Biology of Human Tumors

Alterations Associated with Androgen Receptor Gene Activation in Salivary Duct Carcinoma of Both Sexes: Potential Therapeutic Ramifications

Yoshitsugu Mitani1, Pulivarthi H. Rao2, Sankar N. Maity3, Yu-Chen Lee4, Renata Ferrarotto5, Julian C. Post1, Lisa Licitra6, Scott M. Lippman7, Merrill S. Kies5, Randal S. Weber8, Carlos Caulin8, Sue-Hwa Lin4, and Adel K. El-Naggar1,8

Abstract

Purpose: To investigate the molecular events associated with the activation of androgen receptor (AR) as a potential therapeutic target in patients with salivary duct carcinoma (SDC).

Experimental Design: Comprehensive molecular and expression analysis of the AR gene in 35 tumor specimens (20 males and 15 females) and cell lines derived from SDC using Western blotting and RT-PCR, FISH analysis, and DNA sequencing was conducted. In vitro and in vivo animal studies were also performed.

Results: AR expression was detected in 70% of the tumors and was mainly nuclear and homogenous in both male and female SDCs, although variable cytoplasmic and/or nuclear localization was also found. We report the identification of ligand-independent AR splice variants, mutations, and extra AR gene copy in primary untreated SDC tumors. In contrast to prostate cancer, no AR gene amplification was observed. In vitro knockdown of AR in a female derived SDC cell line revealed marked growth inhibition in culture and in vivo androgen-independent tumor growth.

Conclusions: Our study provides new detailed information on the molecular and structural alterations associated with AR gene activation in SDC and sheds more light on the putative functional role of AR in SDC cells. On the basis of these data, we propose that patients with SDC (male and female) can be stratified for hormone-based therapy in future clinical trials.

Clin Cancer Res; 20(24); 6570–81. ©2014 AACR.

Introduction

Salivary duct carcinoma (SDC), a rare and aggressive epithelial malignancy of major and minor salivary glands, presents de novo or more commonly as carcinoma transformation of pleomorphic forms in elderly patients of both sexes (1–3). The tumor afflicts more males than females and runs a progressively fatal course (4, 5). Patients with primary resectable tumors are treated by complete surgical resection, lymph node dissection, and postoperative radiotherapy (6). Therapeutic options for patients with an advanced unresectable primary, recurrent, and metastatic disease, however, are markedly limited (6, 7). Several chemotherapeutic agents, including SDC have been conducted with disappointing results (8–11). To advance the management of patients with SDCs, extensive efforts are being taken to characterize their molecular composition of this entity and to identify biologic targets for therapy.

A unique characteristic of SDCs is their remarkable phenotypic and biologic resemblance to high-grade mammary ductal carcinoma. Moreover, several immunohistochemical (IHC) studies have demonstrated androgen receptor (AR) gene activation in SDC, as in prostate and breast carcinomas (12–17). Interestingly, although AR is expressed in epithelial cells of reproductive organs including prostate and breast, it is undetected in normal salivary glands (18). These findings together with the selective induction of AR in SDC, exclusive of other salivary carcinoma subtypes, and the reported response to AR suppression therapy, commonly used in patients with primary prostate carcinoma (19), in several patients with
SDC (20, 21) provide a compelling rationale for the potential use of hormone-based therapy in a subset of patients with this cancer. However, aberrant AR gene activation is frequently associated with complex regulatory modifications and alterations, including growth factors phosphorylation (22, 23), gene amplification (24, 25), mutations (26, 27), and isoform formation (28–32) which have been linked to hormonal resistance in patients with prostate cancer.

AR is a type-I transcriptional factor that regulates downstream response genes associated with the normal homeostasis of reproductive organs including prostate, endometrial, and breast (33, 34). AR differs from other steroid receptors in having a single functional copy in females and one allele on the X chromosome in males. The AR gene is located on chromosome Xq 11–12 and spans 180-kb segment of DNA that contains 8 canonical exons (33, 34). The full-length AR gene encodes a 110-kDa protein with four major functional domains; the N-terminal transactivation domain (NTD) encoded by exons 5–8, the DNA-binding domain encoded by exons 1 and 2, the hinge region encoded by exon 4 and the ligand-binding domain (LBD) encoded by exons 5–8 (35). Upon androgen binding to the LBD, the AR undergoes conformational changes, translocates from the cytosol to the nucleus, and binds to specific androgen-responsive elements to induce gene expression, activating transcription of AR-responsive genes (36). As the AR target gene activation has been shown to be dependent on cell and organ context (37), detailed analysis of the AR gene in SDC is fundamental in hormonal therapy planning of male and female patients with SDC.

In this study, we comprehensively investigated the molecular alterations associated with AR activation in SDC from female and male patients and performed in vitro and animal studies using the only available SDC cell line.

Materials and Methods

SDC tissue specimens

Patients were treated at The University of Texas MD Anderson Cancer Center (Houston, TX) between 1981 and 2011. The study was approved by the MD Anderson Cancer Center Institutional Review Board. A search of the head and neck tissue banks for SDC either de novo or as a Ca ex-PA yielded 35 sufficient frozen specimens for tumor and matching normal with sufficient fresh frozen tissue specimens. All fresh tumor specimens were collected from primary tumors before any treatments and their corresponding archived tumor blocks were retrieved. All fresh tissue samples had been immediately harvested from surgical specimens and placed in liquid nitrogen, then transferred and stored at –80°C until used.

Immunohistochemistry

AR immunohistochemical staining was performed on 4 μm thick sections of tissue microarray blocks using the AR mouse monoclonal antibody to the NTD (clone AR441; Dako) diluted with 1 to 50 dilutions. The AR expression was scored on the basis of the extent and intensity of nuclear and/or cytoplasmic staining in tumor cells in a binary fashion. Tumors were categorized as negative if no staining and/or faint and heterogeneous nuclear and/or cytoplasmic staining in <10% cells and positive if strong and homogeneous nuclear, and/or cytoplasmic staining was found in >70% tumor cells.

Western blotting

Protein was extracted as a whole-cell lysates from fresh tumor tissues and cell lines using NP-40 buffer. Aliquots of 30 μg of protein were loaded on SDS-PAGE gel and Western blotting was performed using anti-AR (N-20; Santa Cruz Biotechnology), anti-AR (EP670Y; Abcam), or anti-ACTB (Sigma-Aldrich) antibodies.

RT-PCR for AR isotype characterization

Total RNA was extracted using RNeasy Universal Kit (Qiagen). The first-strand cDNA was synthesized using 2 μg of total RNA by oligo(dT) primer and the SuperScript III reverse transcriptase (Invitrogen). The RT-PCR was performed then using the variant-specific primers (Supplementary Table S1) for detection of AR mRNA splice variants. The quantitative RT-PCR was performed using the Applied Biosystems 7900HT Real-Time PCR Systems (Applied Biosystems) with KAPA SYBR FAST kit (KAPA Biosystems). AR-fl, AR-45, and AR-V7/AR3 primers (Supplementary Table S1) were used for the target and the ACTB gene was used as an internal control; 5’-TCACCGAGCGC-GGT-3’ and 5’-TAATGTCACGCAGATTCCC-3’. Duplicate samples were analyzed and ΔCt method [ΔCt = (Ct of target genes) – (Ct of internal control gene (ACTB))] was done for the quantification of target genes. Relative expression was calculated using AR-fl expression level in LNCaP as one, arbitrarily.

AR copy number status

To screen for AR copy number abnormality, FISH was performed on touch preparations of fresh SDC/adenocarcinoma specimens using vysis LSI Androgen receptor probe Qx12 spectral red and centromeric X chromosome probe DXZ1 spectral green (Abbot Laboratories). To determine the AR amplification status, 200 individual nuclei were analyzed for each case and amplification was
mitani et al.

defined when the presence of ≥10 copies/tumor cell in ≥20% of cells was observed. The interphase nuclei were captured and processed using the Quantitative Image Processing System (Applied Imaging).

TaqMan Copy Number Assay (Applied Biosystems) for AR gene (Hs00034522_cn) was performed using the 7900HT Fast Real-Time PCR Systems (Applied Biosystems) according to the manufacturer’s protocol. The RNaseP gene was used as an internal standard. Triplicate samples for each tumors and normal human male genomic DNA (G1471, Promega) as a reference control were analyzed. Relative AR copy number for each tumor was estimated by using the Copy-Caller-Software, v.1.0 (Applied Biosystems).

**AR mutation analysis**

Genomic DNA was isolated from the fresh frozen tissues and cell lines using the Gentra Puregene tissue kit (Qiagen) according to the manufacturer’s protocols. Quality of genomic DNA was checked by an A260/A280 ratio of more than 1.8 by Nanodrop and quality checked by agarose gel electrophoresis. Sanger DNA sequencing was performed for all exons of AR gene mutation analysis. PCR primer sets were described in Supplementary Table S1. Genomic DNAs were amplified by PCR using KAPA 2G fast (KAPA biosciences), purified using Exo-Sup and S1. Genomic DNAs were amplified by PCR using KAPA 2G fast (KAPA biosciences), purified using Exo-Sup and then analyzed by Applied Biosystems 3730×1 DNA analy- lyzer at GENEWIZ, Inc.

**Cell culture and treatments**

The RET981 developed by our group, is the only cell line, currently available in the field. RET981 was derived from a female patient with metastatic poorly differentiated, mixed malignant tumor of salivary gland (38). We tested the STR analysis and indicated the unique profile without any contamination (Supplementary Fig. S1). RET981 and LNCaP prostate cancer cell line (ATCC) were maintained in RPMI1640 medium with 10% FBS. A253 salivary epi- dermoid carcinoma cell (ATCC) and VCaP prostate cancer cell line (ATCC) were cultured in DMEM with 10% FBS. For androgen treatment, cells were cultured in phenol red–free medium supplemented with 10% charcoal-stripped FBS (CSS; Invitrogen) for 24 hours and then treated with or without 1 nmol/L DHT.

**Immunofluorescent staining**

Cells were seeded on Nunc Lab-Tek II Chamber slides in regular culture medium, and then transferred to CSS medi- um after 24 hours for androgen deprivation test. Cells were then cultured for 3 days under the CSS condition, and then cells were fixed using fresh 4% paraformaldehyde. Fixed cells were incubated with the primary anti-AR (N-20) anti- body at 4°C for overnight. Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen) was used as a secondary antibody.

**AR knockdown by siRNA**

Cells were transfected with siRNAs designed AR exon 1 (E1-3, 5'-CCUUUCAAGGGAGGUUACA-3'; E1-4, 5'-CAA-

GGAGGUUACACAAA-3'), AR exon 6 (E6; 5'-CACUG-

CIALCILICUCAGCAU-3'), AR exon 7 (E7-3; 5'-GGA-

ACUCCGAUCAUCAU-3') using jetPRIME reagent (Polyplus transfection). The MISSION siRNA Universal Negative Control (SIC001; Sigma) was used as a control.

**Cell growth assay**

Cells were seeded at a density of 5,000 cells per well in 96-well plates. Cells were transfected with AR siRNAs under the regular condition or treated with 1 nmol/L DHT under the androgen-depleted conditions after 24 hours seedling (0 day), and monitored at 1, 2, 4, and 6 days by MTT assay.

**Soft agar colony formation assay**

Cells were plated in 0.3% agarose mixed complete media mixed with 0.3% agarose. Cells were incubated at 37°C and fed twice a week for 2 weeks. Colonies were inspected and stained with crystal violet.

**In vivo studies**

RET cells (1 × 10⁶ cells/mouse) were mixed with or without equal volume of Matrigel (BD Biosciences) and injected subcutaneously in the flanks of 6- to 8-week-old male CB17 (SCID) mice (Charles River Laboratories). Tumor development was monitored and tumor sizes measured by caliper. Surgical castration was performed at 5 weeks after tumor inoculation. Animal studies were performed in accordance with regulations and standards of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, the NIH, and The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

**Results**

**Clinical and pathologic findings of SDC samples used in this study**

Table 1 presents the demographic clinicopathologic parameters and the AR alteration in patients with SDC in this study. Ages of the (20 males and 15 females) patients ranged from 42 to 86 years with a median age of 65 years. Tumors were located in the parotid in 32 patients: in each of the remaining 3 patients’ one tumor was located in the submandibular gland, one in the oral cavity, and one in the maxilla. Tumor size ranged from 1.0 to 7.0 cm (mean 3.5 cm). Complete staging information was available for 30 patients, 23 had stage IV, 4 had stage III, 1 had stage II, and 2 had stage I; 5 patients lacked staging information. The follow-up period ranged from 18 to 99 months with a median of 30 months. Of the 35 patients, 32 underwent additional postoperative radiotherapy and/or chemotherapy. Only one female patient (case # 610B8; Table 1) was treated with anti-AR therapy.

**AR expression and localization (IHC)**

AR IHC staining revealed positive staining in 27 (77%) of the 35 tumors. The majority of AR-positive tumors...
showed intense nuclear staining with faint cytoplasmic expression. Four tumors from 3 females and 1 male patient expressed strong cytoplasmic staining (Fig. 1A; Table 1 and Supplementary Table S2). None of the normal salivary ductal or acinar structures showed AR expression (Fig. 1A).

### Table 1. Clinicopathologic and androgen receptor status in salivary duct carcinoma in male and female patients

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Size (cm)</th>
<th>Metastasis(^a) (site)</th>
<th>Stage (TNM)</th>
<th>R(^a)</th>
<th>FU(^b)</th>
<th>AR Alterations in Salivary Duct Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>401G8</td>
<td>73</td>
<td>3</td>
<td>LN</td>
<td>IV</td>
<td>S, X</td>
<td>74</td>
<td>1-2 WT, V7, V3</td>
</tr>
<tr>
<td>458A1</td>
<td>79</td>
<td>1.5</td>
<td>LN</td>
<td>N/A</td>
<td>S, X</td>
<td>13</td>
<td>1 WT</td>
</tr>
<tr>
<td>563F1</td>
<td>54</td>
<td>3</td>
<td>LN, skin, brain</td>
<td>IV</td>
<td>S, X, C</td>
<td>34</td>
<td>1 WT, V7</td>
</tr>
<tr>
<td>560A1</td>
<td>69</td>
<td>3.5</td>
<td>LN</td>
<td>IV</td>
<td>S, X</td>
<td>39</td>
<td>1-2 WT, V7, V3</td>
</tr>
<tr>
<td>544C3</td>
<td>70</td>
<td>5</td>
<td>LN, bone</td>
<td>IV</td>
<td>S, X, C</td>
<td>27</td>
<td>2 WT, V7, V3</td>
</tr>
<tr>
<td>565E4</td>
<td>52</td>
<td>6</td>
<td>LN, brain</td>
<td>IV</td>
<td>S, X</td>
<td>25</td>
<td>2-3 WT</td>
</tr>
<tr>
<td>599B2</td>
<td>68</td>
<td>1</td>
<td>LN, lung, brain</td>
<td>N/A</td>
<td>S, X</td>
<td>21</td>
<td>1 WT</td>
</tr>
<tr>
<td>623E5</td>
<td>71</td>
<td>3.5</td>
<td>LN, skin, lung</td>
<td>IV</td>
<td>S, X, C</td>
<td>26(^a)</td>
<td>1 WT, 45, V7, V3</td>
</tr>
<tr>
<td>629D3</td>
<td>62</td>
<td>1.7</td>
<td>LN, bone, liver</td>
<td>IV</td>
<td>S, X, C</td>
<td>24(^a)</td>
<td>1 WT, 45</td>
</tr>
<tr>
<td>650E2</td>
<td>51</td>
<td>2</td>
<td>LN</td>
<td>IV</td>
<td>S, X</td>
<td>17(^a)</td>
<td>2 WT, V7</td>
</tr>
<tr>
<td>430A8</td>
<td>42</td>
<td>7</td>
<td>LN, lung, bone</td>
<td>IV</td>
<td>S, X, C</td>
<td>42</td>
<td>1 WT</td>
</tr>
<tr>
<td>465A4</td>
<td>54</td>
<td>2.2</td>
<td>I</td>
<td>S, X</td>
<td>97(^a)</td>
<td>1 WT</td>
<td></td>
</tr>
<tr>
<td>518F1</td>
<td>72</td>
<td>3.2</td>
<td>LN, lung, kidney</td>
<td>III</td>
<td>S, X</td>
<td>21</td>
<td>2 WT</td>
</tr>
<tr>
<td>530D2</td>
<td>58</td>
<td>2.5</td>
<td>LN</td>
<td>IV</td>
<td>S, X</td>
<td>57(^a)</td>
<td>2-3 WT, 45, V7, V3</td>
</tr>
<tr>
<td>540E3</td>
<td>71</td>
<td>3</td>
<td>LN, skin, bone</td>
<td>III</td>
<td>S, X, C</td>
<td>32</td>
<td>1 WT, 45, V7, V3</td>
</tr>
<tr>
<td>548A6</td>
<td>86</td>
<td>3.2</td>
<td>LN</td>
<td>IV</td>
<td>S, X</td>
<td>49</td>
<td>1 WT, V7, V1, V3</td>
</tr>
<tr>
<td>49F1</td>
<td>65</td>
<td>5</td>
<td>LN, colon</td>
<td>N/A</td>
<td>S, X</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>532H4</td>
<td>58</td>
<td>5</td>
<td>LN, lung, brain</td>
<td>IV</td>
<td>S, X</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>648B4</td>
<td>59</td>
<td>2.5</td>
<td>LN, lung, bone</td>
<td>N/A</td>
<td>S, C</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>167H1</td>
<td>84</td>
<td>7</td>
<td>LN, skin</td>
<td>N/A</td>
<td>S, X</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>495E6</td>
<td>85</td>
<td>5</td>
<td>LN</td>
<td>IV</td>
<td>S, X</td>
<td>21</td>
<td>2 WT</td>
</tr>
<tr>
<td>545F2</td>
<td>69</td>
<td>6</td>
<td>LN</td>
<td>IV</td>
<td>S, X, C</td>
<td>47(^a)</td>
<td>2 WT, 45, V7, V1</td>
</tr>
<tr>
<td>340H1</td>
<td>65</td>
<td>1</td>
<td>LN, bone</td>
<td>IV</td>
<td>S, X</td>
<td>36</td>
<td>2 WT, 45, V7</td>
</tr>
<tr>
<td>527E5</td>
<td>55</td>
<td>4</td>
<td>—</td>
<td>II</td>
<td>S, X</td>
<td>62(^a)</td>
<td>3-6 WT</td>
</tr>
<tr>
<td>610B8</td>
<td>53</td>
<td>4</td>
<td>LN</td>
<td>IV</td>
<td>S, X, C</td>
<td>36(^a)</td>
<td>2-3 WT</td>
</tr>
<tr>
<td>619C7</td>
<td>53</td>
<td>2</td>
<td>—</td>
<td>IV</td>
<td>S, X, C</td>
<td>27(^a)</td>
<td>2 WT, 45, V7</td>
</tr>
<tr>
<td>570F3</td>
<td>54</td>
<td>3</td>
<td>LN</td>
<td>IV</td>
<td>S, X</td>
<td>8</td>
<td>2 WT, 45, V7</td>
</tr>
<tr>
<td>636A8</td>
<td>68</td>
<td>1.5</td>
<td>—</td>
<td>I</td>
<td>S</td>
<td>15(^a)</td>
<td>Mul WT</td>
</tr>
<tr>
<td>438E6</td>
<td>58</td>
<td>3</td>
<td>LN</td>
<td>III</td>
<td>S, X</td>
<td>99(^a)</td>
<td>1 WT</td>
</tr>
<tr>
<td>688A5</td>
<td>74</td>
<td>N/A</td>
<td>LN, bone, spleen</td>
<td>IV</td>
<td>S</td>
<td>3</td>
<td>2 WT</td>
</tr>
<tr>
<td>625D3</td>
<td>66</td>
<td>2.5</td>
<td>LN, lung, brain</td>
<td>IV</td>
<td>S, X</td>
<td>26</td>
<td>2 WT</td>
</tr>
<tr>
<td>544B8</td>
<td>68</td>
<td>2.5</td>
<td>LN, lung</td>
<td>IV</td>
<td>S, X</td>
<td>29</td>
<td>2-3</td>
</tr>
<tr>
<td>652G7</td>
<td>51</td>
<td>3.5</td>
<td>LN, lung</td>
<td>IV</td>
<td>S</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>403H1</td>
<td>64</td>
<td>3.5</td>
<td>LN, brain</td>
<td>IV</td>
<td>S, X, C</td>
<td>31</td>
<td>3-5</td>
</tr>
<tr>
<td>548B6</td>
<td>47</td>
<td>5</td>
<td>—</td>
<td>III</td>
<td>S, X, C</td>
<td>32</td>
<td>2</td>
</tr>
</tbody>
</table>

**NOTE:** All SDCs arose in parotid gland, except 563F3 (maxilla), 560A1 (submandibular), and 532H4 (tongue).

\(^a\)R, treatment; S, surgery; X, radiation; C, chemotherapy. Patient 610B8 was treated with androgen blockage therapy (Lupron + Casodex).

\(^b\)Follow-up (FU) period (months), survival months from diagnosis to present.

\(^c\)LN, locoregional lymph node. —, no metastasis.

\(^d\)AR status by IHC. N, nuclear staining; C, cytoplasmic staining. —, negative. AR and X chromosome copy numbers (CN) determined by FISH. AR copy number increased with X chromosome gain. No amplification of AR gene was detected. Mul, multiple copy number variation was detected in 636A8.

\(^e\)AR isoforms determined by Western blotting and RT-PCR. —, no isoforms.

\(^f\)Patients are still alive.

www.aacrjournals.org Clin Cancer Res; 20(24) December 15, 2014 6573

Downloaded from clincancerres.aacrjournals.org on April 14, 2017. © 2014 American Association for Cancer Research.
Figure 1. Screening of AR expression and variants in SDC tumors. A, AR protein expression by IHC. Composite panel of normal salivary tissue and different tumor specimens of males (top) and females (bottom). Left, negative AR immunostaining of normal salivary duct structures. Middle, mainly nuclear and faint cytoplasmic staining in tumor cells. Right, both cytoplasmic and nuclear AR staining in SDCs. B, a schematic cartoon of the AR gene. Arrows denote the RT-PCR primers used to identify AR splice variants. The nucleotide sequences and genomic organization of full-length AR (NM_000044) and N-terminal truncated AR from (NM_001011645) were obtained from the NCBI website. The AR splice variants, AR-V7/AR3 and AR-V3, were reported previously (29, 30). C, representative RT-PCR gel image of the AR transcripts in both male and female SDCs. AR-fl were expressed in all SDCs that were AR positive by IHC. D, quantitative RT-PCR analysis revealed that AR-V7/AR3 variants were highly expressed in both male and female cases. Relative expressions were calculated using AR-fl expression level in LNCaP as 1 (arbitrary).
AR splice variants by RT-PCR analysis

Recent studies in prostate cancer indicated that AR gene activation by forming AR splice variants has been linked to hormonal therapy resistance (28–32). To search for the presence of splice variants, we performed RT-PCR (AR splice structures and the primer sets that were used are depicted in Fig. 1B and Supplementary Fig. S2A). The most frequent variant was the AR-V7/AR3 which was detected in 9 male and 4 female tumors (Fig. 1C). The AR-V3 variant was identified in 7 male tumors (weak PCR band; Fig. 1C). The AR-V1/AR4 variant was confirmed in the tumors of 1 male and 1 female patient each (Supplementary Fig. S2B). A faint band corresponding to the AR-45 variant was noted in the tumors of the patients (4 males and females each). All AR-45 variants except in one tumor (629D3) were concurrently found with the AR-V7 variant. Furthermore, we performed quantitative RT-PCR using selective cases (4 AR-positive and one AR-negative in each male and female) and then confirmed AR-V7/AR3 expression in the tumors of both male and female patients (Fig. 1D). The ARV567ca variant (Supplementary Fig. S2B) was not found in any of the tumors.

Screenings for AR splice variants (Western blotting)

To confirm for the PCR bands that correspond to recognized AR splice variants, we performed Western blotting analysis on all 35 tumors with the use of anti-AR antibodies that recognizes the N-terminal (N-20) and the C-terminal (EP670Y) on all 35 tumors. VCaP prostate cell lines reported to express multiple splice variants (29) was used as a control. Western blotting with use of N-terminal AR antibody showed that VCaP expressed few extra bands in addition to full AR (Fig. 2A). In contrast, RET981 cell line, representing the SDC subtype, expressed AR without any extra band by N-terminal AR antibody and AR is present in nuclear (Fig. 2A). Figure 2B presents selective examples of the tumors analyzed. We identified multiple and variable molecular size bands in addition to the AR full-length (AR-fl) by the N-terminal antibody in all AR-expressing tumors. An 80-kDa band was detected by the N-terminal antibody in several tumors from both male and females. The C-terminal antibody showed multiple bands with notable bands of 70 and 55 kDa sizes (Fig. 2B, asterisk marks) which were also detected in VCaP cells. Several additional bands at 87 and 60 kDa were identified in cell lines and SDC tumors and these were considered to represent proteolytic products (39, 40).

Detection of AR gene copy number change

To examine whether the aberrant AR gene expression is due to gene amplification or gain of chromosome, we performed FISH analysis by using probes for AR gene (Xq12, red signal) and centromeric X chromosome (green signal) on touch preparations of fresh specimens from all 35 tumors. No amplification of AR was detected in any tumors. In AR-positive cases by IHC, copy number analysis by FISH revealed a gain of X chromosome and extra AR gene copy with heterogeneity of clones present in the tumors of 7 of 16 (44%) male patients and 3 of 11 (27%) female patients (Fig. 2C, left; Table 1 and Supplementary Table S2); three SDCs from females had gained 3 copies of the X chromosome. In AR-negative cases, one of 4 male and two of 4 female patients had the gain of X chromosome. One tumor from a female patient had loss of one X chromosome copy (Table 1). RET981 SDC cells showed the extra copy with X chromosome and AR (Fig. 2A). The TaqMan copy number assay also revealed increased levels of the AR gene in both male and female patients (Fig. 2C, right).

Mutation analysis of AR gene in SDCs

We performed Sanger sequence analysis of all AR exons in the 35 SDCs and the RET981 cell line. Two different synonymous mutations in exon 1 (p.E213E and p.Q24Q) were detected (Table 1 and Supplementary Table S3); one tumor had mutation of the p.Q24Q and nine at the p. E213E site. p.E213E (c.639G>A, dbSNP re#; rs6152) has been sequenced in 1000 Genome project as SNP and p. Q24Q (c.72G>A, dbSNP re#; rs199644815) was included in refSNP cluster database (http://www.ncbi.nlm.nih.gov/snp/). Our CAG (range 18–27; median 21) and GGN (range 19–24; median 23) repeats analysis showed a within general range counted (41, 42).

AR analysis in SDC cell line

To determine the tumorigenic and potential biologic role of AR in SDCs, we analyzed the effect of AR down-regulation by siRNAs targeted NTD (exon 1, siAR-E1-3 and E1-4) and LBD (exon 6, siAR-E6 and exon 7, siAR-E7-3). Transient knockdown of AR protein by all siRNAs was confirmed in RET981 and LNCaP by Western blotting (Fig. 3A). Interestingly, siRNAs targeting exon-1 inhibited cell growth of the RET981 more drastically than targeting the LBD domain and cleaved PARP was increased after treatment for all siRNAs used (Supplementary Fig. S3). In contrast, LNCaP cell growth was inhibited by all AR siRNAs (Fig. 3A). AR siRNAs had no impact on growth of A253 cells.

To evaluate the effect of androgen on RET981, the MTT assay was performed under the androgen-depleted condition. The androgen-sensitive LNCaP cell line was used as a control. The RET981 growth showed androgen-independent growth in contrast to the LNCaP. The addition of DHT to the CSS medium showed no increase in the RET981 cell growth (Fig. 3B, left). The soft agar analysis showed no difference in colony formation of the RET981 in both regular and CSS conditions (Fig. 3B, right). In addition, we tested the AR subcellular localization in RET981 cell comparing the regular FBS media and CSS conditions. The addition of DHT to the CSS medium increased levels of the AR gene in both male and female patients (Fig. 2C, right).

In vivo tumor growth model using SDC cell

We injected RET981 cells to 5 SCID mice (2 sides per mouse with and without the Matrigel) subcutaneously
Figure 2. AR splice variants by Western blotting and AR genomic abnormality in SDCs. A, left, RET981 cells have a nuclear positive AR immunostaining. RET981 cell line shows the full-length AR (AR-fl) band (middle). Right, FISH analysis in RET981 cells using dual AR and chromosome X probes. B, Western blot of IHC AR-positive and -negative tumors using an N-terminal (N-term) and C-terminal (C-term) antibodies (top and bottom panels). In both (top) AR-positive male and female tumors, a band near the 110 kDa representing the full-length AR (AR-fl) was identified. Note that multiple AR-positive tumors showed extra bands at approximately 85 kDa that may represent the AR splice variants (AR-Vs). Bottom (C-terminal), multiple bands of unknown identity. C3, the bands detected at around 70 and 55 kDa were also identified in VCaP cells. C, left, representative FISH images of dual AR and chromosome X probes in male (top) and female (bottom) tumors. No abnormality of AR gene in both sexes (left) and gains of both AR and chromosome X in both male and females tumors in the right two panels. Right, TaqMan copy number analysis for AR gene identified the increase of AR copy number in both male and female SDCs.
Figure 3. SDC cell growth in response to AR knockdown. A, RET981, LNCaP, and A253 cells were transfected with AR siRNAs targeted exon 1 (E1-3 and E1-4) and LBD (E6 and E7-3). Western blot analysis with anti-AR (N-20) antibody demonstrated the knockdown of AR in RET981 and LNCaP cells by all siRNAs. Cell growth was monitored by MTT assay at indicated day points, and AR siRNAs inhibited the cell growth in both RET981 and LNCaP cells. B, left, cell growth curves were determined by MTT assay under the regular medium (FBS) or AR-depleted (CSS) or CSS with 1 nmol/L DHT conditions. The cell growth of RET981 showed the androgen-independent manner. Right, top, colony formation in soft agar with (FBS)/without androgen (CSS). Bottom, histogram indicates the mean colony numbers in 3 different wells of a 6-well plate. RET981 had no impact of colony formation with/without androgen. C, AR immunofluorescence under the FBS and CSS conditions. AR localization was nuclear in both conditions. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). D, RET981 cell were injected subcutaneously with or without an equal volume of Matrigel in the flanks of SCID mice. Left, the histogram shows the individual tumors grew before and after castration. Tumor formation rate was 100% in this study. Surgical castration was performed at 5 weeks after tumor inoculation. The tumor growth was monitored weekly. Right, average tumor size in response to castration. All tumors grew after the surgical castration.
and found that all of them grew tumors (Fig. 3D, left). Although tumors with Matrigel grew more than did those without Matrigel, the differences between the two were not statistically significant. After confirmation of 100% tumor taking rate, we then castrated these mice and monitored the growth of these tumors. After castration of the mice, 6 tumors continued to grow requiring animal sacrifice after only a week (Fig. 3D). Smaller tumors also continued to grow in 4 other tumors and were sacrificed 3 weeks after castration. Figure 3D (right) is representing the average tumor size in response to castration, and the increase in tumor growth after castration was statistically significant ($P < 0.05$), suggesting castrate resistance.

**Clinicopathologic and AR status correlation**

Table 2 presents the clinicopathologic correlation of males and females with SDC. As expected for this high-grade malignant and aggressive entity, there were no statistically significant differences between AR expression and alterations with the major clinicopathologic parameters. Both males and females SDCs expressed comparable AR. Although no significant statistical correlation was found, the results show that male AR-positive tumors manifest a higher incidence of splice variants (50%) in contrast to (26.6%) in female tumors. As expected the high stage at presentation and the aggressive clinical course and the small number of patients with negative AR led to the lack of association between these factors. Detailed therapy review of patients revealed that only one female patient with AR-positive tumor underwent anti-AR therapy (lupron and casodex). The patient did not respond to treatment.

<table>
<thead>
<tr>
<th>Table 2. Clinicopathologic correlations of androgen receptor alteration in patients with salivary duct carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>&gt;60</td>
</tr>
<tr>
<td>≤60</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>I or II</td>
</tr>
<tr>
<td>III or IV</td>
</tr>
<tr>
<td>Follow-up</td>
</tr>
<tr>
<td>DOD</td>
</tr>
<tr>
<td>Alive</td>
</tr>
</tbody>
</table>

$^a$G (gain), L (loss), and N (normal) of AR copy number by FISH.

$^b$P value was calculated by the Fisher exact test.

$^c$Follow-up was performed on 30 patients for a minimum of 3 years. DOD, died of disease.

**Discussion**

Our study identified variable molecular and structural alterations of the AR gene in SDCs from both male and female patients. The spectrum of the alterations consisted of variable compartmental cellular expression, extra gene copy, synonymous mutations, and alternative splicing. However, a subset of tumors from both sexes was found to contain gene alteration without AR expression. The underlying factors for the lack of AR activation in these tumors are being investigated. In this study, in contrast to prostate cancer studies (43), no activating mutations, gene amplification, or CAG repeat length abnormalities were detected in advanced therapy-naïve SDCs. Moreover, the finding of these alterations in tumors of female patients raises the possibility that AR transcriptional activation and biologic effect could be induced by either a ligand-independent mechanism or intracrine androgen production (44, 45). Our in vivo and in vitro analyses of the female derived tumor cell line RET981, however, lend support to potential ligand-independent AR activation hypothesis. This is further underscored by the consistent nuclear translocation of AR in tumor cells under androgen deprivation conditions. Although, the precise mechanism of AR biologic role in female tumors and cell lines remain uncertain, we contend that low level androgen and/or undetected receptor modifications may lead to persistent AR nuclear translocation and ligand-dependent activation.

We report, for the first time, the presence of multiple AR isoforms in both male and female patients with SDCs with a splicing pattern similar to those reported in hormone-resistant prostate carcinoma (28–30, 46, 47). The most...
common isoform in both male and female patients is AR-V7/AR3. This isoform develops as a result of an intragenic splicing at cryptic exon 3, and lacks the LBD region of the AR gene (29, 30). Interestingly, another isoform, AR-V3, which results from cryptic splicing at exon 2 with loss of the LBD (29, 30), was detected in a subset of tumors from male patients and but not in AR-positive tumors from female patients. We also identified the previously described short AR-45 isoform (46) in a few tumors from males and females. This isoform lacks exon-1 and the NTD region and has been shown to act independently or in combination with the full-length AR in a dominant-negative manner (46, 47). The role and effect of this and other isoforms on the AR transcriptional activation and response to anti-hormonal therapy in patients of SDC remain unknown. Future availability of male and female derived cell lines expressing these isoforms will allow for determining the biologic role of these isoforms. We, however, posit that the identification of AR isoforms in primary untreated SDCs could affect tumor response to antiandrogen agents and may potentially guide the stratification of patients for hormonal-based therapy. This possibility has recently been supported by the successful response of isoform-expressing tumors to targeted therapy (48) and the results of knockdown AR and its variants in prostate cancer cells demonstrating distinctive expression profile in ligand-independent growth and aggressive morphologic features (30, 49). These findings, nonetheless, must also be considered in view of the possible synergistic interaction between the full-length transcript and splice variants in the activation of AR (29, 31, 32), through binding to certain coregulatory factors. This possibility is particularly cogent in SDC, in which organ, sex, and constitutive hormonal context, are characteristically different from those types in reproductive organ–derived tumors.

Our in vitro functional analysis using the RET981 cells showed that SDC cells required AR for cell growth based on the effective proliferative inhibition by AR siRNAs targeted exon1. Similar results in mammary carcinoma of human and animal models have been reported (50, 51). Interestingly, the pattern of inhibition to targeted exon-1 and LBD siRNAs was distinctly different from that observed in LNCaP cell line further underscoring the organ context nature of AR activation. This together with our evidence for a ligand-independent growth of tumors in castrated mice under anchorage-independent condition is consistent with the fact that the RET981 cell line is derived from a female tumor. In that context, female patients that express strong nuclear AR expression either due to the presence of low level circulation androgen or to receptor modifications could be treated as androgen-resistant prostate cancer. Further investigations to validate these findings along with studies of male derived cell lines are being conducted.

The extra copy of the X chromosome and AR gene was found in almost 40% of SDCs from both sexes. Interestingly, an extra AR gene copy was also found in the RET981 cell line derived from a female tumor. The biologic effect and the functionality of the extra AR copy are currently uncertain. However, our findings resemble those reported in other prostate cancer studies in which the extra copy of the AR gene was found in CRPCs (24) and in hormone-naïve prostate carcinoma (52). In contrast to advanced hormonal-refractory prostate carcinomas, however, no evidence for AR gene amplification was found in primary SDC (24, 25). Although, the biologic effect of elevated AR level in tumors with extra copy is unknown, a possible dose related effect is likely. We, however, observed weak AR expression in a few tumors with X chromosome and AR copy gain raising the possibility that either inactivation and/or epigenetic modification of the AR expression may play a role. (53–55). Of interest is the identification of the p.E213E SNP, as in prostatic hyperplasia (56), in 4 of our female patients. The significance of this finding is currently unknown. Similarly, our AR expression analysis indicates that although ligand-based nuclear AR translocation and activation are dominant, considerable cytoplasmic components remained untransported in certain tumors. The findings suggest that either a lack of ligand and/or a disruption of receptor–ligand binding underlie the cytoplasmic sequestration in these tumors. Whether cytoplasmic AR induces a nongenomic transcriptional activation is currently unknown.

Although limited, our clinicopathologic findings, as anticipated, showed no significant correlation between AR gene expression and alterations and clinicopathologic factors. We observed, however, that patients with AR-negative tumors run a more lethal course than AR-positive with and without gene aberrations. Similar observations linking the lack of AR expression to aggressive behavior of patients with prostate cancer has also been reported (57, 58). It is interesting that only one patient with SDCs, treated postoperatively with antiandrogen therapy, was a female with AR positive and failed to respond. Assuming similarity to prostate carcinoma, tumors from male patients with splice variants along with females, can potentially be considered refractory to hormone deprivation treatment (59, 60). More importantly, the potential association of the AR aberration, as in prostate, with response and development of resistance to hormonal therapy highlights their significance in future clinical trials for SDCs (20, 21).

In summary, the identification of multiple AR isoforms and extra copy of the AR gene in primary untreated SDCs of male and female patients provide new findings that may guide future use of hormonal- based targeted therapy. AR analysis allows for patients with AR (IHC)-positive tumors to be triaged on the basis of their sex, isoform, and/or copy number status to traditional treatment (for male) or to be managed as patients with castration-resistant (for female) prostate cancer.

Disclosure of Potential Conflicts of Interest
L. Licitra is a consultant/advisory board member for Astrazeneca, Boehringer Ingelheim, Eisai, Merck, and Sobi. No potential conflicts of interest were disclosed by the other authors.
Mitani et al.

Disclaimer
The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the NIH.

Authors' Contributions
Development of methodology: Y. Mitani, Y.-C. Lee, L. Licitra
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Mitani, Y.-C. Lee, I.C. Post, L. Licitra, S.A. Lippman, S.-H. Lin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Mitani, P.H. Rao, Y.-C. Lee, R. Ferrarotto, L. Licitra, S.M. Lippman, M.S. Kies, C. Caulin, S.-H. Lin
Writing, review, and/or revision of the manuscript: Y. Mitani, S.N. Maity, R. Ferrarotto, L. Licitra, S.M. Lippman, M.S. Kies, R.S. Weber, C. Caulin, S.-H. Lin, A.K. El-Naggar
Study supervision: L. Licitra, A.K. El-Naggar

References
Writing, review, and/or revision of the manuscript: Y. Mitani, S.N. Maity, R. Ferrarotto, L. Licitra, S.M. Lippman, M.S. Kies, R.S. Weber, C. Caulin, S.-H. Lin, A.K. El-Naggar
Study supervision: L. Licitra, A.K. El-Naggar

Acknowledgments
The authors thank Yan Cai, Deborah A. Rodriguez, and Stella U. Njoku for material retrieval and follow-up information, Courtney Johnson Miller for her secretarial and administrative assistance, and Ms. Tamara Locke for her valuable editorial review of the manuscript.

Grant Support
The study is supported in part by the NIH National Institute of Dental and Craniofacial Research and the NIH Office of Rare Diseases Research grant number U01DE019765, the Head and Neck SPORE program grant number P50CA097007, The Kenneth D. Muller Professorship, and an NCI CA-16672 grant.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 9, 2014; revised September 11, 2014; accepted September 17, 2014; published OnlineFirst October 14, 2014.


Ramifications
Salivary Duct Carcinoma of Both Sexes: Potential Therapeutic Alterations Associated with Androgen Receptor Gene Activation in


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-1746

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/10/14/1078-0432.CCR-14-1746.DC1

Cited articles
This article cites 60 articles, 24 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/24/6570.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/20/24/6570.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.