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Authors

Mitani, Yoshitsugu
Rao, Pulivarthi H
Maity, Sankar N
et al.

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

Yoshitsugu Mitani[1], **Pulivarthi H. Rao**[2], **Sankar N. Maity**[3], **Yu-Chen Lee**[4], **Renata Ferrarotto**[5], **Julian C. Post**[1], **Lisa Licitra**[6], **Scott M. Lippman**[7], **Merrill S. Kies**[5], **Randal S. Weber**[8], **Carlos Caulin**[8], **Sue-Hwa Lin**[4], and **Adel K. El-Naggar**[1,8]

[1]Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[2]Department of Pediatrics, Baylor College of Medicine, Houston, Texas, USA

[3]Genitourinary Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[4]Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[5]Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

[6]Head and Neck Cancer Medical Oncology Unit, Department of Medical Oncology, Fondazione IRCCS “Istituto Nazionaledei Tumori”, Milan, Italy

[7]Moores Cancer Center, University of California San Diego, San Diego, California, United States of America

[8]Head and Neck Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

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 were conducted. *In vitro* and *in vivo* animal studies were also performed.

Corresponding Author: Adel K. El-Naggar, Department of Pathology, Unit 85, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030; phone number: 713-792-3109; fax number: 713-745-3356; anaggar@mdanderson.org.

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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 shed more light on the putative functional role of AR in SDC cells. Based on these data, we propose that patients with SDC (male and female) can be stratified for hormone-based therapy in future clinical trials.

Keywords

Salivary duct carcinoma; Androgen receptor; Splice variants; Copy number alterations; Androgen Resistance

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 patients, however, are markedly limited (6, 7). Several chemo- and/or radio therapy-based targeted clinical trials of patients with advanced and metastatic salivary carcinomas 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 biological targets for therapy.

A unique characteristic of SDCs is their remarkable phenotypic and biological 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 isoforms formation (28–32) which have been linked to hormonal resistance in prostate cancer patients.

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 1 and 2, the DNA-binding domain (DBD) encoded by exons 2 and 3, the hinge region encoded by exon-4 and the ligand binding domain (LBD) encoded by exons 5 to 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). Since 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 SDCs.

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 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 prior to 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 (IHC)

AR immunohistochemical staining was performed on 4-μm thick sections of TMA blocks using the AR mouse monoclonal antibody to the N-terminal domain (clone AR441, DaKo) diluted with 1 to 50 dilutions. The AR expression was scored based on the extent and intensity of nuclear and/or cytoplasmic staining in tumor cells in a binary fashion. Tumors were scored negative if no staining and/or faint and heterogeneous nuclear and/or cytoplasmic staining in <10% cells and positive if strong and homogenous 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 RN easy 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, Carlsbad, CA, USA). The RT-PCR was performed then using the variants 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′-TCACCGAGCGCGGCT-3′ and 5′-TAATGTCACGCACGATTTCCC-3′. Duplicate samples were analyzed and ΔCT method (ΔC_t= [C_t of target genes] – [C_t of internal control gene (ACTB]) was done for the quantification of target gens. 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, fluorescent in situ hybridization (FISH) was performed on touch preparations of fresh SDC/adeno carcinoma specimens using vysis LSI Androgen receptor probe Xq12 spectral red and centromeric X chromosome probe DXZ1 spectral green (Abbot Laboratories, Des Plaines, IL). To determine the AR amplification status, 200 individual nuclei were analyzed for each case and amplification was 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 GentraPuregene tissue kit (QIAGEN, Valencia, CA) 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 then analyzed by Applied Biosystems 3730×1 DNA analyzer at GENEWIZ, Inc. (South Plainfield, NJ).

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 Figure 1). RET981 and LNCaP prostate cancer cell line (ATCC) were maintained in RPMI 1640 medium with 10% FBS. A253 salivary epidermoid carcinoma cell (ATCC) and VCaP prostate cancer cell line (ATCC) were cultured in DMEM medium 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 1nM DHT.

Immunofluorescent staining

Cells were seeded on Nunc® Lab-Tek™ II Chamber slides in regular culture medium, and then transferred to CSS medium after 24 hours for androgen depravation 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 (N20) antibody at 4°C for overnight. Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen) was used as a secondary antibody.

AR knock-down by siRNA

Cells were transfected with siRNAs designed AR exon 1 (E1-3, 5′-CCUUUCAAGGGAGGUUACA-3′; E1-4, 5′-CAAGGGAGGUUACACCAAA-3′), AR exon 6 (E6; 5′-CACUGCUACUCUUCAGCAU-3′), AR exon 7 (E7-3; 5′-GGAACUCGAUCGUAUCAUU-3′) using jet PRIME 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 5000 cells per well in 96- well plates. Cells were transfected with AR siRNAs under the regular condition or treated with 1nM DHT under the androgen-depleted conditions after 24 hours seeding (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 (1×10^6 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, Wilmington MA). Tumor development was monitored and tumor sizes measured by caliper. Surgical castration was performed at 5-weeks post 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 National Institutes of Health, 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 cm to 7.0 cm (mean 3.5 cm). Complete staging information was available for 30 patients, 23 had stage IV, four had stage III, one had stage II, and two had stage I; five patients lacked staging information. The follow-up period ranged from 18 to 99 months with a median of (30 months). All 35 patients and 31 underwent additional postoperative radio therapy (XRT) 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 three females and one male patient expressed strong cytoplasmic staining (Figure 1A, Table 1, and Supplementary table S2). None of the normal salivary ductal or acinarstructures showed AR expression (Figure 1A).

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 Figure 1B and Supplementary Figure S2A). The most frequent variant was the AR-V7/AR3 which was detected in (9 male and 4 female tumors, (Figure 1C). The AR-V3 variant was identified in seven male tumors (weak PCR band, Figure 1C). The AR-V1/AR4 variant was confirmed in the tumors of one male and one female patient each (Supplementary Figure S2B). A faint band corresponding to the AR-45 variant was noted in the tumors (4 males and females each) patients'. 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 (Figure 1D). The AR^{v567es} variant (Supplementary Figure 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 (Figure 2A). In contrast, RET981 cell line, representing the SDC subtype, expressed AR without any extra b and by N-terminal AR antibody and AR is present in nuclear (Figure 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 70kDa and 55kDa sizes (Figure 2B, asterisk marks) which were also detected in VCaP cells. Several additional bands at 87 kDa and 60kDa 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 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 (Figure 2C left panel, 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 had the gain of X chromosome. One tumor from a female patient had loss of one X chromosome copy (Table 1). Only three tumors with an extra-copy of the gene were negative for AR expression (Table 1 and Supplementary Table S2). RET981 SDC cells showed the extra copy with X chromosome and AR (Figure 2A). The TaqMancopy number assay also revealed increased levels of the AR gene in both male and female patients (Figure 2C right panel).

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 biological role of AR in SDCs, we analyzed the effect of AR down regulation by siRNAs targeted N-terminal domain (NTD) (exon 1, siAR-E1- 3 and E1-4) and Ligand binding domain (LBD) (exon 6, siAR-E6 and exon 7, siAR-E7-3). Transient knock down of AR protein by all siRNAs was confirmed in RET981 and LNCaP by Western blotting (Figure 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 Figure 3). In contrast, LNCaP cell growth was inhibited by all AR siRNAs (Figure 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 charcoal-stripped serum medium showed no increase in the RET981 cell growth (Figure 3B left panel). The soft agar analysis showed no difference in colony formation of the RET981 in both regular and CSS conditions (Figure 3B right panel). Additionally, we tested the AR subcellular localization in RET981 cell comparing the regular FBS media and CSS treated after 3 days. Interestingly AR remains translocated in the nuclei under both FBS and CSS conditions (Figure 3C).

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 and found that all of them grew tumors (Figure 3D, left panel). 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 (Figure 3D). Smaller tumors also continued to grow in 4 other tumors and were sacrificed 3 weeks after castration. Figure 3D right panel 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.

Clinico-pathologic and AR status correlation

Tables 1 and 2 present the clinicpathologic correlation of males and females with SDC. As expected for this high grade malignant and aggressive entity, there were no significant statistical difference between AR expression and alterations with the major clinicpathologic parameters. Both males and females SDCs expressed comparable AR. Although no significant statistical correlation was found, the results show that male 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 & Casodex). The patient did not respond to treatment.

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 biological 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 biological 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 (28, 29), 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 biological role of these isoforms. We, however, posit that the identification of AR isoforms in primary untreated SDCs could affect tumor response to anti-androgen 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 it's 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 with the caveat that synergistic interaction between the full-length transcript and splice variants in the activation of AR (29, 31, 32), through binding to certain co-regulatory factors, might occur. This possibility is particularly cogent in SDC, in which cell, sex, and constitutive hormonal context, are characteristically different from those types in reproductive organs derived tumors.

Our *in vitro* functional analysis using the RET981 cells showed that SDC cells required AR expression for cell growth as evidenced by the effective growth inhibition by AR siRNAs targeted exon 1. 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 biological 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 frequent gain of AR gene extra-copy was found in CRPCs (24) and in hormone-naïve prostate carcinoma (50). In contrast to advanced hormonal refractory prostate carcinomas, however, no evidence for *AR* gene amplification was found in primary SDC (24, 25). Although, the biological 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. (52–55). Of particular interest is the identification of the p.E213E SNP, as in prostatic hyperplasia (56), in four of our female patients. The significance of this finding is uncertain. Similarly, our phenotypic expression analysis indicates that although Ligand-based nuclear AR translocation and activation dominate the phenotypic expression of the receptor, considerable cytoplasmic component remained untransported. 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 non-genomic transcriptional activation is currently unknown.

Although limited, our clinicpathologic findings, as anticipated, showed no significant correlation between AR gene expression and alterations and clinicpathologic 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 prostate cancer patients has also been reported (57, 58). It is interesting that only one patient with SDCs, treated post-operatively with anti-androgen 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 (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 castration resistant (for female) prostate cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

The study is the first to define the molecular alterations associated with Androgen Receptor (AR) gene activation in primary untreated salivary duct carcinoma of both male and female patients. The findings together with evidence for an AR growth effect in a female tumor derived cell line and a Ligand-independent tumor progression in castrated mice provide novel information for AR-based targeted therapy in patients with SDC.

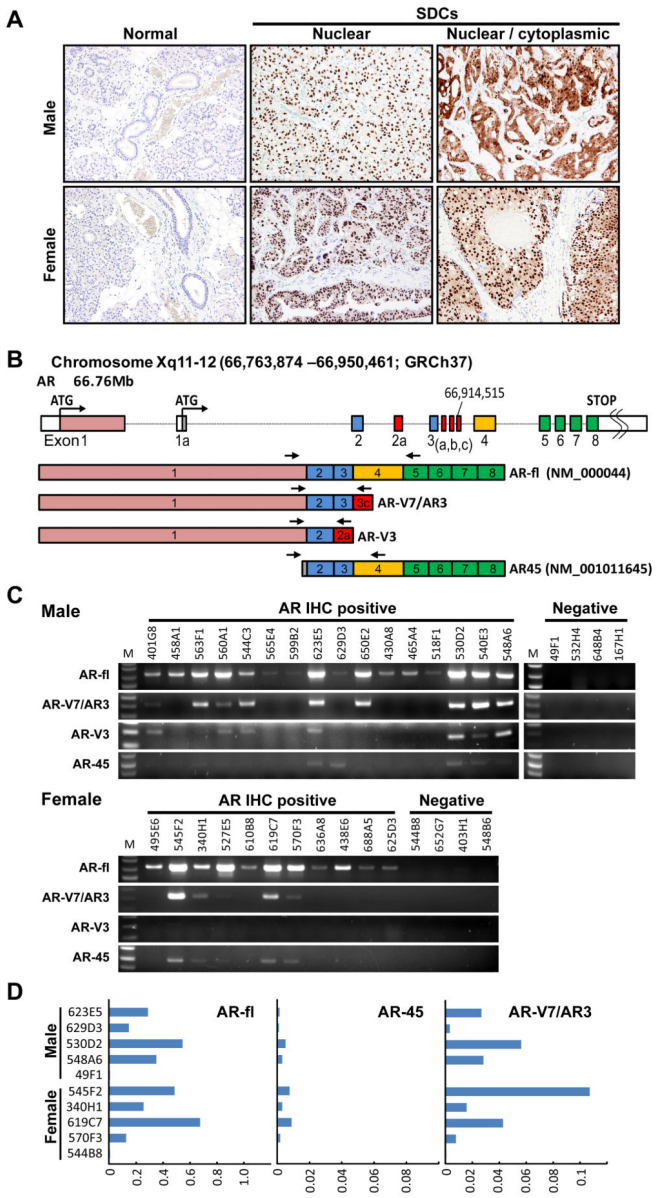

Figure 1. Screening of AR expression and variants in SDC tumors

(A) AR protein expression by IHC. Composite panel of normal salivary tissue and different tumor specimens of males (upper) and females (lower). Left panels; negative AR immunostaining of normal salivary duct structures. Middle panels depict mainly nuclear and faint cytoplasmic staining in tumor cells. Right panels illustrate 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 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

expressed high in both male and female cases. Relative expressions were calculated using AR-fl expression level in LNCaP as one, arbitrary.

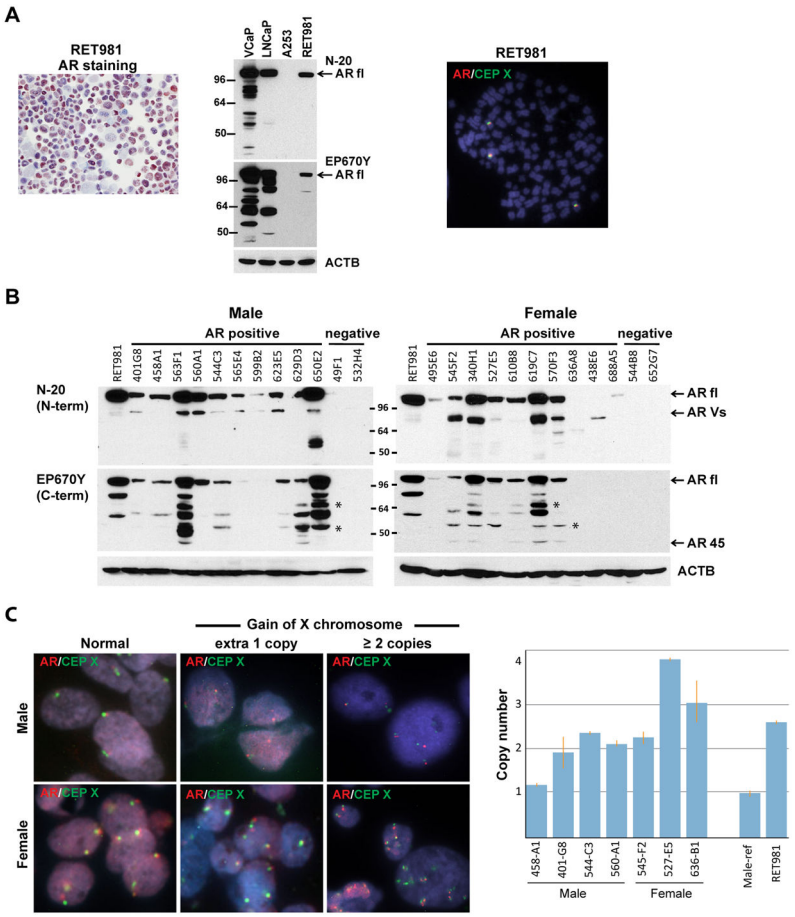

Figure 2. AR splice variants by Western blotting and AR genomic abnormality in SDCs

(A) Left panel show RET 981 cells have a nuclear positive AR immunostaining. RET981 cell line shows the full-length AR (AR-fl) band on middle panel. Right panel; FISH analysis in RET981 cells using dual *AR* and chromosome X probes. (B) A western blot of IHC AR positive and negative tumors using an N-terminal (N-term) and C-terminal (C-term) antibodies (upper and lower panels). In both (upper) 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 hands at approximately 85 kDa that may represent the AR splice variants (AR-Vs). Lower panel (C-terminal) show multiple bands of unknown identity. Note three tumors (two male and one female) show a distinct band at 45 kDa, which may represent the N-terminal truncated AR isoform. (C) Left panels; Representative FISH images of dual AR and chromosome X probes in male (upper) and female (lower) tumors. No abnormality of *AR* gene in both sexes (left panels) and gains of both *AR* and chromosome X in both male and females tumors in the right 2 panels. Right panel; TaqMan copy number analysis for *AR* gene was identified the increase of AR copy number in both male and female SDCs.

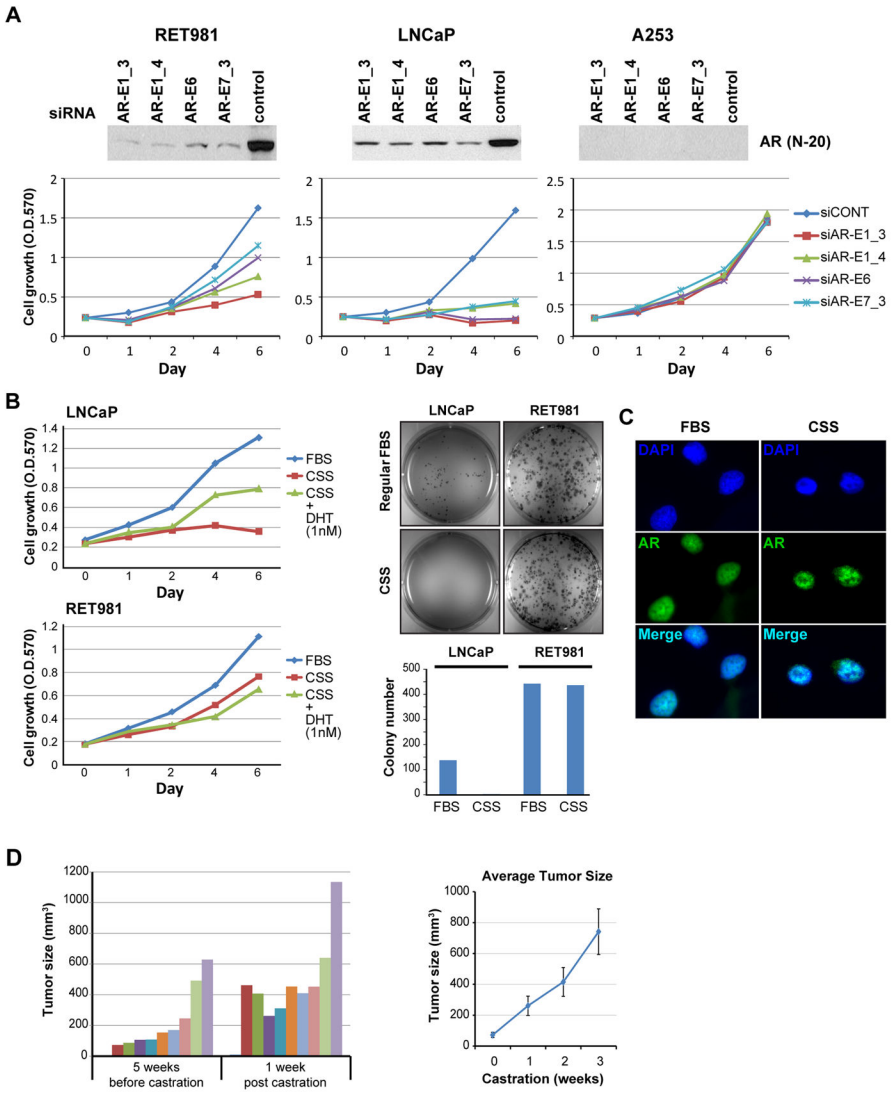

Figure 3. SDC cells growth in response to AR knock-down

(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 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 panels; Cell growth curve were determined by MTT assay under the regular medium (FBS) or AR-depleted (CSS) or CSS with 1nM DHT condition. The cell growth of RET981 showed the androgen independent manner. Right panels; Upper panels represent the image of Colony formation in soft agar with (FBS)/without Androgen (CSS). Lower panel of the histogram indicates the mean colony numbers in 3 different wells of 6-well plate. RET981 had no impact of colony formation with/without androgen. (C) AR immunofluorescence under the FBS and CSS condition. AR localized in nuclear in both condition. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Blue). Green; AR. (D) RET981 cell were injected

subcutaneously with or without equal volume of Matrigel in the flanks of SCID mice. Left panel; 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 post tumor inoculation. The tumor growth was monitored weekly. Right panel; average tumor size in response to castration. All tumors grew after the surgical castration.

Table 1

Clinico-pathologic and Androgen Receptor Data in Patients with Salivary Duct Carcinoma.

Sample	Age	Size (cm)	Metastasis[c] (Site)	Stage (TNM)	R/[a]	FU[b]	AR			
							IHC[d]	CN[e]	Variants[f]	mutation
MALE										
401G8	73	3	LN	IV	S, X	74	N	1–2	WT, V7, V3	-
458A1	79	1.5	LN	N/A	S, X	13	N	1	WT	-
563F1	54	3	LN, Skin, Brain	IV	S, X, C	34	N	1	WT, V7	-
560A1	69	3.5	LN	IV	S, X	39	N	1–2	WT, V7, V3	-
544C3	70	5	LN, Bone	IV	S, X, C	27	N	2	WT, V7, V3	p.E213E
565E4	52	6	LN, Brain	IV	S, X	25	N/C	2–3	WT	p.E213E
599B2	68	1	LN Lung, Brain	N/A	S, X, C	21	N	1	WT	-
623E5	71	3.5	LN, Skin, Lung,	IV	S, X, C	*26	N	1	WT, 45, V7, V3	p.Q24Q
629D3	62	1.7	LN, Bone, Liver	IV	S, X, C	*24	N	1	WT, 45	-
650E2	51	2	LN	IV	S, X, C	*17	N	2	WT, V7	-
430A8	42	7	LN, Lung, Bone	IV	S, X, C	42	N	1	WT	p.E213E
465A4	54	2.2	-	I	S, X	*97	N	1	WT	-
518F1	72	3.2	LN, Lung, Kidney	III	S, X, C	21	N	2	WT	-
530D2	58	2.5	LN	IV	S, X	*57	N	2–3	WT, 45, V7, V3	-
540E3	71	3	LN Skin, Bone,	III	S, X, C	32	N	1	WT, 45, V7, V3	-
548A6	86	3.2	LN	IV	S, X	49	N	1	WT, V7, V1, V3	p.E213E
49F1	65	5	LN colon	N/A	S, X	42	-	1	-	-
532H4	58	5	LN, Lung, Brain	IV	S, X, C	37	-	2	-	-
648B4	59	2.5	LN, Lung, Bone	N/A	S, C	3	-	1	-	p.E213E
167H1	84	7	LN, Skin	N/A	S, X	64	-	1	-	-
FEMALE										
495E6	85	5	LN	IV	S, X	21	N	2	WT	-
545F2	69	6	LN	IV	S, X, C	*47	N	2	WT, 45, V7, V1	-
340H1	65	1	LN, Bone	IV	S, X, C	36	N	2	WT, 45, V7	-

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Sample	Age	Size (cm)	Metastasis[c] (Site)	Stage (TNM)	R/[a]	FU[b]	AR			
							IHC[d]	CN[e]	Variants[f]	mutation
527E5	55	4	-	II	S, X	*62	N/C	3–6	WT	-
610B8	53	4	LN	IV	S, X, C	*36	N	2–3	WT	p.E213E
619C7	53	2	-	IV	S, X, C	*27	N	2	WT, 45, V7	p.E213E
570F3	54	3	LN	IV	S, X	8	N	2	WT, 45, V7	-
636A8	68	1.5	-	I	S	*15	N	Mul	WT	-
438E6	58	3	LN	III	S, X	*99	N/C	1	WT	p.E213E
688A5	74	N/A	LN, Bone, Spleen	IV	S	3	N	2	WT	-
625D3	66	2.5	LN, Lung, Brain	IV	S, X	26	N/C	2	WT	-
544B8	68	2.5	LN, Lung	IV	S, X	29	-	2–3	-	-
652G7	51	3.5	LN, Lung	IV	S	16	-	2	-	-
403H1	64	3.5	LN, Brain	IV	S, X, C	31	-	3–5	-	p.E213E
548B6	47	5	-	III	S, X, C	32	-	2	-	-

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

[a] R/= Treatment, S; Surgery, X; Radiation, C; Chemotherapy. 610B8 patient was treated by with androgen blockage therapy (Lupron + Casodex),

[b] Follow-Up (FU) period (months), survival months from diagnosis to present.

* Patients are still alive.

[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.

[f] AR variants determined by Western blotting and RT-PCR. (−): No variants.

Table 2

Clinicopathologic features of Androgen Receptor (AR) in Salivary Duct Carcinoma

Parameter	Expression			Copy Number[a]			Splice Variants		
	(+)	(−)	*p*[b]	G/L	N	*p*[b]	(+)	(−)	*p*[b]
Age									
>60	16	4		7	13		9	11	
≤60	11	4	0.70	7	8	0.73	5	10	0.73
Sex									
Male	16	4		8	12		10	10	
Female	11	4	0.7	6	9	1.0	4	11	0.30
Stage									
I or II	3	0		2	1		0	3	
III or IV	22	5	1.0	12	15	0.59	14	13	0.23
Follow up[c]									
DOD	12	5		5	12		5	12	
Alive	10	3	1.0	7	6	0.26	5	8	0.71

[a] G (gain), L (loss), and N (normal) of AR copy number by FISH

[b] *P* value was calculated by Fisher's exact test

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