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5αDH-DOC (5α-dihydro-deoxycorticosterone) activates androgen receptor in castration-resistant prostate cancer

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Prostate cancer often relapses during androgen-depletion therapy, even under the castration condition in which circulating androgens are drastically reduced. High expressions of androgen receptor (AR) and genes involved in androgen metabolism indicate a continued role for AR in castration-resistant prostate cancers (CRPCs). There is increasing evidence that some amounts of 5α-dihydrotestosterone (DHT) and other androgens are present sufficiently to activate AR within CRPC tissues, and enzymes involved in the androgen and steroid metabolism, such as 5α-steroid reductases, are activated in CRPCs. In this report, we screened eight natural 5αDH-steroids to search for novel products of 5α-steroid reductases, and identified 11-deoxycorticosterone (DOC) as a novel substrate for 5α-steroid reductases in CRPCs. 11-Deoxycorticosterone (DOC) and 5α-dihydro-deoxycorticosterone (5αDH-DOC) could promote prostate cancer cell proliferation through AR activation, and type 1 5α-steroid reductase (SRD5A1) could convert from DOC to 5αDH-DOC. Sensitive liquid chromatography-tandem mass spectrometric analysis detected 5αDH-DOC in some clinical CRPC tissues. These findings implicated that under an extremely low level of DHT, 5αDH-DOC and other products of 5α-steroid reductases within CRPC tissues might activate the AR pathway for prostate cancer cell proliferation and survival under castration. (*Cancer Sci* 2010; 101: 1897–1904)

Prostate cancer (PC) is the most common malignancy in men and the second-leading cause of cancer-related death in Western countries.[1] The androgen–androgen receptor (AR) signaling pathway plays a central role in PC development and progression, and PC growth is androgen-stimulated. Androgen depletion (castration) is usually effective for a limited duration and eventually PC evolves to regain the ability to grow despite low levels of circulating androgens.[2] This more aggressive and castration-resistant phenotype has been termed castration-resistant prostate cancer (CRPC). Treatment options for CRPC are an unmet need with docetaxel being the only agent that has been shown to prolonged survival,[3,4] but its survival benefit is limited. Hence, many groups are now attempting various approaches to identify novel molecular targets or signaling pathways that contribute to the CRPC phenotype and some strategies are currently being tested in clinical trials in CRPC.[5,6]

Several clinical observations have been offering clues that AR signaling is still active and required in most CRPC. PSA (prostate specific antigen) declines after the initiation of androgen-depletion therapy (ADT), but a subsequent rise of PSA is commonly the first sign of disease progression. This indicates that reactivation of AR signaling accompanies the development of CRPC.[2,5,6] Within several androgen-target tissues such as the prostate, testosterone is converted to 5α-dihydrotestosterone (DHT), which is the most potent natural androgen. Although ADT can lead to a drastic reduction of the serum circulating testosterone level (to <5%), the intraprostatic concentration of DHT remains at ~40% in CRPCs,[7–10] indicating that CRPC cells preserve their DHT level to maintain their AR signaling pathways and survive under the castrated condition. The conversion from testosterone to DHT is catalyzed by 5α-steroid reductase enzymes, and three types of human 5α-steroid reductase enzymes have been reported so far.[11] In the normal prostate and benign prostate diseases, type 2 isozyme is expressed dominantly and responsible for DHT production. On the other hand, in prostate cancer tissues, the type 2 isozyme expression is dramatically down-regulated and the expressions of types 1 and 3 isozymes are up-regulated, indicating that type 1 and type 3 isozymes are mainly responsible for DHT production in PC tissues, especially in CRPCs under the castration condition.[10,11] Although the biological significance and the mechanism of this switching toward types 1 and 3 isozymes in CRPCs remain completely unknown, the question then arises whether these 5α-steroid reductase enzymes can produce other 5αDH steroids to activate AR or other steroid receptors as well as DHT and may provide some survival advantages to CRPC cells in the castration condition.

In this study, we screened eight natural 5αDH steroids for candidates that could be produced by 5α-steroid reductases to activate the AR signaling pathway in CRPCs, and identified 11-deoxycorticosterone (DOC) as a novel substrate for 5α-steroid reductases in CRPCs. We demonstrated that DOC and 5α-dihydro-deoxycorticosterone (5αDH-DOC) could stimulate AR activity in CRPC cells and type 1 5α-steroid reductase could convert from DOC to 5αDH-DOC *in vitro* and *in vivo*. Furthermore, we measured 5αDH-DOC levels in clinical CRPC tissues, and detected 5αDH-DOC in some clinical CRPC tissues. These findings implicated that under the castration condition, CRPC cells might take advantage of 5αDH-DOC and other products of 5α-steroid reductases to maintain their AR pathway for their survival, and also they provide new insights in molecular mechanisms of CRPC progression and some clues to develop new therapeutic strategies for CRPC.

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Materials and Methods

Cell lines and clinical PC tissues. COS7 cell and four PC cell lines (LNCaP, 22Rv1, PC-3, and DU-145) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). They were grown in Delbecco's modified Eagle's medium with or without phenol red (Invitrogen, Carlsbad, CA, USA); these media were supplemented with 10% fetal bovine serum (FBS) or 10% charcoal/dextran-treated FBS (Hyclone, Logan, UT, USA) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 37°C in atmospheres of humidified air with 5% CO_2. Clinical CRPC tissues were obtained by autopsy and transurethral resection of prostate (TURP) at Kochi University Medical School, Iwate Medical University, Kyoto Prefectural University of Medicine, and Okayama University Medical School with the appropriate informed consents, as described before,[12] and the tissues included one liver metastasis, two lymphnode metastases, and two bone metastases from autopsies. All patients were undergoing the treatment of LH-RH agonist even after the emergence of CRPC.

Steroid compounds. Steroid compounds used in this study were purchased from Sigma or Steraloids (Newport, RI, USA) and included: androstanedione (Sigma), cholestanone (Steraloids), 5α-dihydrocorticosterone (Steraloids), 5α-dihydrocortisone (Steraloids), 5α-dihydroprogesterone (Sigama), 5α-dihydroDOC (Steraloids), 5α-dihydrocortisol (Steraloids), 5α-dihydro-17OH-progesterone (Steraloids), corticosterone (Sigma), cortisone (Sigma), progesterone (Sigma), DOC (Sigma), cortisol (Sigma). All steroids were dissolved by pure ethanol.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays. Prostate cancer (PC) cells were cultured in the medium containing 10% charcoal/dextran-treated FBS. Cell proliferation assay was performed by plating 1.5×10^5 of 22Rv1 cells, 2.0×10^5 of LNCaP cells, 4.0×10^4 of DU-145 cells, or 5.0×10^4 of PC-3 cells on six-well plates. Twenty-four hours later, cells were treated with the indicated concentration of each steroid and cultured for additional 72 h. Cell viability was measured by MTT assay using Cell-Counting kit-8 (Dojindo, Kumamoto, Japan). Absorbance at 490 nm and at 630 nm as a reference was measured with a multi-label counter ARVO MX (Perkin Elmer, Fremont, CA, USA). Each experiment was performed six times.

Dual luciferase assays. Twenty-four hours after plating 3×10^5 cells of 22Rv1, DU-145, and PC-3 or 5×10^5 cells of LNCaP on six-well plates in the media with 10% charcoal/dextran-treated FBS, 22Rv1, DU-145, or PC-3 cells were co-transfected by FuGENE6 (Roche, Basel, Switzerland) with 0.5 μg of pGL3-PSA luciferase reporter plasmid and 0.1 μg of *Renila* luciferase expression plasmid serving as an internal control. As for LNCaP, Lipoectamine 2000 (Invitrogen) was used as a transfection reagent. Human AR expression plasmid pSG5-AR (wild type), pSV-ARmut T877S, or mock vector (provided by Dr. Chang at University of Rochester Medical Center) was co-transfected to PC-3 cells, and 24 h after transfection, cells were treated with the indicated concentration of each steroid. Cells were harvested 48 h after addition of the steroid and lysed by the lysis buffer (Dual-Luciferase Reporter Assay System; Promega, Madison, WI, USA). The luciferase activity was quantified by a luminometer, and the results were normalized by *Renila* luciferase activity. To examine the specificity of AR activation by each steroid, 10 μM of bicalutamide (Sigma-Aldrich) was added 30 min before the cells were treated with each steroid.

Small-interfering RNA (siRNA) to *SRD5A1*. To inhibit *SRD5A1* expression in PC cells efficiently and specifically, we synthesized RNA duplexes corresponding to the target sequence of *SRD5A1* (siSRD5A1: 5′-GAGCCAUUGUGCAGUGUAUTT-3′ and 5′-AUACACUGCACAAUGGCUCTT-3′), as well as control RNA duplex (siEGFP: 5′-GAAGCAGCACGACUUCUUCTT-3′ and 5′-GAAGAAGUCGUGCUGCUUCTT-3′). 1.8×10^6 of 22RV1 cells onto a 10-cm dish were transfected with a final concentration of 100 nmol/L of RNA duplex using Lipofectamin RNAiMAX (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, total RNAs were extracted from the transfected cells to evaluate the knockdown effects by semi-quantitative RT-PCR. The primer sequences were 5′-TTGGCTTGACTCAGGATTTA-3′ and 5′-ATGCTATCACCTCCCCTGTG-3′ for *β-actin* (*ACTB*) as an internal control; 5′- CCTGTTTGTTCTTTGTTGATTGAA-3′ and 5′-CCAGATGAGATGATAAGGCAAAG-3′ for *SRD5A1*; and 5′-TTTAATCAGGCCCTGTCTGC-3′ and 5′-GGGGTATAGAAATGGAATGGAGA-3′ for *SRD5A3*. Forty-eight hours after transfection, MTT assays were performed as described above. For 5α-steroid reductase assay *in vitro*, 48 h after transfection, the cells were treated with 10^{-6} M DOC, and after 1 h of incubation, their conditioned media were collected to measure their 5αDH-DOC levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described below.

***In-vivo* 5α-steroid reductase reaction.** To construct the expression vectors for SRD5A1 and SRD5A3, the entire coding sequences of *SRD5A1* (GenBank accession no. NM_001047) and *SRD5A3* (GenBank accession no. NM_024592) were amplified by PCR using Prime STAR DNA polymerase (Takara, Kyoto, Japan). COS7 cells were transfected with each of HA-tagged expression vectors (SRD5A1, SRD5A3, and mock). The expressions of exogenous SRD5A1 and SRD5A3 were evaluated by western blot analysis using anti-HA tag antibody (Sigma-Aldrich). Forty-eight hours after the transfection, cells were treated with 10^{-6} M DOC, and after 1 h of incubation, the conditioned media were harvested. 5α-Dihydro-deoxycorticosterone (5αDH-DOC) levels were measured by LC-MS/MS described below. To inhibit 5α-steroid reductase activity, we added 1 μM dutasteride (provided by GlaxoSmithKline, Middlesex, UK) to the media 30 min before DOC treatment.

Quantitative analysis of 5αDH-DOC and DHT by LC-MS/MS. 5α-Dihydrotestosterone (DHT) measurement by LC-MS/MS was described previously.[11] One ng of DHT-d3 [17,16,16-$^2H_3$]-DHT and 1 ng of Corticosterone-d8 as an internal standard was added to the individual homogenized cells or the conditioned media, which was extracted with ether. The organic layer was evaporated and the extracts were dissolved in 1 mL of 20% acetonitrile–H_2O in an ultrasonic bath and applied to a 3-mL Bond Elut C18 cartridge column (Varian, Harbor City, CA, USA). These columns were then washed successively with 1 mL water and 3 mL of 30% acetonitrile–H_2O, and the steroidal fraction was eluted with 2.5 mL of 70% acetonitrile–H_2O and dried using a centrifugal evaporator. To increase the sensitivity of MS analysis, the dried steroidal fraction was reacted with 50 μL reagent mixture (50 mg 2-methyl-6-nitrobenzoic anhydride, 20 mg 4-dimethyl-aminopyridine, and 50 mg picolinic acid in 1 mL tetrahydrofuran) and 15 μL triethylamine for 60 min at room temperature. The reaction mixture diluted with 1% acetic acid was applied to a 3-mL Bond Elut C18 cartridge column. The columns were washed with distilled water and 30% acetonitrile–H_2O, and the steroidal fraction was eluted with 3 mL of 70% acetonitrile–H_2O. The collected fraction was evaporated and dissolved in 100 μL of 40% acetonitrile–H_2O. Ten millilitres was applied to the LC-MS/MS instrument: API4000 QTRAP (Applied Biosystems, Foster City, CA, USA) equipped with an ESI ion source and a Shimadzu high-performance liquid chromatography (HPLC) system (Shimazu, Kyoto, Japan). The HPLC column was a Cadenza CD-C18 (150 × 2 mm, inner diameter 3 μm; Imtakt, Kyoto, Japan). The mobile phase consisting of acetonitrile–methanol (50:50 v/v, solvent A) and 0.1% formic acid (solvent B) was

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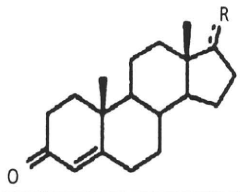
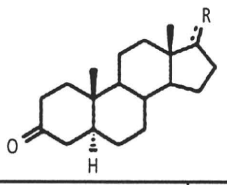
Table 1. Summary of the growth-promoting effects of eight 5αDH steroids and their precursors





4-ene-3-oxosteroid	22Rv1	LNCaP	DU1-45	PC3	5α-DH-3-oxosteroid	22Rv1	LNCaP	DU-145	PC3
Androstenedione					Androstenedione				
Cholestenone					5α-Dihydrocholestanone				
Corticosterone					5α-Dihydrocholestanone				
Cortisone					5α-Dihydrocortisone				
Progesterone					5α-Dihydrocholestanone				
DOC					5α-DihydroDOC				
Cortisol					5α-Dihydrocortisol				
17OH-progesterone					5α-Dihydro-17OH-progesterone				

10^{-10} 10^{-9} 10^{-8} 10^{-7} $>10^{-7}$ or NS

The gradient color in each column indicates the concentration of each steroid which shows a statistically significant growth-promoting effect on prostate cancer cells. White indicates a statistically significant growth-promoting effect at the concentration of 10^{-6} or not significant (NS). DOC, 11-deoxycorticosterone.

used with a gradient elution of A:B = 60:40–100:0) at a flow rate of 0.4 mL/min. The electrospray (ESI)/MS conditions were as follows: spray voltage, 3300V; Collison gas, 1.5 psi (gas pressure) nitrogen; curtain gas nitrogen, 11 psi (gas pressure); ion source temperature, 600°C; and ion polarity, positive. For 5αDH-DOC determination, m/z 333.3 was activated as a precursor ion, and the product m/z 279.2 ions were monitored. The produced ion mass monitored for the internal standards was m/z 125.1. The assay was validated to ensure that the result was within the 20% range of accuracy and precision. The lower limit value for 5αDH-DOC was 1 pg.

Results

5αDH Steroids stimulated PC cell growth. For this analysis, we selected eight 5α-DH-oxosteroids (5αDH steroids) that were reported to be present naturally in the human body (Table 1). To investigate whether they could stimulate PC cell proliferation, we treated four sets of PC cell lines (22Rv1, LNCaP, DU-145, and PC-3) with serial concentration of eight 5α-DH-oxosteroids. Simultaneously, we treated the four PC cell lines with their precursors 4-ene-3-oxosteroids, which were expected to be converted to 5α-DH-oxosteroids by endogenous 5α-steroid reductases in PC cells, and examined their growth-promoting effect in the same way. As shown in Figure 1a,c treatment of 5αDH-DOC and 5αDH-progesterone at 10^{-7} M or lower concentration showed some growth-promoting effect on both 22Rv1 and LNCaP cells which expressed AR, but not on DU-145 and PC-3 cells which did not express AR. Their precursor steroids (DOC and progesterone) also showed significant growth-promoting effect on AR-positive 22Rv1 and LNCaP cells, but not on AR-negative PC-3 and DU-145 cells (Fig. 1b,d). These findings indicate that 5αDH-DOC and 5αDH-progesterone could be good candidates to stimulate PC proliferation through AR activation. On the other hand, corticosterone and cortisol had a positive effect in proliferating cells of AR-negative DU-145, as well as LNCaP or 22RV1 cells (Fig. S1a,c), and this effect on AR-negative DU-145 cells might be mediated by other steroid receptors, rather than AR. Their 5αDH steroids did not show any significant growth-promoting effect on PC cell lines (Fig. S1b,d). These data are summarized in Table 1.

5α-Dihydro-deoxycorticosterone (5αDH-DOC) activated wild-type AR more efficiently than DOC. Focusing on 5αDH-DOC and 5αDH-progesterone, to evaluate their direct ability to stimulate AR transactivation activity, we transfected LNCaP cells and 22Rv1 cells with a luciferase plasmid (pGL3-PSA) driven by the PSA enhancer which included androgen response elements, and compared the luciferase activity reflecting AR transactivation activity by the treatment of these 5αDH-steroids and their putative precursors. LNCaP cells and 22Rv1 cells expressed mutant AR (T877A and H874Y, respectively) and mutant AR has been reported to respond to steroids differently from wild-type AR.[14,15] Then, to evaluate their effects on wild-type AR, we also co-transfected AR-null PC-3 cells with wild-type AR or mutant AR (T877S) and examined the transactivation activity of

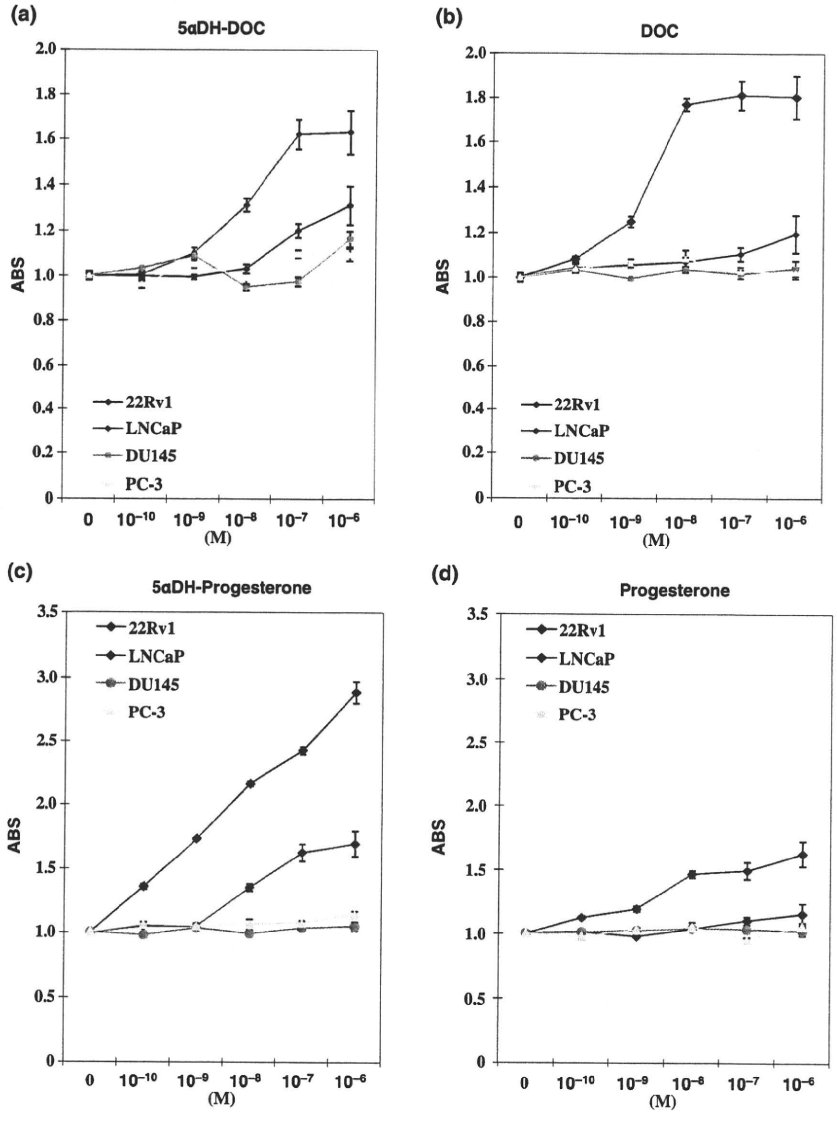


Fig. 1. Cell growth assays of 5αDH steroids and their precursors. 5α-Dihydro-deoxycorticosterone (5αDH-DOC) (a) and 5αDH-progesterone (c) at 10^{-7} M or lower concentration showed growth-promoting effect on both androgen receptor (AR)-positive prostate cancer (PC) cell lines 22Rv1 and LNCaP, but did not on AR-negative PC cells DU-145 and PC-3. (b,d) Their 4-ene-3-oxosteroids (DOC and progesterone) had also some ability to stimulate cell proliferation. Prostate cancer (PC) cells were treated with the indicated concentration of each steroid (*x*-axis, 10^{-10}–10^{-6} M) and 72 h later, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed. *Y*-axis: absorbance (ABS) at 490 nm (MTT assay), and at 630 nm as a reference, measured with a microplate reader. Each assay was tested six times and the means ± SD were plotted.

AR by each of the steroids. As shown in Figure 2(a), both 5αDH-DOC and DOC at 10^{-7} M or lower concentration showed some level of AR transactivation in LNCaP cells (mutant AR: T877A) and 22Rv1 cells (mutant AR: H874Y). 5α-Dihydro-deoxycorticosterone (5αDH-DOC) and DOC treatment showed transactivation of wild-type AR (Fig. 2b, left) and mutant AR (Fig. 2b, right) which was introduced in AR-null PC-3 cells, although they did not show any transactivation activity in mock-transfected PC-3 cells (Fig. 2b, lower). These transactivation activities were blocked by the AR antagonist bicalutamide (BCL), confirming that they were caused via AR, regardless of its mutation status. Interestingly, 5αDH-DOC showed more impact on the transactivation activity of wild-type AR than DOC (Fig. 2b, left), while 5αDH-DOC did not show any advantageous impact on the transactivation activity of mutant AR (T877S) over DOC (Fig. 2b, right). This was consistent with the findings that 5αDH-DOC showed less effect on the transactivation of mutant ARs in LNCaP cells and 22Rv1 cells than DOC. On the other hand, 5αDH-progesterone and other 5α-DH-oxosteroids did not show such a characteristic feature to induce AR transactivation (Fig. 2c,d for 5αDH-progesterone, and data are not shown as for other 5αDH-steorids), except for DHT. These findings suggest that the conversion from DOC to 5αDH-DOC by 5α-steroid reductases could provide some advantageous effects on AR transactivation to PC cells with wild-type AR, as well as the conversion from testosterone to DHT. Most of CRPC cells showed overexpression of wild-type AR, not mutant AR,[13] and these effects of 5αDH-DOC on wild-type AR might be beneficial for CRPC cells. Thus we focused on 5αDH-DOC and DOC in further experiments.

Type 1 5α-steroid reductase (SRD5A1) was responsible for 5αDH-DOC production in PC cells. In PC tissues, especially CRPC tissues, type 1 (SRD5A1) and type 3 (SRD5A3) isozymes are up-regulated and dominant[11,14] and also their expressions were observed in all available PC cell lines (Fig. S2). They are likely to be responsible for DHT and other 5αDH-steroid production in PC tissues. To investigate which of type 1 and/or type 3 is responsible for 5αDH-DOC production in PC cells, COS7 cells were transfected with each of HA-tagged expression vectors (SRD5A1, SRD5A3, and mock, Fig. 3a) and the cells were treated with 10^{-6} M DOC. One hour after incubation under 10^{-6} M DOC, the conditioned media were harvested to measure the amounts of 5αDH-DOC by sensitive LC-MS/MS analysis. As a result, a much higher amount of 5αDH-DOC was observed in the cells that overexpressed SRD5A1 than in the SRD5A3 or mock-transfected (Fig. 3b) cells. The production of 5αDH-DOC

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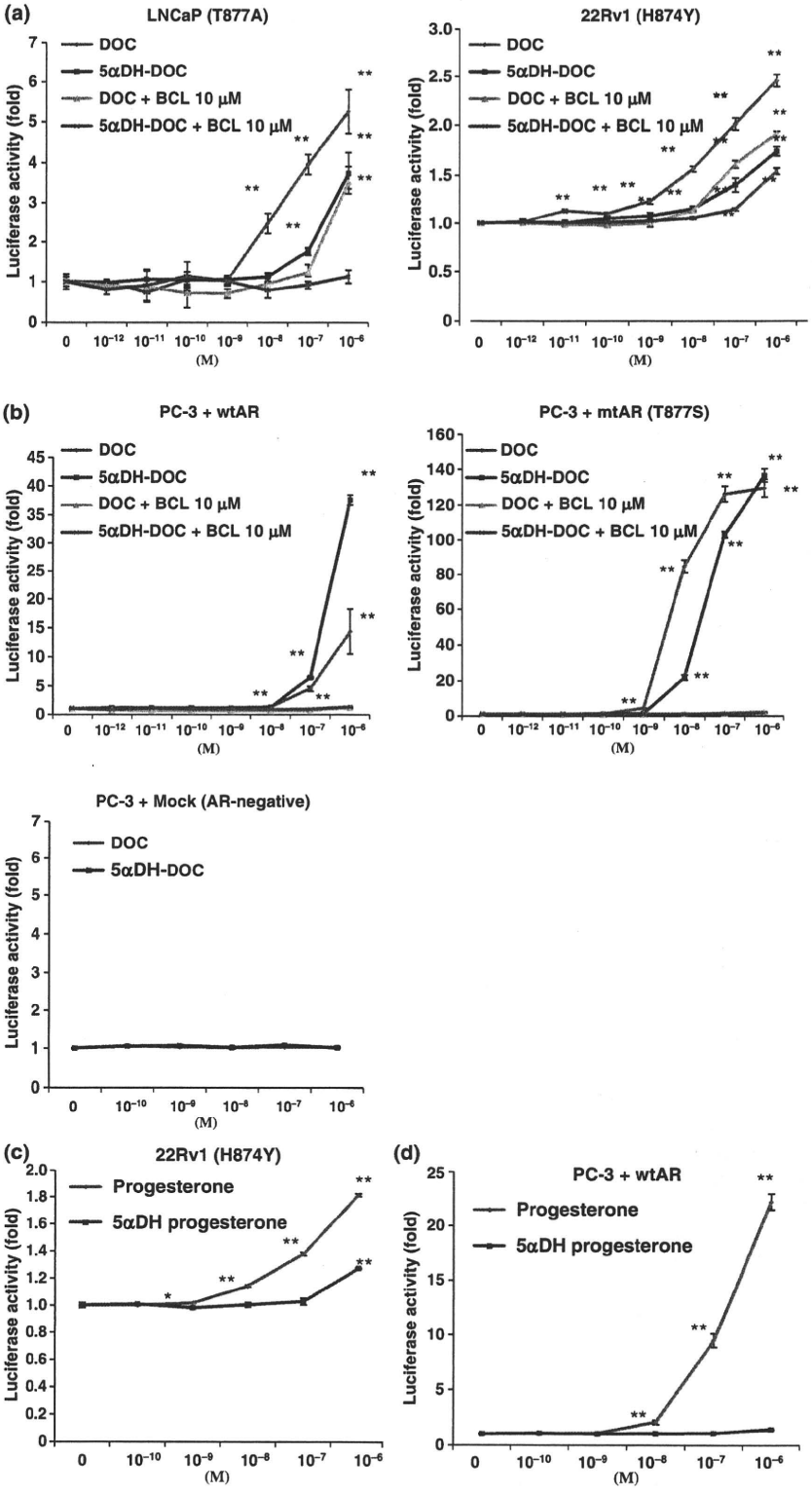

Fig. 2. Luciferase assays of 5αDH steroids and their precursors for androgen receptor (AR) transactivation activity. (a) LNCaP cells and 22Rv1 cells were transfected with pGL3-PSA and the luciferase activity in the presence of indicated concentration (*X*-axis) of 5α-dihydro-deoxycorticosterone (5αDH-DOC) and 11-deoxycorticosterone (DOC) was compared. LNCaP cells and 22Rv1 cells expressed mutant AR, T877A, and H874Y, respectively. (b) Androgen receptor (AR)-null PC-3 cells were co-transfected with wild-type AR (left), mutant AR-T877A (right), or mock (lower) and pGL3-PSA and the luciferase activity in the presence of indicated concentration (*x*-axis) of 5αDH-DOC and DOC was compared. These luciferase activities were blocked by antiandrogen bicalutamide (BCL). Each assay was tested six times and the means ± SD were plotted. $*P < 0.05$, $**P < 0.01$ by Student's *t*-test. (c) 22Rv1 cells were transfected with pGL3-PSA and the luciferase activity in the presence of indicated concentration (*x*-axis) of 5αDH-progesterone and progesterone was compared. (d) Androgen receptor (AR)-null PC-3 cells were co-transfected with wild-type AR and pGL3-PSA and the luciferase activity in the presence of indicated concentration (*x*-axis) of 5αDH-progesterone and progesterone was compared. Each assay was tested six times and the means ± SD were plotted. $*P < 0.05$, $**P < 0.01$ by Student's *t*-test.

was inhibited by pre-treatment of 1 μM dutasteride which could inhibit the activity of SRD5A1 (Fig. 3c). These findings indicated that SRD5A1 could be responsible for 5αDH-DOC production *in vitro*.

Subsequently, to evaluate the endogenous activity of SRD5A1 for 5αDH-DOC, 22Rv1 cells were treated with siRNA duplex specific to *SRD5A1* (siSRD5A1) or the control siRNA duplex (siEGFP). Reverse transcription (RT)-PCR validated the knockdown effect by siSRD5A1 (Fig. 4a), and siSRD5A1 treatment suppressed the cell viability of 22Rv1 cells, compared with the control siEGFP (Fig. 4b, $P < 0.01$). The amount of 5αDH-DOC production was also significantly decreased in the conditioned media of 22Rv1 of which *SRD5A1* was knocked down (Fig. 4c, $P < 0.01$). These findings suggest that SRD5A1 is likely to play some important roles in converting DOC to 5αDH-DOC in PC cells, as well as PC cell viability.

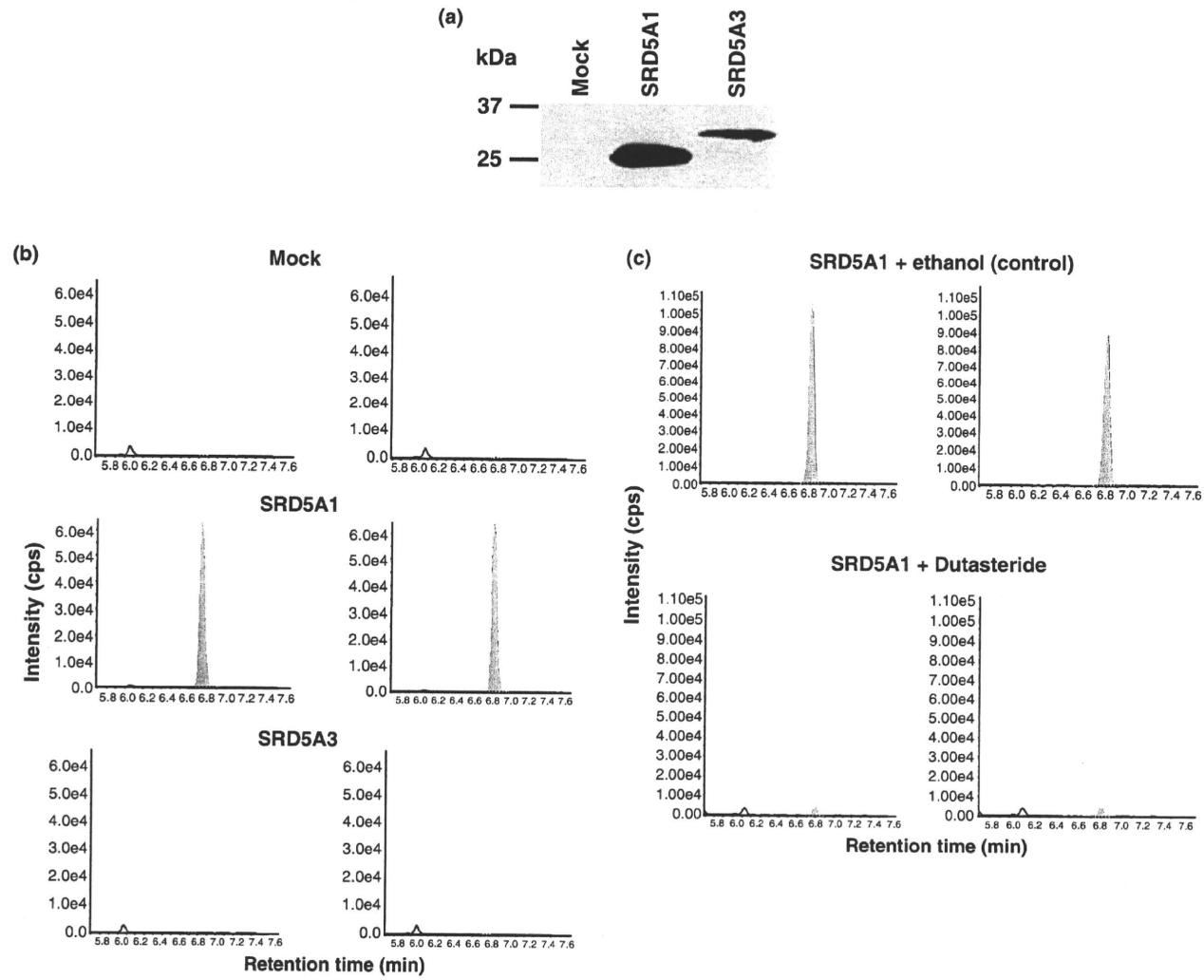

Fig. 3. Type 1 5α-steroid reductase (SRD5A1) was responsible for the production of 5α-dihydro-deoxycorticosterone (5αDH-DOC). (a) COS7 cells were transfected with SRD5A1, SRD5A3, or mock vector and the expression of exogenous SRD5A1 and SRD5A3 were evaluated by western blot analysis using anti-HA tag antibody. (b) The transfected cells were treated with 10^{-6} M 11-deoxycorticosterone (DOC), and after 1 h of incubation, the conditioned media were harvested. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the media specifically detected the production of 5αDH-DOC which was converted from DOC in COS7 cells overexpressing SRD5A1 (middle panel), but not in COS7 cells overexpressing SRD5A3 (lower panel) or mock cells (upper panel). These experiments were performed in duplicate (right and left panels). (c) 1-μM Dutasteride treatment inhibited 5αDH-DOC production in COS7 cells transfected with SRD5A1 vector. 5α-Dihydro-deoxycorticosterone (5αDH-DOC)was detected by LC-MS/MS and these experiments were performed in duplicate (right and left panels).

5α-Dihydro-deoxycorticosterone (5αDH-DOC) was detected in clinical CRPC tissues by LC-MS/MS. To prove the presence of 5αDH-DOC in clinical CRPC tissues, we extracted steroid fractions from 13 fresh frozen CRPC tissues and measured the intratumoral 5αDH-DOC and DHT levels by the sensitive LC-MS/MS analysis we established here. As expected, the intratumoral DHT level in all CRPC samples was below 1000 pg/g tissues, and 5αDH-DOC was detected in eight out of 13 CRPC tissues (Fig. 4d). We did not find any clinical features of these 5αDH-DOC-positive CRPCs. Interestingly, 5αDH-DOC-positive CRPCs had a comparatively lower level of DHT, and the level of 5αDH-DOC was significantly inversely correlated to the DHT level within CRPC tissues (Pearson $r = -0.5727$, $P < 0.05$).

Discussion

Several molecular events that drive the progression to the castration-resistant state have been proposed and extensively reviewed.[5,6,15–17] One of the key and main mechanisms is overexpression of wild-type AR with or without amplification of the *AR* gene, which is commonly observed in clinical CRPCs.[6,12,18,19] On the other hand, AR mutations allow AR activation by low androgen levels or by other endogenous steroids such as corticosteroids and antiandrogens,[20–23] but the incidence of AR mutation is <20% in clinical CRPCs.[13] In this study, we searched for novel 5αDH steroids that could have any potential to stimulate PC cell growth as well as AR activity, and among them, we here focused on 5αDH-DOC and demonstrated that it could stimulate wild-type AR preferentially rather than mutant AR, and type 1 5α-steroid reductase overexpressed in CRPC cells could be responsible for 5αDH-DOC production. These findings indicate that 5αDH-DOC and other unknown products of 5α-steroid reductases (type 1 and type 3) could activate AR under extremely low levels of DHT in some CRPC cases and provide some survival advantages to PC cells, although the concentration of 5αDH-DOC we here showed was relatively lower than that of DHT. *In situ* steroidogenesis

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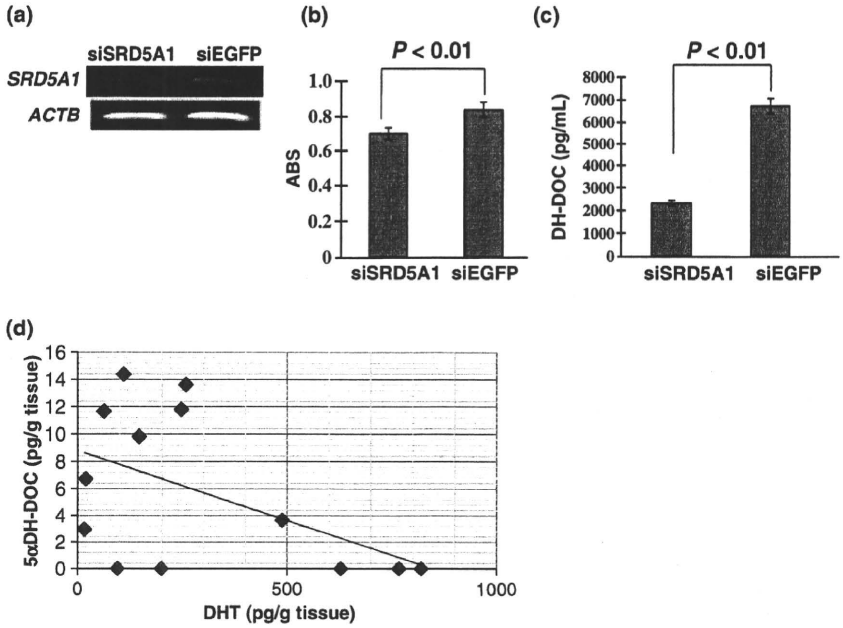

Fig. 4. (a) Reverse transcription (RT)-PCR confirmed knockdown effect on type 1 5α-steroid reductase (*SRD5A1*) expression by siSRD5A1 in 22Rv1 cells. β-Actin (*ACTB*) was used to quantify the input RNAs. (b) Knockdown of *SRD5A1* expression by siSRD5A1 suppressed the proliferation of 22Rv1 cells, compared with the control RNA duplex siEGFP ($P < 0.01$, Student's *t*-test). *Y*-axis, absorbance (ABS) at 490 nm (MTT assay), and at 630 nm as a reference, measured with a microplate reader. (c) Suppression of *SRD5A1* expression by siSRD5A1 reduced 5α-dihydro-deoxycorticosterone (5αDH-DOC) production in 22Rv1 cells ($P < 0.01$, Student's *t*-test). 5α-Dihydro-deoxycorticosterone (5αDH-DOC) in the media was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. (d) Detection of 5αDH-DOC and DHT in 13 clinical CRPC tissues. 5α-Dihydro-deoxycorticosterone (5αDH-DOC) was detected by sensitive LC-MS/MS in clinical CRPC tissues. 5α-Dihydrotestosterone (DHT) was also measured by sensitive LC-MS/MS in the same tissues, and the level of 5αDH-DOC was inversely correlated with the low level of DHT concentration (Pearson $r = -0.5727$, $P < 0.05$).

including this 5α-steroid reduction is likely to be one of the key mechanisms of CRPC development and a good molecular target for CRPC treatment.

In the standard steroidogenesis pathway, the testes provide the major source of androgens, particularly testosterone. Alternatively, prostate cells can convert adrenal-derived steroids, such as androstenediol and DHEA (dehydroepiandrosterone), to testosterone by 17β-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase.[17,24] Currently, there is increasing evidence that PC cells can synthesize androgens *de novo* instead of receiving them through the bloodstream.[17,25] This concept is supported by the fact that expressions of several enzymes responsible for steroid synthesis were up-regulated in CRPCs[26–28] and they are likely to play some critical roles in the CRPC phenotype. Among them, cytochrome P17 (CYP17) catalyzes the key reactions to androgen and estrogen biosynthesis, and its selective inhibitor, abiraterone, is now in clinical trials for CRPCs, showing significant antitumor activity in CRPCs,[29] indicating that CRPCs remain dependent on ligand-activated AR signaling, and also these findings suggest that such steroids or androgen metabolism enzymes could be potential molecular therapeutic targets for CRPCs. Among several steroid metabolism enzymes altered in CRPCs, 5α-steroid reductases mostly contribute to both the standard steroidogenesis pathway (testosterone to DHT), and the alternative steroidogenesis pathway which is likely to be activated when testosterone are not available from the blood circulation.[16,24] In addition to DHT, other various types of steroids can be subject to the reduction of 5α-steroid reductases to acquire more potentials as functional steroids or modify/change their functions.[30] Neuroendocrine cells in the brain highly express type 1 5α-steroid reductase (SRD5A1) and produce several neurosteroids such as 5αDH-progesterone, allopregnolone (ALLO), and tetrahydrodeoxycorticosterone (THDOC), which modulate GABA action at $GAGA_A$ receptors to contribute to neuroendocrine transmission.[31] In this point, 5αDH-DOC production by SRD5A1 in CRPCs may reflect the neuroendocrine-like phenotype of CRPCs,[32,33] and in addition to AR activation, it may regulate or stimulate other signaling pathways similar to how other 5αDH neurosteroids to contribute to the CRPC phenotype.

The source of DHT in prostatic tissue after ADT is likely to be intracrine production within the prostate, which converts adrenal androgens to DHT.[9] Interestingly, we observed that the level of 5αDH-DOC was significantly inversely correlated to the DHT level within CRPC tissues, although the concentration of 5αDH-DOC was quite lower than that of DHT. This might implicate that when DHT levels were declined within CRPC tissues, there might still be compensatory increases in 5α-DH-DOC and other unknown 5αDH steroids to activate AR by taking advantage of overexpressing SRD5A1 or SRD5A3. There is no evidence obtained so far by clinical trials to support the sole treatment with dutastride; dual inhibitor of type 1 and 2 5α-steroid reductases could effectively suppress CRPC growth, although the DHT level within PC tissues was not assessed.[34,35] Several other ligands can activate the AR pathway or other pathways to support the survival of PC cells under the castrated and 5α-steroid reductase-free environment, and the combination hormonal or AR-targeting therapy with dutastride might be required to deplete intratumoral active steroids in CRPCs and suppress CRPC growth effectively.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Cell growth assays of 5αDH steroids and their precursors. Corticosterone (a) and cortisol (c) had positive effect on the proliferation of androgen receptor (AR)-negative DU-145 cells, as well as LNCaP or 22RV1 cells. 5αDH-Corticosterone (b) and 5αDH-cortisol (d) did not show any significant growth-promoting effect on prostate cancer (PC) cell lines. Prostate cancer (PC) cells were treated with indicated concentration of each steroid (*x*-axis, 10^{-10}–10^{-6} M) and 72 h later, MTT assay was performed. *Y*-axis: absorbance (ABS) at 490 nm (MTT assay), and at 630 nm as a reference, measured with a microplate reader. Each assay was tested six times and the means ± SD. were plotted.

Fig. S2. Semi-quantitative RT-PCR showed that all of the prostate cancer (PC) cell lines we used expressed type 3 5α-steroid reductase (SRD5A1) and *SRD5A1*. Expression of β-actin (*ACTB*) served as the quantitative control.

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Original Article: Clinical Investigation

Community-based prostate cancer screening in Japan: Predicting factors for positive repeat biopsy

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Objectives: To assess possible predictors in determining criteria for repeat biopsy in a prostate cancer screening population.

Methods: A total of 50 207 men over 55 years-of-age have participated in a prostate cancer screening program in Otokuni, Kyoto, Japan for 12 years. Transperineal systematic biopsy was carried out in case of positive digital rectal examination (DRE) or positive transrectal ultrasonography (TRUS) or a prostate-specific antigen (PSA) value greater than 10.0 ng/mL. For those with a PSA level from 4.1 to 10.0 ng/mL, and negative DRE and TRUS findings, biopsy was indicated only when PSA density (PSAD) was greater than 0.15. The same indication was applied for the repeat biopsy.

Results: A repeat biopsy after an interval of more than 2 years was carried out in 140 patients and was positive in 50 (36%) patients. The PSA value at the diagnosis of cancer declined from the initial value in six (12%) patients. On multivariate logistic regression analysis, PSA velocity (PSAV) as well as PSAD and DRE findings at latest screening were independent predictors for positive repeat-biopsy outcome. The odds ratio (95% confidence intervals) of PSAV >0.48, latest PSAD >0.33 and positive latest DRE were 4.17 (1.05–18.5), 4.15 (1.31–14.0), and 3.62 (1.06–13.2), respectively. A combination of three variables defined as positive if any of these were positive, reduced 31% of unnecessary biopsies while missing 8% of low volume, low grade cancers.

Conclusions: A combination of latest PSAD, PSAV and positive DRE at latest screening might help to reduce unnecessary repeat biopsies in high-risk patients with an initial negative biopsy.

Key words: prostate cancer screening, prostate-specific antigen density, prostate-specific antigen velocity, repeat biopsy.

Introduction

Although prostate cancer is one of the most frequent cancers and accounts for approximately 20% of cancer mortalities for men living in Western countries, it is the fifth leading incidence of male neoplasm and accounts for just 4.5% of cancer mortalities in Japanese males.[1] However, with rapid population aging and Westernization of the diet, the prostate cancer mortality rate has increased in Japan, and it is expected to increase by 2.8-fold in 2020 compared with 2000.[1] The number of patients with prostate cancer is also expected to become the second most frequent cancer in Japanese males, counting about 80 000 in 2020.[1]

The rapid increase in the number of patients with prostate cancer is partly as a result of the marked rise in the implementation of a prostate cancer screening program by the local government. According to a survey by the Japanese Foundation for Prostate Research, the prostate cancer screening program was provided for approximately 14% of districts in 2000, but this percentage increased to 71% in a similar survey after 6 years. With this rapid spread of prostate-specific antigen (PSA)-based screening and the increase in the number of screened patients, a necessity arose to establish an appropriate quality control scheme for the follow-up of screened patients with a negative initial prostate biopsy.

There have recently been a number of reports on predictors for a positive repeat prostate biopsy in patients on an outpatient-referral basis. On prostate mass screening, unlike outpatient referral basis, a clarification to select screened patients for repeat biopsy needs to be balanced by cost and survival benefit. For this purpose, a validation of the screening system and its modification, if necessary, are extremely important.

We have carried out primary prostate cancer screening based on the PSA level in fixed local government since 1995. The objective of the present study was to extract predictors of positive repeat biopsy in a screening population and to obtain future guidance for our screening system.

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Methods

Screening system

Prostate screening is carried out annually in the Otokuni District, which is located to the south of Kyoto City and consists of two cities and one town (Nagaokakyo City, Muko City and Oyamazaki Town). According to a census, the total population of the Otokuni District as of 1 October 2005 was 148 567, of whom 72 222 were males, and 23 114 (32%) of the males were aged 55 years and above.

The subjects of the present study were males aged 55 years or more who desired screening for prostate cancer who had undergone general health screening in September to October each year since 1995. Primary prostate cancer screening used the examination of serum PSA alone with a cut-off level of 4.0 ng/mL. The serum PSA level was determined using Delfia PSA assay kit in all subjects. The health administrative organization or private medical facilities in the Otokuni District advised the screened patients with a serum PSA level of 4.1 ng/mL or above to undergo secondary screening at a core hospital (Kyoto Saiseikai Hospital). The second screening, including prostate biopsy, was carried out by urologists in Kyoto Saiseikai Hospital under the health insurance system. In the secondary screening, digital rectal examination (DRE) was carried out by one urologist (K.K.) who was a voting member of the Japanese Urological Association. Transrectal ultrasonography (TRUS) of the prostate was carried out using an ultrasound machine equipped with a chair-type scanner (SSD-520, Aloca, Tokyo, Japan), and prostate volume (PV) was obtained by the step-sectioned method. For screened patients with a PSA level between 4.1 and 10.0 ng/mL, PSA density (PSAD)[2] was calculated as PSA divided by PV.

When screened patients had positive DRE or positive TRUS or a PSA value greater than 10.0 ng/mL, prostate biopsy was routinely carried out. For a screened patient who had a PSA level from 4.1 to 10.0 ng/mL, and negative DRE and TRUS findings, a biopsy was indicated only when his PSAD was greater than 0.15. For screened patients with a negative DRE, negative TRUS and PSAD equal to or less than 0.15, consecutive annual prostate screening participation was recommended. The same indication was applied for the repeat biopsy.

For those indicated for prostate biopsy, transperineal prostate biopsy was carried out under local anesthesia. Systematic sextant biopsy technique was applied between 1995 and 2001. Since 2002, an additional sample has been taken from the far lateral region in each lobe (systematic octant biopsy).

Because this screening system was established in 1995, free to total PSA ratio and high-grade prostatic intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP) on the initial biopsy were not considered to indicate repeat biopsy for patients with a negative initial biopsy. Consequently, age and PV at initial biopsy, DRE findings, TRUS findings, serum PSA levels, and PSAD values at the initial and latest biopsies, as well as the time interval and PSA velocity (PSAV) between the initial and latest biopsies were evaluated as predictors for positive repeat biopsy.

Calculation of PSAV

Similar to the definition by Etzioni *et al.*,[3] PSAV was defined as an absolute increase in PSA level per year from the initial biopsy to the latest biopsy. Therefore, if a patient underwent biopsies three times or more, and cancer was detected at the latest biopsy, PSAV was calculated using PSA values at the initial and latest biopsies. Additionally, if the PSA level at the latest measurement was lower than the initial one, PSAV was recorded as a negative value.

Statistical analysis

Analyses were carried out with JMP 8 software (SAS Institute, Cary, NC, USA). We used Wilcoxon rank-sum test to compare a continuous variable within two populations. For categorical parameters, Fischer's exact test was applied. $P < 0.05$ was considered to indicate a significant difference. Receiver operating characteristic (ROC) curves and the corresponding areas under the ROC curves (AUC) were obtained to predict the positive repeat biopsy. The threshold value for optimal sensitivity was defined using Youden index (sensitivity + specificity − 1). Uni- and multivariate analysis was carried out using logistic regression model and odds ratios (OR) and 95% confidence intervals (CI) were given for selected variables.

Results

A total of 50 207 people attended primary PSA screening during 12 years between 1995 and 2006. As previously described, the estimated exposure rate for PSA screening was 65%.[4] Of those, the serum PSA concentration was 4.1 ng/mL or above in 3212 (6%) of all screened patients. Of the screened patients with elevated PSA levels, 2176 (68%) underwent secondary screening, and prostate cancer was finally detected in 383 (18%). Of the 2176 screened patients with elevated PSA levels, 1905 men (88%) visited a core hospital (Kyoto Saiseikai Hospital) to receive secondary screening. After DRE and TRUS, a total of 836 men (1136 times) underwent a prostate biopsy over a period of 12 years, and 340 men (41%) were finally diagnosed with prostate cancer at the hospital (Table 1).

Of the 836 men who underwent a prostate biopsy at the hospital, repeat biopsies were carried out in 179 men (21%). Of these, 72 (39%) were finally diagnosed with prostate cancer. In the present study, we enrolled 140 of the 179 men (78%) with a biopsy interval greater than or equal to 2 years

Table 1 Number of biopsies and cancer detection rate

Number of biopsies (times)	PC	Non-PC	Total (screened patients)	Total number of biopsies (times)
1	268	389	657	657
2	53	58	111	222
3	11	26	37	111
4	6	11	17	68
5	1	8	9	45
6	1	2	3	18
7	0	1	1	7
8	0	1	1	8
Total number of screened patients	340	496	836	1136
Number of screened patients who underwent biopsy two or more times	72	107	179	479
Number of screened patients who underwent biopsies at an interval of 2 years or longer	50	90	140	401

PC, prostate cancer.

Table 2 Clinical parameters of positive and negative biopsy groups whose biopsy interval was longer or equal to 2 years

	Cancer	Non-cancer	*P*-value	statistics
No. patients	50	90		
Mean ± SD age (years)	73.8 ± 5.6	72.8 ± 6.4	0.36	Wilcoxon rank-sum test
Mean ± SD initial PSA (ng/mL)	6.8 ± 3.2	7.0 ± 3.9	0.82	Wilcoxon rank-sum test
Mean ± SD latest PSA (ng/mL)	15.1 ± 19.5	10.2 ± 6.9	0.034	Wilcoxon rank-sum test
Mean ± SD PSAV (ng/mL/year)	1.92 ± 3.1	0.82 ± 1.5	0.0011	Wilcoxon rank-sum test
Mean ± SD initial PSAD (ng/mL/cc)	0.30 ± 0.20	0.22 ± 0.08	<0.0001	Wilcoxon rank-sum test
Mean ± SD latest PSAD (ng/mL/cc)	0.55 ± 0.51	0.27 ± 0.21	<0.0001	Wilcoxon rank-sum test
Mean ± SD time interval (years)	3.9 ± 2.1	4.3 ± 2.2	0.23	Wilcoxon rank-sum test
Mean ± SD prostate volume (cc)	28.6 ± 12.6	41.0 ± 20.9	<0.0001	Wilcoxon rank-sum test
No. initial DRE positive (%)	14 (28%)	11 (12%)	0.023	Fischer's exact test
No. latest DRE positive (%)	16 (32%)	9 (10%)	0.0022	Fischer's exact test
No. initial TRUS positive (%)	17 (34%)	23 (26%)	0.33	Fischer's exact test
No. latest TRUS positive (%)	14 (28%)	9 (10%)	0.0085	Fischer's exact test

DRE, digital rectal examination; PSA, prostate-specific antigen; PSAD, prostate-specific antigen density; PSAV, prostate-specific antigen velocity; TRUS, transrectal ultrasonography.

in order to exclude patients with a negative initial biopsy by sampling error. Of the 140 men, 50 (36%) were finally diagnosed with prostate cancer (Table 1). In the light of the consecutive change in PSA values in the 140 cases, the latest PSA value increased or decreased from the initial PSA value in 118 (84%) and 22 (16%), respectively. Among patients with decreased PSA from the initial value, cancer was detected in 6 (12%) out of 50 cases.

Table 2 compares the clinical parameters between patients with positive and negative biopsies. Of the variables evaluated, significant differences were observed in PV at initial biopsy, DRE findings and PSAD values at the initial and latest biopsies, TRUS findings and serum PSA levels at latest biopsies as well as PSAV between the initial and latest biopsies.

Uni- and multivariate analyses to predict the positive biopsy outcome are shown in Table 3. On univariate analysis, a positive biopsy was significantly related to latest PSA >7.4, initial PSAD >0.23, latest PSAD >0.33, PV <28.2 mL, PSAV > 0.48, increased PSAD, and positive latest DRE and TRUS findings. On multivariate analysis, a positive biopsy was significantly related to latest PSAD >0.33, PSAV >0.48 and positive latest DRE. The odds ratio (95% CI) with a threshold of PSAV >0.48 was the highest (4.17: 95% CI

Table 3 Uni and multivariate logistic regression analysis to predict positive biopsy outcome in men undergoing repeat biopsy

Variables	Univariate *P*-value	Optimal cut-off value	AUC	Multivariate *P*-value	Odds ratio	95% Confidence intervals
Age	0.361	66	0.54689			
Initial PSA	0.7336	6.8	0.51044			
Latest PSA	0.0235	7.4	0.60867	0.5246	1.53	0.41, 5.95
Initial PSAD	0.0001	0.23	0.70722	0.0673	2.5	0.94, 6.80
Latest PSAD	<0.0001	0.33	0.78133	0.0154	4.15	1.31, 14.0
Interval (years)	0.1937	3	0.5633			
Prostate Volume	<0.0001	28.2	0.72322	0.1289	2.23	0.79, 6.34
PSAV	0.004	0.48	0.66611	0.0417	4.17	1.05, 18.5
Latest DRE	0.0014			0.0397	3.62	1.06, 13.2
Latest TRUS	0.007			0.4523	1.61	0.47, 5.90
Increased PSA	0.3598					
Increased PSAD	0.0028			0.7788	1.19	0.35, 4.08

AUC, area under receiver operating characteristics curve; DRE, digital rectal examination; PSA, prostate-specific antigen; PSAD, prostate-specific antigen density; PSAV, prostate-specific antigen velocity; TRUS, transrectal ultrasonography.

Table 4 Diagnostic performance for prostate-specific antigen velocity >0.48, latest prostate-specific antigen density >0.33, positive latest digital rectal examination, and combination of the three variables to predict positive repeat biopsy

Parameters	Sensitivity	Specificity	PPV	NPV	No. Bx saved	No. Ca missed
PSAV >0.48	40/50, 80%	46/90, 51%	40/84, 48%	46/56, 82%	56/140, 40%	10/50, 20%
Latest PSAD >0.33	34/50, 68%	75/90, 83%	34/49, 69%	75/91, 82%	91/140, 65%	16/50, 32%
Latest DRE (+)	16/50, 32%	81/90, 90%	16/25, 64%	81/115, 70%	115/140, 82%	34/50, 68%
Combination (+)	46/50, 92%	39/90, 43%	46/97, 47%	39/43, 91%	43/140, 31%	4/50, 8%
PSA increase (+)	44/50, 88%	16/90, 18%	44/118, 37%	16/22, 73%	22/140, 16%	6/50, 12%
PSAD increase (+)	41/50, 82%	38/90, 42%	41/93, 44%	38/47, 81%	47/140, 34%	9/50, 18%

Bx, biopsies; Ca, cancers; DRE, digital rectal examination; NPV, negative predictive value; PPV, positive predictive value; PSA, prostate-specific antigen; PSAD, prostate-specific antigen density; PSAV, prostate-specific antigen velocity.

1.05–18.5), followed by 4.15 (1.31–14.0) with a threshold of PSAD >0.33 and 3.62 (1.06–13.2) with a positive latest DRE.

Consequently, these three significant variables to predict positive repeat biopsies were evaluated by using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), number of biopsies saved and number of cancers missed (Table 4). A combination of three variables was defined as positive if any of them were positive. As the result, a combination of the variables displayed 92% sensitivity while saving 31% of biopsies. Among four patients missed by the combination variable, three underwent radical prostatectomy and showed low grade and small (<1 cc) focus of organ confined cancers. Although the other patient did not undergo prostatectomy because of his advanced age, the biopsy result showed one positive core out of six for low grade disease. In contrast, simple use of PSA increase and PSAD increase missed six (12%) and nine (18%) cancers, respectively, including high grade and high volume cancers.

Discussion

For the quality control of the prostate cancer screening system that has been developed over many years, the following are thought to be important: (i) the determination of the screening interval based on the baseline PSA values at primary screening; (ii) validity of the use of PSA-related indices to enhance the diagnostic accuracy; and (iii) appropriate continual guidance to undergo screening for generally asymptomatic patients who showed negative results on the initial secondary screening (including prostate biopsy). The Japanese Urological Association Guidelines on PSA-Based Screening for Prostate Cancer was published in 2008,[1] which recommended the use of 4.0 ng/mL as a cut-off PSA

Table 5 Reports concerning the significant parameters used for the prediction of prostate cancer by repeat biopsy

Authors	Screening or referral basis	PSA range at the repeat biopsy	Indication for the repeat biopsy	Biopsy methods at the repeat biopsy	Detection rate	Variable predictor
Keetch *et al.* (*J Urol* 1996)[6]	R	More than 4.0 ng./mL	Persistently elevated serum PSA (more than 4.0 ng./mL.), and positive DRE and/or TRUS	from 4 to 6 cores	25% (81/327)	PSAD and PSA slope
Morgan *et al.* (*Prostate* 1996)[7]	R	Mean 9.6 ng/mL; range 4.1–24.8 ng/mL	Positive DRE and/or an elevated PSA level	8 cores	16% (11/67)	F/T PSA (<10%)
Djavan *et al.* (*J Urol* 2000)[8]	R	From 4 to 10	Mandatory	8 cores	10% (83/820)	F/T PSA and PSATZ
Borboroglu *et al.* (*J Urol* 2001)[9]	R	NA	HG-PIN and ASAP	6 cores, or an extended biopsy technique	47% (47/100)	HG-PIN and ASAP
Lopez-Corona *et al.* (*J Urol* 2003)[10]	R	Varied	Physician's decision	from 6 to 22 cores	19.5% (67/343) at 2nd Bx	Nomogram
Zackrisson *et al.* (*Eur Urol* 2003)[11]	S	More than or equal to 3	PSA elevated	6 cores	26% (84/456)	Persistently elevated PSA and small PV (<20)
Park *et al.* (*Int J Urol* 2003)[12]	R	7.6 ± 9.5	Persistent elevated PSA, increased PSAV, positive DRE or TRUS, and/or previous suspicious biopsy findings such as prostatic intraepithelial neoplasia (PIN).	from 6 to 12 cores	21.2% (22/104)	PSA and PSAD, PSAV, DRE and TRUS findings
Singh *et al.* (*J Urol* 2004)[13]	R	6.6 (4.9–9.4)	Percent of PSA 15.0 or less with total PSA persistently greater than 3.0 ng/mL and/or PSAV 0.75 ng/mL yearly or greater.	12 cores	21.2% (21/99)	HGPIN, advanced age and PSATZ
Garzotto *et al.* (*Cancer* 2005)[14]	R	NA	NA	minimum of 6 cores	28.9% (107/373)	PSADT, PSAD >0.25, age, elevated PSA, DRE, HGPIN, Positive family history
Yanke *et al.* (*J Urol* 2005)[15]	R	0.28–123	Persistently increased PSA or positive DRE, and PSA slope greater than 0.75 ng/mL yearly, including HGPIN and/or ASAP on a previous biopsy,	from 6 to 12 cores	34% (78/230)	Nomogram (Age, DRE, No. neg cores, HGPIN, ASAP, PSA, PSA slope, family history, interval)
Walz *et al.* (*Eur Urol* 2006)[16]	R	3.3–125	High PSA level (10 ng/mL) Significant rising PSA level (0.75 ng/mL) ASAP in previous biopsy HGPIN in previous biopsy	at least 18 cores	41% (66/161)	Nomogram (age, PSA, F/T PSA, PSAD, PSATZ, PV, TZV, No. prev. Bx, No. neg cores)
Chun *et al.* (*Eur Urol* 2007)[17]	R	NA	NA	10 or more cores	40.6% (1176/2900)	Nomogram (Age, PSA, DRE, F/T PSA, No. prev. Bx, PV)
Yuasa *et al.* (*BMJ Urol* 2008)[18]	R	NA	NA	from 6 to 10 cores	18% (23/127)	PSAV and PSAD
Present study (2009)	S	More than 4.0 ng./mL	Positive DRE and/or TRUS, PSA >10.1, PSAD >0.15 and PSA from 4.1 to 10.0	8 cores	39% (72/179)	PSAV, DRE and PSAD

ASAP, atypical small acinar proliferation; Bx, biopsy; DRE, digital rectal examination; F/T, free to total PSA ratio; HGPIN; high-grade prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; PSAD, prostate-specific antigen density; PSATZ, prostate-specific antigen density of the transition zone; PSAV, prostate-specific antigen velocity; PV, prostate volume; TRUS, transrectal ultrasonography.

level or age-specific PSA criterion for primary screening. For the screening system in the Otokuni District, the cut-off PSA level was set at 4.0 ng/mL, conforming to the recommendation of the guidelines. However, the guidelines failed to show the validity of PSA-related indices to select candidates for repeat biopsy because of the insufficient evidence, even in Western countries.[5] Akakura[6] discussed the current status regarding the follow-up of screened patients with negative biopsy results in Japan. Unfortunately, repeat biopsy strategy in the screening basis is left to the discretion of each screening facility. The establishment of original guidelines in Japan is necessary to improve the quality control of prostate cancer screening.

A feature of our screening system is that the indication of biopsy is set according to PSAD along with DRE and TRUS findings. When this screening system was implemented in 1995, PSAD was reported to be an effective PSA-related index to avoid unnecessary biopsies, and therefore, was adopted to reduce the excessive number of screened patients indicated for biopsy without a sufficient number of prostate pathologists operating in the district.

After 12 years from the beginning of screening, an adoption of PSAD as a screening criterion had been validated in screened patients without indication for biopsy. Our previous results showed that needle biopsy was avoided as a result of a low PSAD in 32% (289/894) of the screened patients, and localized prostate cancer was detected in 23 of them by subsequent biopsy.[4] The use of PSAD as a criterion contributed to avoiding a substantial number of biopsies, and the diagnosis of cancer could be made in a curable stage for all patients exempt from biopsy. Therefore, the use of PSAD to select patients for initial biopsy appeared to be useful as an intervention factor for the quality control of screening.

Table 5 shows factors reported to be predictive for positive repeat biopsy on a screening or outpatient-referral basis.[7–19] Most of the reports involved biopsies carried out on an outpatient-referral basis, and recent reports have recommended the use of various nomograms.[16–18] Although these nomograms would give a percentage of the chance of positive repeat biopsy, they should be useful only for counseling patients on whether they need repeat biopsy or not. Our screening system requires a definite criterion to select patients for repeat biopsy with acceptable sensitivity and specificity. For this sense, our newly proposed criteria will provide a guide to selecting patients for repeat biopsy, maintaining a reasonable sensitivity while preventing a considerable number of unnecessary biopsies.

As for a screening population, only a few studies have reported predictors for a positive repeat prostate biopsy. Zackrisson *et al.*[12] reported that persistently elevated PSA and low PV are useful predictors for positive repeat biopsy. In some reports, PSA kinetics, including the PSAV, PSA slope, and PSA doubling time (PSADT), were suggested to be useful predictors. The result in the current study showing PSAD, PSAV and DRE as independent predictors for positive repeat biopsy corresponds to previous reports with regard to an importance of prostate volume-related indices and PSA kinetics. We did not include PSADT as a predictor, because PSADT is calculated only for patients with increased PSA. Furthermore, we found six (12%) cancers missed in patients with decreased PSA. The use of DRE is generally limited by various quality levels of the examiner; however, the present study involved only one examiner for DRE and therefore, the latest DRE appeared to be one of the significant predictors.

Several limitations might apply to the present study. We did not account for ASAP or HGPIN at initial biopsy as well as free to total PSA ratio, which have been reported to be risk factors for cancer on repeat biopsy. Thus, they should be included in future studies. The present study included a relatively small number of screened patients who underwent repeat biopsies and our predictions are not perfect. Nevertheless, a combination of latest DRE findings, latest PSAD >0.33 and PSAV >0.48 could reduce 31% of unnecessary biopsies, while missing 8% of low volume, low grade cancers and, therefore, seems to be applicable to selecting candidates for repeat biopsy in our prostate cancer screening system.

Conclusion

A decrease in the repeated PSA value cannot predict a negative biopsy outcome under the established biopsy criteria. Latest PSAD, PSAV and positive DRE at latest screening appeared to be significant predictors for prostate cancer. The combination of these three variables might help to reduce unnecessary repeat biopsies in the high-risk cohort of patients with negative initial biopsies.

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前立腺がん検診の有効性評価を目的とした症例対照研究（第１報）

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はじめに

厚労省がん研究助成金「がん検診の評価とあり方に関する研究」班（垣添班）のなかで、「前立腺PSA検診の有効性評価に関する研究」小班（三木小班）が進めようとしている症例対照研究の概要と進捗状況を報告する。また、わが国のPSA検診の評価を目的とした、症例対照研究を遂行するための、課題点に関しても言及する。

対象と方法

1）説明と同意

本研究は後ろ向きの観察的な疫学研究であり、研究対象者に対して薬剤の投与や試料の採取を一切行わない。したがって、『疫学研究における倫理指針』の第7章「研究対象者からインフォームド・コンセントを受ける手続き等（2）観察研究を行う場合②人体から採取された試料を用いない場合」に該当する。特に症例はすべて死亡者であり、本人に説明と同意を行うことはできない。また前立腺がん死亡者は高齢者に偏るため、症例と同一生年の対照候補者のうちインフォームド・コンセントを得られたもののみを研究対象として採用した場合、健康意識の高いものに偏ってしまうため、適切な評価ができないことになる。よって上記倫理指針に基づき、研究対象者に対して、インフォームド・コンセントを行わないものとする。ただし研究計画はホームページ上に公開し、対象者に周知を図り、異議申し立てを受け、参加拒否を受け付けるものとする。

2）「症例」と「対照」の選定

症例の選択：厚生労働省から人口動態調査死亡小票の調査票情報の使用許可を得た上で、該当する京都府乙訓保健所内で、平成15～20年の人口動態調査死亡小票を閲覧し、前立腺がん死亡者を抽出する。なお死亡時年齢は50歳以上とする。この症例候補者を、該当する住所地の住民基本台帳と氏名・生年月日・住所で照合し、平成7～14年まで住民登録されていたものを症例とする。予定症例数は、20例を目標とする。該当期間内の当該地区での前立腺がん死亡は年間約3～5例程度であり、本期間内では最大30例が見込まれるものの、除外例が存在しうることから20例を目標とする。

対照の選択：症例1例につき、10例の対照候補者を該当する住所地の住民基本台帳から選択する。この選択にあたっては、各症例と生年が±2歳以内とし、ほぼ同一居住区であるこ

とを条件とする。上記作業で選択された対照候補者を、該当する住所地の住民基本台帳と氏名・生年月日・住所で照合し、平成7～14年まで住民登録されていたものを対照とする。

症例と対照の除外条件：平成7～14年の間での住所地外への短期間での転出後再転入に関しては、検診の受診機会が損なわれることから、これは除外対象とする。具体的には、自宅のリフォームや老健施設への入所等が考えられる。一方、住所地での住民登録状況については、同一市町内での異動については、検診受診機会が損なわれないことから、これは除外対象とはしない。死亡時住所が、介護老健施設等である症例候補者に対しても、評価する検診が行われた平成7～14年の間に該当市町の他の住所（介護老健施設等を除く）であることが確認された場合は、症例として採用する。

検診受診歴の把握：症例および対照を、該当地区の保健センターが保管する検診受診者名簿と、氏名・生年月日・住所を用いて照合し、平成7～14年の検診受診歴、受診時のPSA検査測定値を把握する。上記作業が終了した時点で、氏名・生年月日・住所などの個人識別情報を削除した、解析ファイルを作成し、以後の解析はこの解析ファイルを用いて、京都府立医科大学泌尿器科教室内および国立がんセンターがん対策情報センターがん情報・統計部部長室内で作業する。

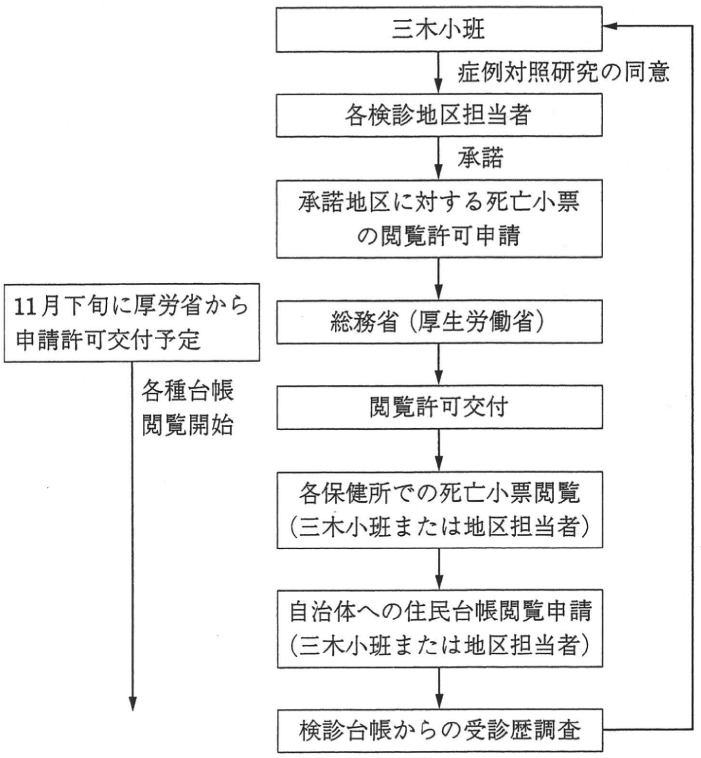

図1●症例・対照研究の流れ

3）進捗状況

研究の流れを図1に示す。11月下旬に死亡小票の閲覧許可がおりれば、各台帳の閲覧申請に移行する予定である。

4）今後の展望と課題点

わが国の死亡率減少効果のエビデンスを高めるためには、100例の「症例」の蓄積が必要と考えられ、乙訓地区以外の解析も必要となることが予想される。したがって、臨床系の研究協力者は、幅広く採用したほうがよいと考える。また、死亡小票閲覧許可後、行政区が保管する名簿（住民基本台帳・1次検診受診者名簿）の閲覧には2市1町の首長の許可が必要である。

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DISEASES OF THE ESOPHAGUS

Original article

Pattern of lymph node metastases of esophageal squamous cell carcinoma based on the anatomical lymphatic drainage system

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SUMMARY. **The recent anatomical studies of the esophagus showed that submucosal longitudinal lymphatic vessels connect to the superior mediastinal and the paracardial lymphatics and lymphatic routes to periesophageal nodes originate from the muscle layer. Using clinical data for lymph node metastasis, we verify these anatomical bases to clarify the rational areas of lymph node dissection in esophageal cancer surgery. Analysis was performed on 356 consecutive patients who underwent esophagectomy with three-field dissection. Patients were divided into those with tumor limited within the submucosal layer and those with tumor invading or penetrating the muscle layer. Frequency of node metastasis was compared according to supraclavicular, upper mediastinum, mid-mediastinum, lower mediastinum, perigastric and celiac areas. In patients with tumor limited to the submucosal layer, node metastasis was more frequent in the upper mediastinum and perigastric area than the mid- or lower mediastinum. Even in patients with tumor located in the lower esophagus, node metastasis was more frequent in the upper mediastinum than the mid-mediastinum or lower mediastinum. In patients with tumor located in the mid-esophagus, node metastasis was more frequent in the supraclavicular area than the mid-mediastinum or lower mediastinum. In patients with tumor invading or penetrating the muscle layer, node metastasis in the mid- and lower mediastinum increased dramatically, but was still less frequent than those in the upper mediastinum or the perigastric area. Postoperative survival curves did not differ among the involved areas. The most predictive factor associated with lymph node metastasis for postoperative survival was not the area of involved nodes, but the number of involved nodes by multivariate analyses. These clinical results verify recent anatomical observations. The lack of difference in survival rates among the involved areas suggests that these areas should be staged equivalently. For adequate nodal staging, the upper mediastinum should be dissected for the lower esophageal tumor and supraclavicular areas should be dissected for the mid-esophageal tumor even in patients with tumor limited to within the submucosal layer.**

KEY WORDS: **esophageal cancer, lymph node excision, lymphatic metastasis, neoplasm staging.**

INTRODUCTION

Despite recent advances in multidisciplinary approaches including radio- and chemotherapy,[1] surgical resection remains the standard treatment for potentially resectable esophageal carcinoma. Considerable controversy remains regarding the extent of lymph node dissection in esophageal cancer surgery. Some favor more limited resection strategies such as transhiatal resection or transthoracic resection with minimal nodal dissection and hold that extended nodal dissection offers no meaningful improvement in survival.[2,3] Others have argued that three-field lymphadenectomy entailing nodal dissection along the recurrent laryngeal nerves in the thoracic cavity extending to the 'third' field in the neck improves local disease control and possibly enhances survival.[4–7] A consistent operative strategy has yet to be established.

Knowing the anatomical lymphatic drainage system of the esophagus is crucial to achieving an adequate field of dissection. The abundant lymphatic channels in the lamina propria mucosae and submucosa of the esophagus are well known from classic descriptions.[8–10] A recent anatomical study by Kuge *et al.* showed that long longitudinal lymphatic extension in the esophageal submucosa is very evident.[11] Another anatomical study showed a morphological connection between submucosal lymphatic vessels in

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the proximal esophagus and recurrent nerve nodes in the superior mediastinum.[12] These morphologies suggest an explanation for anatomically distant lymph nodes metastasis known as 'skip metastasis' in thoracic esophageal cancer. Kuge *et al.* also reported that lymphatic routes to periesophageal lymph nodes usually originate from the intermuscular area of the muscularis propria and lymphatic communication between the submucosa and intermuscular area was rarely apparent histologically.[11]

The present retrospective study attempted to verify these anatomical bases using clinical data of lymph node metastasis. We analyzed the frequency of involved nodes according to the areas of dissection. We also analyzed the survival effects of dissection for involved nodes to clarify the rational extent of lymph node dissection during esophagectomy.

PATIENTS AND METHODS

We conducted a retrospective review of a prospective database to identify patients with surgically resected esophageal carcinoma at our institution from January 2001 to December 2005. Analysis was performed on 356 consecutive patients with esophageal squamous cell carcinoma who underwent esophagectomy with three-field dissection to compare areas including the supraclavicular and celiac. During the 5-year study period, the incidence of adenocarcinoma was <3% in our institution. The presence of supraclavicular and celiac nodal involvement was not considered a contraindication for resection whenever potentially curative resections could be performed. Patients who underwent palliative resection were excluded, as three-field dissection was avoided. Six patients with T4 tumor (pericardium, $n = 3$; diaphragm, $n = 2$; lung, $n = 1$) who underwent curative resection with three-field dissection were included. Patients who had a past history of neck dissection or gastric surgery were excluded. Patients who had received preoperative chemo- or chemoradiotherapy were also excluded. To analyze the lymphatic routes of the submucosa and intermuscular area, patients were divided into those with tumor limited within the submucosal layer (pT1) and those with tumor invading or penetrating the muscle layer (pT2-4).

Surgical resection consisted of transthoracic esophagectomy with three-field lymphadenectomy, which included all lymphovascular tissue along with mediastinal lymph nodes from the superior mediastinal nodes and nodes along both recurrent laryngeal nerves to the hiatus. Upper abdominal lymphadenectomy was performed to include the paracardial, lesser curvature, left gastric, common hepatic, celiac, and splenic nodes. Neck lymphadenectomy included supraclavicular nodes located lateral to the jugular veins.

Table 1 Patient demographics and tumor characteristics for 356 patients who underwent three-field esophagectomy for squamous cell carcinoma of the esophagus

	pT1 $n = 127$ (100%)	pT2-4 $n = 229$ (100%)
Mean age (range)	62.7 (41–80)	62.8 (45–83)
Male/Female	115/12	199/30
Tumor location		
Upper	22 (17.3)	33 (14.4)
Mid	67 (52.8)	106 (46.3)
Lower	38 (29.9)	90 (39.3)
T classification (pathological)		
pT1	127 (100)	–
pT2	–	40 (17.5)
pT3	–	183 (79.9)
pT4	–	6 (2.6)
N classification (pathological)		
pN0	69 (54.3)	41 (17.9)
pN1	40 (31.5)	76 (33.2)
pN2	15 (11.8)	66 (28.8)
pN3	3 (2.4)	46 (20.1)
M classification (pathological)		
pM0	116 (91.3)	19 (83.0)
pM1 (supraclavicular node)	11 (8.7)	39 (17.0)

The frequency of lymph node metastasis was compared according to the areas, comprising supraclavicular area, upper mediastinum including paratracheal nodes, nodes along both recurrent laryngeal nerves and posterior mediastinal nodes, mid-mediastinum including middle paraesophageal nodes and subcarinal nodes, lower mediastinum from the caudal margin of the inferior pulmonary vein including lower paraesophageal nodes and diaphragmatic nodes, perigastric area including paracardial nodes and left gastric nodes and the celiac area including common hepatic nodes, splenic nodes and celiac nodes. The efficacy of dissection was estimated by postoperative survival of patients with involved nodes according to these areas.

Statistical analysis was performed using SPSS statistical software (SPSS, Chicago, IL, USA). Overall survivals were analyzed using Kaplan–Meier methods including all causes of death. The log-rank test was used to determine the significance of survival distributions among groups. Multivariate analyses were performed by Cox regression. Results were considered significant for values of $P < 0.05$.

RESULTS

Patient demographics and tumor characteristics are shown in Table 1. Frequency of lymph node metastasis including supraclavicular node was 47.2% in patients with pT1 tumor and 82.1% in patients with pT2-4 tumor. Fifty patients were staged pM1 by supraclavicular node metastasis.