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14. ABSTRACT

We hypothesized that dehydroepiandrosterone metabolites or their synthetic derivatives are able to bind to the androgen receptor with low, if any, agonist activity and thus function as better antiandrogens than currently available ones. We previously identified three potential compounds with marginal androgenic activity. Using different prostate cancer cell lines, we showed that these compounds could inhibit androgen-induced growth of androgen receptor-positive tumors *in vitro*. In tolerance tests, the mice appeared to suffer from no adverse effects from injections of the steroids. We then assessed the anti-tumor activities of these compounds in mouse xenograft models for prostate cancer. Inconsistent with our *in vitro* data, the dehydroepiandrosterone metabolites only slightly reduced the growth of inoculated tumors as well as the expression of angiogenesis- and metastasis-related genes in the tumors. Thus, we plan to perform further *in vivo* experiments (*e.g.* higher doses of the compounds, different cell lines).

15. SUBJECT TERMS
Antiandrogen, androgen receptor, dehydroepiandrosterone, mouse xenograft model

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Introduction

Although antiandrogens that can block androgen action through the androgen receptor (AR) have been widely used for the treatment of prostate cancer, the majority of available ones possess agonist activity, resulting in increases in serum prostate-specific antigen (PSA) levels, known as the antiandrogen withdrawal syndrome [1,2]. In addition, we previously found that androstenediol (Adiol), a physiological metabolite from dehydroepiandrosterone (DHEA) and a precursor of testosterone, has an intrinsic androgenic activity which was not completely antagonized by two antiandrogens clinically used, hydroxyflutamide (HF) and bicalutamide (BC) [3]. Therefore, new and more effective antiandrogenic compounds with marginal androgenic activities need to be identified. Our hypothesis in the current project was that DHEA metabolites or their synthetic derivatives are able to bind to the AR with low, if any, agonist activity and thus function as better antiandrogens than currently available ones. We previously screened DHEA derivatives/metabolites for their androgenic and antiandrogenic activities and found that three compounds, 3β-acetoxyandrost-1,5-diene-17-ethylene-ketal (ADEK), 3β-hydroxyandrost-5,16-diene (HAD), and 3-oxo-androst-1,4-diene-17-ketal (OADK), show only marginal agonist effects and suppress significantly 5α-dihydrotestosterone (DHT)- and Adiol-induced AR transactivations [4-6]. Thus, ADEK, HAD, and OADK have the potential to function as potent antiandrogens that carry fewer risks of withdrawal response if used for therapy in patients with prostate cancer.

We have previously assessed the effects of ADEK, HAD, and OADK on prostate cancer cell growth *in vitro*. The tasks in the approved Statement of Work in this period (months 25-36) would be to evaluate the effects of these DHEA derivatives *in vivo* (*Task 2* for months 19-48), including *Task 2-a* (to test the tolerance and toxicity of the compounds in mice), Task *2-b* (to test the anti-tumor effects of the compounds in mouse xenograft models for prostate cancer), and Task *2-c* (to analyze xenograft tumors by immunohistochemistry, reverse transcription (RT)-polymerase chain reaction (PCR), and western blotting).

Body

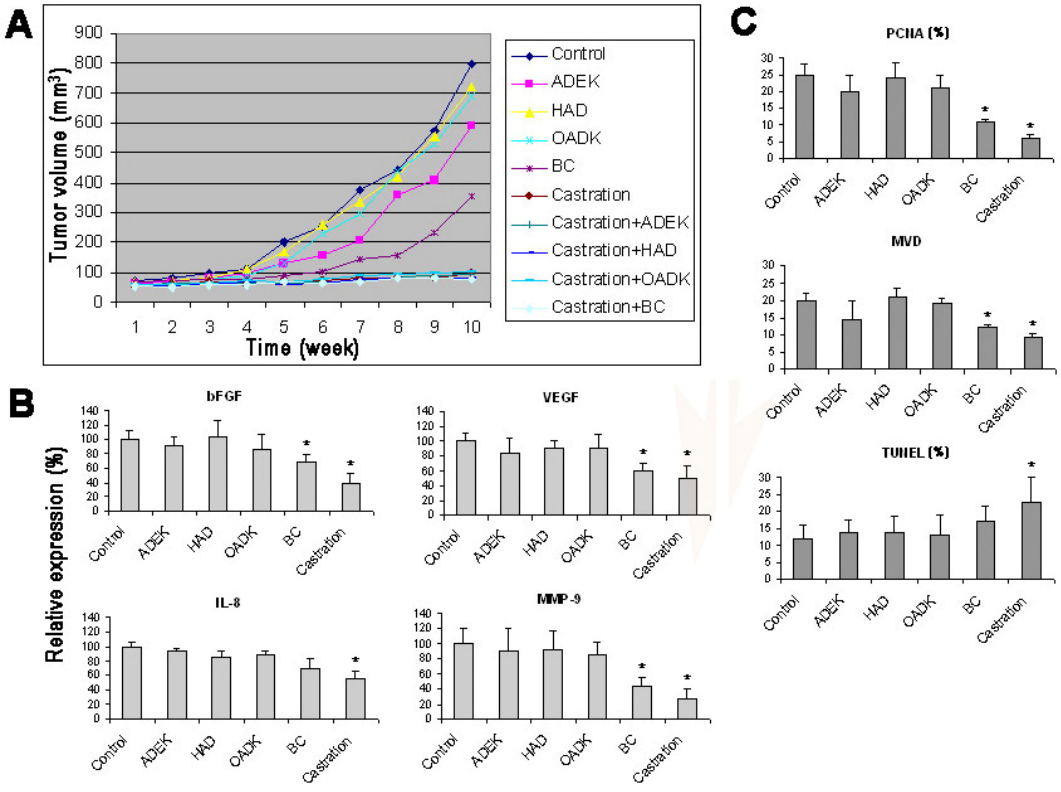
Tolerance/toxicity for ADEK, HAD, and OADK in animals

To determine whether the DHEA derivatives are well tolerated or affected any adverse responses in animals, ADEK, HAD, or OADK (200 mg/Kg daily for 14 days; 2x postulated therapeutic dose [7]) was administered subcutaneously in 6-week-old male C57BL/6 mice. As detailed in the previous report (July 2011), there were no statistically significant differences (by Student's *t*-test) in food intake, weight gain, and the weight of major organs (*e.g.* heart, liver, kidney, adrenal, spleen, testis, and brain) between the control and treatment groups. I, as a board certified pathologist, also confirmed that there were no significant histological changes in the tissues from different groups of the mice. Thus, it was likely that animals with injections of a high dose of ADEK, HAD, or OADK suffered from no adverse effects.

Anti-tumor effects of ADEK, HAD, and OADK in mouse xenograft models for prostate cancer

Inhibitory effects of DHEA derivatives on tumor growth were assessed in mouse xenograft models for AR-positive prostate cancer. We used LNCaP and CWR22Rv1 because the compounds were found to significantly suppress androgen-mediated cell proliferation *in vitro*. These lines were implanted subcutaneously into the flanks of 7-8-week-old male SCID mice. After 2-4 weeks, when the estimated volumes of all tumors reached 40 mm^3, we started daily injection of the compounds into mice. As shown in Figure 1A, inoculated LNCaP tumors in mice treated with BC (without castration) were significantly smaller (61% reduction) than those in the control mice. However, subcutaneous injection of ADEK (26% at 10 weeks), HAD (9% at 10 weeks), or OADK (13% at 10 weeks) only slightly (P>0.05) reduced the size of the LNCaP tumors in non-castrated mice, compared to control treatment. In addition, castration significantly retarded the growth of LNCaP tumors, and no significant additive effects of DHEA derivatives as well as BC were seen. Similarly, subcutaneous injection of DEHA derivatives resulted in only slight decreases (10-19% at 7 weeks) in CWR22Rv1 tumors (Figure 2A). We then injected intraperitoneally the compounds in LNCaP-implanted mice (Figure 3). The sizes of the tumors were similar among the cohorts (except the BC group) at 1-8 weeks of treatment, although considerable reductions (26-34%, *vs.* control) were seen at 9 weeks.

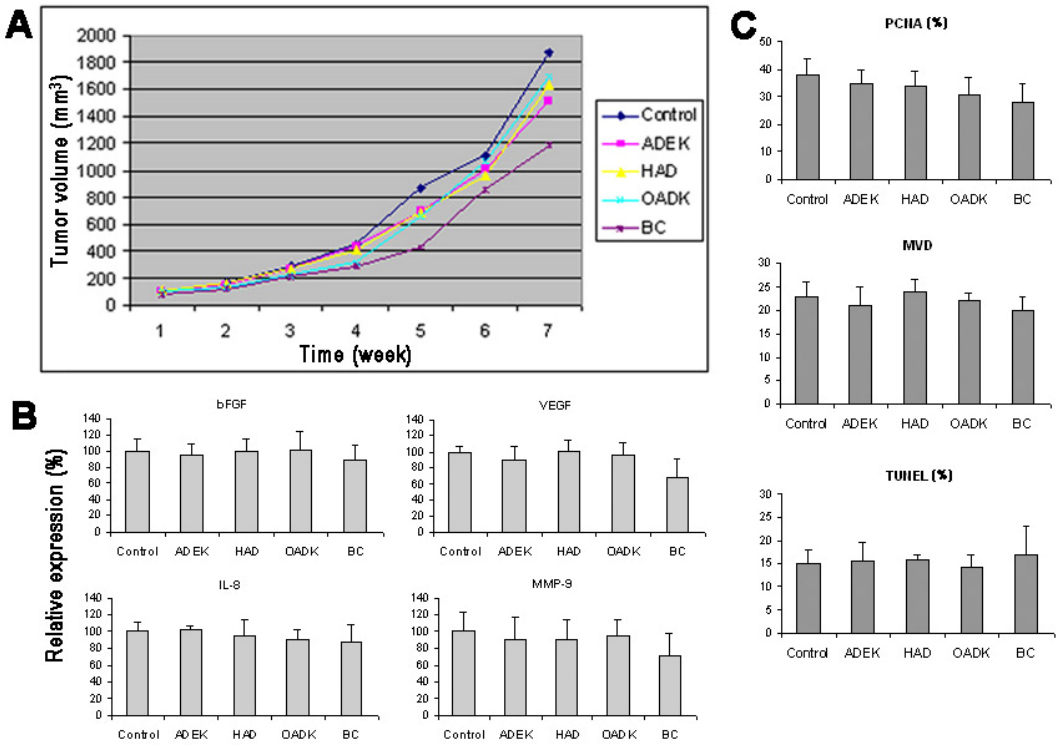
Figure 1



Some of the harvested tumor specimens were also assessed for cell proliferation [by proliferating cell nuclear antigen (PCNA) immunostaining], apoptosis (by TUNEL assay),

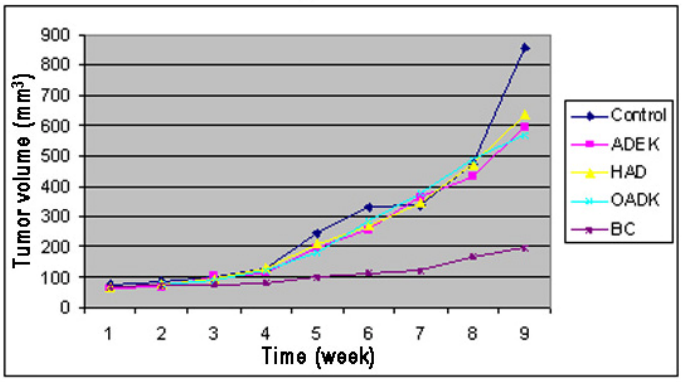
and angiogenesis or metastatic ability [micro-vessel density (MVD) by CD31 immunostaining and the expression of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin (IL)-8, and matrix metalloproteinase (MMP)-9 by quantitative RT-PCR]. Correlating with the sizes of xenograft tumors, ADEK, HAD, and OADK failed to significantly change these factors (Figures 1B, 1C, 2B & 2C). Furthermore, there were no noticeable differences in the expression of VEGF, MMP-9, and E-cadherin detected by immunohistochemistry, as well as that of VEGF, MMP-2, and MMP-9 detected by western blotting, between the tumors from control versus ADEK/HAD/OADK groups.

Figure 2



Figures legends. The effects of ADEK, HAD, and OADK on tumor progression in mouse xenograft models for prostate cancer. LNCaP (**Figures 1A & 3**) or CWR22Rv1 (**Figure 2A**) cells resuspended in Matrigel (2×10^6 cells in 200 µl per site) were implanted subcutaneously into the right and left flanks of SCID mice, and treatment [daily subcutaneous (**Figures 1A & 2A**) or intra-peritoneal (**Figure 3**) injection of 100 mg/Kg each compound) began when estimated tumor volume reached 40 mm^3 calculated by the following formula: tumor weight = tumor length (mm) × [tumor width (mm)]^2 × 0.5 [8]. In the groups of mice (**Figure 3**), bilateral orchiectomy or sham surgery was also performed. Tumor volume (n = 6 tumors in each group)

Figure 3



was monitored twice a week for 7-10 weeks. The mice were then killed, and the tumors were harvested for further analyses. mRNA expression of bFGF, VEGF, IL-8, and MMP-9 in the tumors was analyzed by real-time RT-PCR (**Figures 1B & 2B**). Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of control tumors in each cell line (first lanes; set as 100%). Each value represents the mean + SD from at least three independent experiments. Immunohistochemical and TUNEL analyses in harvested xenograft tumors from mice were also performed (**Figures 1C & 2C**). Mean value + SD of the percentage of PCNA-positive cells, MVD (number of vessels highlighted by CD31 staining per high-power field), and the percentage of TUNEL-positive cells in each group of tumors is shown. **P*<0.05 (*vs.* control by Student's *t*-test).

Thus, in contrast to our *in vitro* data, ADEK, HAD, and OADK did not show significant suppressive effects on AR-positive tumor growth *in vivo*. We will repeat mouse xenograft experiments, using higher doses of the compounds and/or different cell lines [*e.g.* VCaP harboring a wild-type AR we recently obtained from the American Type Culture Collection (ATCC) after performing *in vitro* analyses in this cell line].

Key Research Accomplishments

1. (for *Task 2-a*) ADEK, HAD, and OADK were well tolerated or affected no adverse responses in mice.

2. (for Tasks *2-b* & *2-c*) ADEK, HAD, and OADK only slightly inhibited tumor progression in mouse xenograft models for prostate cancer (LNCaP and CWR22Rv1).

Reportable Outcomes

Promotion

I was promoted to Associate Professor of Pathology and Laboratory Medicine at the University of Rochester School of Medicine and Dentistry on November 1, 2011.

Peer-reviewed Publications

Underlined articles acknowledge the current award.

1. Canacci AM, Izumi K, Zheng Y, Gordetsky J, Yao JL, **Miyamoto H** (corresponding author): Expression of semenogelins I and II and its prognostic significance in human prostate cancer. *Prostate* 71(10): 1108-1114, 2011 (July).

2. Zheng Y, Izumi K, Yao JL, **Miyamoto H** (corresponding author): Dihydrotestosterone upregulates the expression of epidermal growth factor receptor and ERBB2 in androgen receptor-positive bladder cancer cells. *Endocr-Relat Cancer* 18(4): 451-464, 2011 (August).

3. Gordetsky J, Findeis-Hosey J, Erturk E, Messing EM, Yao JL, **Miyamoto H** (corresponding author): Intraoperative frozen section analysis in fibrous pseudotumor of the testicle: a five-case experience. *Can Urol Assoc J (CUAJ)* 5(4): E47-E51, 2011 (August).

4. Lee TK, Chaux A, Karram S, **Miyamoto H**, Miller JS, Fajardo DA, Epstein JI, Netto GJ: Papillary urothelial neoplasm of low malignant potential of the urinary bladder: Clinicopathologic and outcome analysis from a single academic center. *Hum Pathol* 42(11): 1799-1803, 2011 (November).

5. Fajardo DA, **Miyamoto H**, Miller JS, Lee TK, Epstein JI: Identification of Gleason pattern 5 on prostatic needle core biopsy: Frequency of underdiagnosis and relation to morphology. *Am J Surg Pathol* 35(11): 1706-1711, 2011 (November).

6. Chaux A, Karram S, Miller JS, Fajardo DA, Lee TK, **Miyamoto H**, Netto GJ: High-grade papillary urothelial carcinoma of the urinary tract: a clinicopathologic analysis of a post-World Health Organization/ International Society of Urological Pathology classification cohort from a single academic center. *Hum Pathol* 43(1): 115-120, 2012 (January).

7. **Miyamoto H** (corresponding author), Zheng Y, Izumi K: Nuclear hormone receptor signals as new therapeutic targets for urothelial carcinoma. *Curr Cancer Drug Tar* 12(1): 14-22, 2012 (January).

8. Yamashita S, Lai K-P, Chuang K-L, Xu D, **Miyamoto H**, Tochigi T, Pang S-T, Li L, Arai Y, Kung H-J, Yeh S, Chang C: ASC-J9 suppresses castration-resistant prostate cancer growth through degradation of full-length and splice variant androgen receptors. *Neoplasia* 14(1): 74-83, 2012 (January).

9. Choy B, Gordetsky J, Varghese M, Lloyd GL, Wu G, **Miyamoto H** (corresponding author): Mixed epithelial and stromal tumor of the kidney in a 14-year-old boy. *Urol Int* 88(2): 247-248, 2012 (March).

10. **Miyamoto H**: "Urine-based assays complementing cytologic examination in the detection of bladder neoplasm" in Koss's Cytology of the Urinary Tract with Histopathologic Correlations (Koss LG/Hoda RS Eds.), pp.109-121, Springer, New York (ISBN 978-1-4614-2055-2), 2012 (April).

11. Subik MK, Yao JL, di Sant'Agnese PA, **Miyamoto H** (corresponding author): The role of periprostatic and periseminal vesicle lymph node metastasis in the staging and prognosis of prostate cancer. *Histopathology* 60(6): 1009-1010, 2012 (May).

12. **Miyamoto H** (corresponding author), Yao JL, Chaux AM, Zheng Y, Hsu I, Izumi K, Chang C, Messing EM, Netto GJ, Yeh S: Expression of androgen and oestrogen receptors and its prognostic significance in urothelial neoplasm of the urinary bladder. *BJU Int* 109(11): 1716-1726, 2011 (June).

13. Izumi K, Zheng Y, Hsu J-W, Chang C, **Miyamoto H** (corresponding author): Androgen receptor signals regulate UDP-glucuronosyltransferases in the urinary

bladder: A potential mechanism of androgen-induced bladder carcinogenesis. *Mol Carcinogen* (November 15, 2011; Epub ahead of print).

14. Subik MK, Gordetsky J, Yao JL, di Sant'Agnese PA, **Miyamoto H** (corresponding author): Frozen section assessment in testicular and paratesticular lesions suspicious for malignancy: its role in preventing unnecessary orchiectomy. *Hum Pathol* (March 8, 2012; Epub ahead of print).

15. **Miyamoto H** (corresponding author), Izumi K, Yao JL, Li Y, Yang Q, McMahon LA, Gonzalez-Roibon N, Hicks DG, Tacha D, Netto GJ: GATA binding protein 3 is down-regulated in bladder cancer yet strong expression is an independent predictor of poor prognosis in invasive tumor. *Hum Pathol* (May 18, 2012; Epub ahead of print).

16. Izumi K, Li Y, Zheng Y, Gordetsky J, Yao JL, **Miyamoto H** (corresponding author): Seminal plasma proteins in prostatic carcinoma: Increased nuclear semenogelin I expression is a predictor of biochemical recurrence after radical prostatectomy. *Hum Pathol* (May 21, 2012; Epub ahead of print).

17. Li Y, Izumi K, **Miyamoto H** (corresponding author): (Review) The role of the androgen receptor in the development and progression of bladder cancer. *Jpn J Clin Oncol* 42(7): 569-577, 2012.

18. Choy B, Gordetsky J, **Miyamoto H** (corresponding author): Clinicopathologic features of prostate cancer in patients diagnosed by age 45 who underwent radical prostatectomy. *Eur Urol* 62(2): 354-355, 2012.

19. Izumi K, Zheng Y, Li Y, Zaengle J, **Miyamoto H** (corresponding author): Epidermal growth factor induces bladder cancer cell proliferation through activation of the androgen receptor. *Int J Oncol*, in press.

Other Presentations/Abstracts

1. Gordetsky J, Subik K, Choy B, Varghese M, Messing E, **Miyamoto H**, Yeh S: Analysis of tocopherol-associated protein expression in prostate cancer and its correlation with clinicopathologic features. College of American Pathologists 2011 Annual Meeting (CAP'11) at Dallas, Texas (September 2011); Abstract published in *Arch Pathol Lab Med* 135(9): 1125, 2011.

2. Choy B, **Miyamoto H**: Characteristics of prostate cancer in patients aged 45 years or younger. College of American Pathologists 2011 Annual Meeting (CAP'11) at Dallas, Texas (September 2011); Abstract published in *Arch Pathol Lab Med* 135(9): 1127, 2011.

3. Subik MK, Gordetsky J, **Miyamoto H**: Frozen section diagnosis and testicular-sparing surgery for testicular/paratesticular fibrous pseudotumors. College of American Pathologists 2011 Annual Meeting (CAP'11) at Dallas, Texas (September 2011); Abstract published in *Arch Pathol Lab Med* 135(9): 1128, 2011.

4. Gordetsky J, Varghese M, Messing E, **Miyamoto H**, Yeh S: Analysis of tocopherol-associated protein (TAP) expression in prostate cancer and its correlation with clinico-pathologic features. The 63rd Annual Meeting of the Northeastern Section of the American Urological Association at New Orleans, Louisiana (October 27, 2011).

5. **Miyamoto H**, Izumi K, Yao J, Yang Q, McMahon L, Gonzalez-Roibon N, Chaux A, Hicks D, Netto G, Tacha D: GATA3 is down-regulated in bladder cancer yet strong expression is an independent predictor of poor prognosis in invasive tumor. United States & Canadian Academy of Pathology 101st Annual Meeting at Vancouver, Canada (March 20, 2012); Abstract published in *Mod Pathol* 25(Suppl 2): 228A, 2012.

6. Venigalla S, Zhao C, **Miyamoto H**: Features of atypical glands on initial prostate biopsy as positive or negative predictors of malignancy on subsequent prostate biopsy. United States & Canadian Academy of Pathology 101st Annual Meeting at Vancouver, Canada (March 20, 2012); Abstract published in *Mod Pathol* 25(Suppl 2): 248A, 2012.

7. **Miyamoto H**, Yao J, Chaux A, Yang Q, McMahon L, Zheng Y, Izumi K, Netto G: Expression of androgen and estrogen receptors and its prognostic significance in urothelial neoplasm of the urinary bladder. United States & Canadian Academy of Pathology 101st Annual Meeting at Vancouver, Canada (March 21, 2012); Abstract published in *Mod Pathol* 25(Suppl 2): 228A, 2012.

8. Izumi K, Li Y, Zheng Y, Yang Q, McMahon L, Gordetsky J, Yao J, **Miyamoto H**: Seminal plasma proteins in prostate cancer: Increased semenogelin I expression is a predictor of biochemical recurrence after radical prostatectomy. United States & Canadian Academy of Pathology 101st Annual Meeting at Vancouver, Canada (March 21, 2012); Abstract published in *Mod Pathol* 25(Suppl 2): 215A, 2012.

9. Kakiuchi Y, Gordetsky J, **Miyamoto H**: Role of frozen section analysis during radical prostatectomy: a 1,993-case experience. United States & Canadian Academy of Pathology 101st Annual Meeting at Vancouver, Canada (March 21, 2012); Abstract published in *Mod Pathol* 25(Suppl 2): 216A, 2012.

10. **Miyamoto H**, Izumi K, Yao J, Yang Q, McMahon L, Gonzalez-Roibon N, Chaux A, Hicks D, Netto G, Tacha D: GATA3 is down-regulated in bladder cancer yet strong expression is an independent predictor of poor prognosis in invasive tumor. American Urological Association 107th Annual Meeting at Atlanta, Georgia (May 20, 2012); Abstract published in *J Urol* 187(4 Suppl): e159, 2012.

11. **Miyamoto H**, Yao JL, Chaux A, Zheng Y, Hsu I, Izumi K, Netto GJ, Chang C, Messing EM, Netto G, Yeh S: Expression of androgen and estrogen receptors and its prognostic significance in urothelial neoplasm of the urinary bladder. American Urological Association 107th Annual Meeting at Atlanta, Georgia (May 20, 2012); Abstract published in *J Urol* 187(4 Suppl): e159-e160, 2012.

12. Li Y, Zheng Y, Izumi K, Ye B, Li F, **Miyamoto H**: Androgen activates β-catenin signaling in androgen receptor-positive bladder cancer cells. American Urological

Association 107th Annual Meeting at Atlanta, Georgia (May 20, 2012); Abstract published in *J Urol* 187(4 Suppl): e228, 2012.

13. Kakiuchi Y, Choy B, Gordetsky J, Wu G, Rashid H, Joseph J, **Miyamoto H**: Role of frozen section analysis during radical prostatectomy: a 2,641-case experience. American Urological Association 107th Annual Meeting at Atlanta, Georgia (May 20, 2012); Abstract published in *J Urol* 187(4 Suppl): e281-e282, 2012.

14. Zheng Y, Izumi K, Li Y, **Miyamoto H**: Dexamethasone oppositely regulates bladder cancer cell proliferation and invasion through the glucocorticoid receptor pathway. American Urological Association 107th Annual Meeting at Atlanta, Georgia (May 21, 2012); Abstract published in *J Urol* 187(4 Suppl): e359-e360, 2012.

15. Yamashita S, Lai K-P, Chuang K-L, **Miyamoto H**, Tochigi T, Pang S-T, Li L, Arai Y, Kung H-J, Yeh S, Chang C: ASC-J9® suppresses castration-resistant prostate cancer growth via degradation of full-length and splice variant androgen receptors. American Urological Association 107th Annual Meeting at Atlanta, Georgia (May 21, 2012); Abstract published in *J Urol* 187(4 Suppl): e391, 2012.

16. Izumi K, Zheng Y, Zaengle J, Li Y, **Miyamoto H**: Epidermal growth factor induces bladder cancer cell proliferation through activation of androgen receptor. American Urological Association 107th Annual Meeting at Atlanta, Georgia (May 21, 2012); Abstract published in *J Urol* 187(4 Suppl): e431, 2012.

17. Venigalla S, Zhao C, **Miyamoto H**: Features of atypical glands on initial prostate biopsy as positive or negative predictors of malignancy on subsequent prostate biopsy. American Urological Association 107th Annual Meeting at Atlanta, Georgia (May 22, 2012); Abstract published in *J Urol* 187(4 Suppl): e775-e776, 2012.

Invited Speakers

1. Educational lecture at the 76th Annual Meeting of the Eastern Section of the Japanese Urological Association, Yokohama, Japan (October 21, 2011).

2. University of Occupational and Environmental Health (Department of Urology), Kitakyushu, Japan (October 24, 2011).

3. Mie Pathologists' Association, Tsu, Japan (October 25, 2011).

4. Yokohama City University Medical Association, Yokohama, Japan (October 26, 2011).

5. Johns Hopkins University (Department of Pathology), Baltimore, Maryland (April 20, 2012).

Conclusion

Using mouse xenograft models for prostate cancer (LNCaP and CWR22Rv1), we have

assessed the effects of ADEK, HAD, and OADK, in comparison with that of BC, on tumor progression *in vivo*. All these DHEA derivatives, as well as HF and BC, were previously shown to have antiandrogenic properties and significantly inhibited cell proliferation and invasion of prostate cancer lines with different AR statuses *in vitro*. Compared to these *in vitro* analyses, inhibitory effects of the three compounds at a dose of 100 mg/Kg given subcutaneously or intraperitoneally on tumor growth were found to be less significant in animal models. We will first repeat mouse xenograft experiments, using higher doses of the compounds. Concurrently, another useful prostate cancer line, VCaP harboring a wild-type AR available from the ATCC, will be assessed for anti-tumor effects of ADEK, HAD, and OADK *in vitro*. If these compounds are found to exhibit antiandrogenic activities in VCaP cells, we will also perform mouse xenograft experiments using this line.

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1. Miyamoto H, Messing EM, Chang C: Androgen deprivation therapy for prostate cancer: Current status and future prospects. *Prostate* 61: 332-353, 2004.

2. Miyamoto H, Rahman MM, Chang C: Molecular basis for the antiandrogen withdrawal syndrome. *J Cell Biochem* 91: 3-12, 2004.

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7. Lardy H, Marwah A, Zhong W, Moore R, Marwah P, Thompson T, Wilding G: A test of rats' tolerance for 3β-acetoxyandrosta-1,5-dien-17-one ethylene ketal, a new anti-androgen. *J Steroid Biochem Mol Biol* 111: 50-65, 2008.

8. Miyamoto H, Yang Z, Chen Y-T, Ishiguro H, Uemura H, Kubota Y, Nagashima Y, Chang Y-J, Hu Y-C, Tsai M-Y, Yeh S, Messing EM, Chang C: Promotion of bladder cancer development and progression by androgen receptor signals. *J Natl Cancer Inst* 99: 558-568, 2007.