HUMAN	42		38		Kallikrein-2
NGAL_HUMAN	587	67			Neutrophil gelatinase-associated lipocalin

appropriate PDX model to evaluate mechanisms of abiraterone resistance [20].

In conclusion, we established an original PDX model, KUCaP3, with applications in the investigation of prostate cancer biology and biomarkers. It is initially castration-sensitive but acquires castration-resistant character under castrated conditions. KUCaP3 expresses AR and PSA, and shows glandular formation. This original, transplantable PDX has potential as a relevant tool for understanding prostate cancer biology and a preclinical model to evaluate molecular markers.

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