Role for the Aryl Hydrocarbon Receptor and Diverse Ligands in Oral Squamous Cell Carcinoma Migration and Tumorigenesis

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Abstract

Over 45,000 new cases of oral and pharyngeal cancers are diagnosed and account for over 8,000 deaths a year in the United States. An environmental chemical receptor, the aryl hydrocarbon receptor (AhR), has previously been implicated in oral squamous cell carcinoma (OSCC) initiation as well as in normal tissue-specific stem cell self-renewal. These previous studies inspired the hypothesis that the AhR plays a role in both the acquisition and progression of OSCC, as well as in the formation and maintenance of cancer stem-like cells. To test this hypothesis, AhR activity in two oral squamous cell lines was modulated with AhR prototypic, environmental, and bacterial AhR ligands, AhR-specific inhibitors, and phenotypic, genomic, and functional characteristics were evaluated. The data demonstrate that: (i) primary OSCC tissue expresses elevated levels of nuclear AhR as compared with normal tissue, (ii) AhR mRNA expression is upregulated in 320 primary OSCCs, (iii) AhR hyperactivation with several ligands, including environmental and bacterial ligands, significantly increases AhR activity, ALDH1 activity, and accelerates cell migration, (iv) AhR inhibition blocks the rapid migration of OSCC cells and reduces cell chemoresistance, (v) AhR knockdown inhibits tumorsphere formation in low adherence conditions, and (vi) AhR knockdown inhibits tumor growth and increases overall survival in vivo. These data demonstrate that the AhR plays an important role in development and progression of OSCC, and specifically cancer stem-like cells. Prototypic, environmental, and bacterial AhR ligands may exacerbate OSCC by enhancing expression of these properties.

Implications: This study, for the first time, demonstrates the ability of diverse AhR ligands to regulate AhR activity in oral squamous cell carcinoma cells, as well as regulate several important characteristics of oral cancer stem cells, in vivo and in vitro. Mol Cancer Res; 14(8): 696–706. ©2016 AACR.

Introduction

Historically, the majority of studies on environmental chemical carcinogenesis have focused on the ability of genotoxic chemicals to damage DNA and to induce mutations (1, 2). More recent data suggest that environmental carcino-
Over 45,000 new cases of oral squamous cell carcinoma (OSCC) are diagnosed and account for over 8,000 deaths a year in the United States (12). The disease and its treatments are highly debilitating and the 5-year survival rates of regionally invasive tongue and floor-of-the-mouth cancers range from only 38% to 63% (13, 14). It has been suggested that chemoresistant oral cancer stem-like cells (CSCs) contribute significantly to these relatively poor outcomes (15–18). CSCs are defined as a relatively small subset of chemotherapy- and radiotherapy-resistant, invasive, metastatic tumor cells that are highly efficient at tumor initiation (19–22). Oral CSCs also tend to exhibit higher levels of ALDH1, an enzyme associated with chemoresistance and radioresistance, than non-CSCs (16, 20). Indeed, there is a direct correlation between ALDH levels and disease stage, prognosis, and chemotherapeutic resistance (18, 21). Consequently, elucidation of pathways through which CSCs are produced and maintained is an important next step in identifying potential candidates for targeted OSCC therapy.

It has been estimated that 70% to 90% of OSCC are linked to tobacco use, with a linear relationship between the number of smoking years and OSCC risk (23, 24). There are over 60 carcinogens found in tobacco products, including several AhR ligands such as benzo(a)pyrene [B(a)P] and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; refs. 25, 26). B(a)P and another ambient environmental PAHs have been implicated in OSCC through their activation of the AhR, subsequent induction of AhR-regulated P450 enzymes (CYP1A1, CYP1B1), and PAH-dependent production of DNA-reactive metabolic intermediates (27). PAHs also likely play a role in OSCC by altering key cell fate decisions and promoting cell immortalization over senescence (28). Similarly, TCDD, a high affinity and poorly metabolized AhR ligand, likely contributes to carcinogenesis by chronically activating the AhR signaling pathway (29). Furthermore, "constitutive" or chronic AhR activation in other cell types has been implicated in cancer progression even in the absence of environmental ligands (30, 31). Therefore, analysis of AhR signaling in OSCC would both increase our understanding of the etiology of the disease in the presence or absence of environmental stimuli, and identify a novel therapeutic target, that is, the AhR, regardless of disease etiology.

Here, we explore the role of the AhR in OSCC with particular emphasis on outcomes associated with advancing cancer, including production of oral CSCs and increased migration. To address the possible contribution of environmental and microbial AhR ligands to OSCC, several classes of AhR ligands were used including a genotoxic PAH [B(a)P], a nongenotoxic but persistent halogenated hydrocarbon (TCDD), PAH-containing 2.5-μm airborne particles (PM2.5), and products from Pseudomonas aeruginosa and Porphyromonas gingivalis, the latter a primary etiologic agent associated with chronic periodontitis. To assess the feasibility of targeting the AhR signaling pathway as a novel OSCC treatment, we used two OSCC cell lines, a unique OSCC orthotopic xenograft model (32), a novel AhR inhibitor (CB7993113; ref. 33), and molecular knockdown techniques.

**Materials and Methods**

**OSCC tissue specimens**

All studies with surgical OSCC specimens were approved by the Institutional Review Board at the Boston University Medical Campus (Boston, MA). Fresh tissues were obtained from patients with moderately differentiated OSCC of the lateral tongue and base of the tongue. Regions of OSCC and adjacent epithelia (AE), defined by an on-site pathology analysis, were snap-frozen at −80°C. Tissues were divided for hematoxylin and eosin (H&E) analyses, biochemistry, and immunofluorescence staining. Optimal cutting temperature (OCT)-embedded fresh tumor tissues were used for preparation of frozen sections (5 μm). One frozen section was set aside for H&E staining, and the remaining sections were processed for immunofluorescence analyses as described below.

**Immunofluorescence**

For indirect immunofluorescence analyses, sections (5 μm) of OSCC OCT-embedded tissues were blocked with 10% goat serum and incubated with antibodies against AhR followed by secondary antibodies conjugated with FITC. Negative controls lacked primary antibodies and no staining was observed. The slides were mounted in Vectashield and optical sections were analyzed with a Zeiss LSM 510 META confocal microscope. To compare fluorescence intensities between samples, settings were fixed to the most highly stained sample with all other images acquired at those settings. Nuclear and cytosolic AhR were quantified using ImageQuant TL software (GE Healthcare Life Sciences) and calculated as nuclear or cytosolic density/nuclear + cytosolic density × 100%.

**Chemicals**

DMSO, β-naphthoflavone (B-NF), 7,12-dimethylbenz[a]anthracene (DMBA), TCDD, B(a)P, pyocyanin (PYO), indoxyl sulfate (IS), thiazolyl blue tetrazolium bromide (MTT), and cisplatin were obtained from Sigma-Aldrich. 6-Formylindolino(3,2-b)carbazole (FICZ), CH223191, and CB7993113 were provided by Dr. M. Pollastri (Northeastern University, Boston, MA). AhR-antibody was purchased and used from Cell Signaling Technology (product #: 13790).

**Western blotting**

OSCC tissue lysates were fractionated on 4%-12% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, blocked with 10% nonfat dry milk, and incubated with primary antibodies to AhR and β-actin. Protein-specific detection was carried out with horseradish peroxidase–labeled secondary antibodies and Enhanced Chemiluminescence Plus (Amersham Biosciences).

**P. aeruginosa and P. gingivalis culture**

Frozen stocks of P. gingivalis wild-type strain 381 were grown anaerobically at 37°C on blood agar plates (Remel) for 3 to 5 days. Blood-heart infusion medium (BD Biosciences) supplemented with yeast extract (0.5%; BD Biosciences), hemin (10 mg/mL, Sigma-Aldrich), and menadione (1 mg/mL, Sigma-Aldrich) was inoculated with plate-grown bacteria and cultures grown anaerobically at 37°C for 16 to 18 hours. P. gingivalis was then harvested and transferred to DMEM (Mediatech) containing 10% FBS (Sigma-Aldrich) for 48 hours, the supernatant was collected, double sterile filtered, diluted 1:10, and used for experiments (P. gingivalis supernatant; PGS).

For P. aeruginosa, DMEM (Mediatech) containing 10% FBS (Sigma-Aldrich) was inoculated with the PAO1 strain (Perkin-Elmer Xen41; isolated after overnight growth on blood agar...
plates) and cultured while shaking at room temperature at 37°C for 6 hours; a sterile supernatant was collected by removing bacteria using sedimentation (at 1,500 × g) followed by filtration (with 0.22-μm pores). The P. aeruginosa supernatants (PAS) were confirmed sterile and stored at 4°C until used in experiments.

**Isolation of 2.5-μm particulate matter**

Particulate matter samples (2.5 μm; PM2.5) were collected for 1 week from a ventilation exhaust stack in a northeast U.S. urban highway tunnel. The samples were collected on polyurethane foam using a high volume cascade impactor (HVCI). PM2.5 were released from the collection filter by vigorous washing in PBS followed by sonication and used at a final concentration of 5 mg/cm² (34).

**Cell line acquisition, cell culture, and media**

CAL27 cells were purchased from and cultured according to ATCC recommendations. All experiments were performed within 6 months of vial thawing from ATCC, which validates ATCC recommendations. All experiments were performed in accordance with the NIH Animal Care Committee and used in accordance with the NIH Animal Care Committee. HSC-3 or CAL27 cells were treated with 50 mmol/L diethylamino-benzaldehyde (DEAB), an ALDH-specific inhibitor. Samples were incubated for 35 minutes at 37°C, washed, and suspended in PM2.5.

**RT-qPCR**

Cells were dosed as above for 24 hours. mRNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and cDNA prepared using the GoScript Reverse Transcription System (Promega) with a 1:1 mixture of random and Oligo (dT)₁₅ primers. All RT-qPCR reactions were performed using the GoTaq RT-qPCR Master Mix System (Promega). Validated primers were purchased from Qiagen (human CYP1B1 - QTO0209496 and GAPDH - QT011192646). RT-qPCR reactions were performed using a 7900HT Fast Real-Time PCR instrument (Applied Biosystems) with hot-start activation at 95°C for 2 minutes, 40 cycles of denaturation (95°C for 15 seconds), and annealing/extension (55°C for 60 seconds). Relative gene expression was determined using the Pfaff method (35) and the threshold value for GAPDH mRNA was used for normalization.

**Transient transfection**

HSC-3 or CAL27 cells cultured in 24-well plates were cotransfected with 0.5 μg pGfuluc reporter plasmid, provided by Dr. M. Denison (University of California, Davis; Davis, CA), and CMV-green (0.25 μg) using 1 μL TransIT-2020 transfection reagent (Mirus). Transfection medium was replaced after 24 hours. The cells were left untreated or dosed as above and harvested after 24 hours in Glo Lysis Buffer (Promega). Luciferase activity was determined with the Bright-Glo Luciferase System according to the manufacturer’s instructions (Promega). Luminescence and fluorescence were determined using a Synergy2 multifunction plate reader (Bio-Tek).

**Scratch-wound assay**

HSC-3 cells were grown to confluence in 6-well plates. At confluence, media were changed to serum-free media and cells were preseeded for 24 hours as above with FICZ, TCDD, B(a)P, PYY, and/or PM2.5 with or without 10 μmol/L CH223191 or CB7993113. After 24 hours, a 200 μL transfection pipet tip was used to make an “X” scratch in each well and nonadherent cells were removed with three PBS washes. Serum-containing media were added and cells redosed. The scratch surface (Tobias Gebäck and Martin Schulz, ETH Zürich, Switzerland) quantified the closure of the scratch at 0 and 24 hours.

**Orthotopic xenograft mouse model**

CAL27 cells (3 × 10⁶) were injected into the tongues of 6-week-old female NCr nude mice (CtAc:NCr-Foxn1<sup>tm</sup>, Taconic) as we described previously (32). Mice began a daily lavage schedule with 50-μL sesam oil alone or CB7993113 dissolved in sesame oil (50 mg/kg/day in 50 μL) 24 hours before tongue injections. Animals were weighed and tumor size was measured with Vernier calipers over a 26-day period. Animals were sacrificed when mice lost 20% of their total body mass. Surviving mice were sacrificed by day 26. Animals were housed at the Association for Assessment and Accreditation of Laboratory Animal Care–certified Boston University Medical Laboratory Animal Science Center and used in accordance with the NIH.
Guide for the Care and Use of Laboratory Animals. A Boston University Medical Campus Institutional Animal Care and Use Committee–approved protocol was followed.

**MTT assay**

CAL27 cells were plated at 3,500 cells per well in 96-well plates. After 24 hours, cells were treated with a 1 to 500 μmol/L cisplatin with or without 10 μmol/L CH223191. Twenty-four hours later, 10 μL of a 10 mg/mL MTT solution was added to each well, and plates were incubated for 3 hours at 37°C. MTT and media were aspirated off and DMSO was added to solubilize the MTT crystals. After 2 hours of solubilization, the plate absorbance was read at 590 and 620 nm (reference filter) to determine cell viability.

**Statistical analyses**

Statistical analyses were performed with Microsoft Excel. Data are presented as means ± SEs where applicable. Paired and two-sample equal or unequal variance T tests were used to determine significance. For experiments measuring relative fold changes in gene expression, the Pfaffl method (35) was used for normalization to GAPDH mRNA levels.

**Results**

**AhR expression and activity are implicated in OSCC cells**

Previous work implicated the AhR in cancer progression and tissue stem cell biology (36, 37). We, and others, have demonstrated that the AhR is highly expressed and constitutively active in breast cancers and that its activity correlates with tumor aggressiveness (30). However, the role of the AhR in OSCC cells has not been examined thoroughly. First, AhR protein levels in primary tissue were evaluated by immunofluorescence using the Pafull method (35) for normalization to GAPDH mRNA levels.

![Figure 1](image_url)

**Figure 1.** Primary OSCC tumors express increased AhR mRNA and protein levels. A, primary OSCC and adjacent normal tissue were stained for AhR immunofluorescence using AhR-specific antibody (green). Samples were costained with DAPI to identify nuclei (blue). Representative patient-paired samples are shown. Arrows indicate neoplastic cells expressing nuclear AhR. The table below the images summarizes mean percent nuclear or cytoplasmic AhR ± SE (6 fields) for cytologically normal and OSCC samples as determined with ImageQuant software. B, *AhR* mRNA levels in primary OSCC samples (*n* = 320) and adjacent normal tissue (*n* = 52) were compared using data from the TCGA dataset (normalized level 3 gene expression – RNASeqV2) and found to be significantly different, *P* < 1 × 10⁻⁷.
demonstration that \textit{P. gingivalis}, a bacterium commonly found in the oral cavity, produces AhR ligand(s). In all cases, the induced AhR activity was suppressed by addition of 10 µmol/L CH223191. It is important to note that the bacterial media did not induce AhR activity.

To determine whether an endogenous AhR target gene was similarly affected by AhR ligands and inhibitors, HSC-3 cells were treated with CH223191 or CB7993113 and/or the ligands described above and CYP1B1 mRNA levels quantified 24 hours following treatment with vehicle and AhR ligands at concentrations described above. CYP1B1 mRNA was quantified by RT-qPCR and normalized to GAPDH mRNA expression. Values were normalized to CYP1B1 fold change in untreated HSC-3 cells. Data from four to five experiments are presented as mean fold change + SE. Asterisks indicate a significant increase in CYP1B1 mRNA fold-change, * \( P < 0.05; ** \( P < 0.01; *** \( P < 0.005. A cross indicates a significant decrease in reporter activity, \(+ \ P < 0.05; ++ \ P < 0.01; +++ \ P < 0.005. C:\) HSC-3 cells were treated for 24 hours with vehicle and AhR ligands at concentrations described above.
ALDHhigh cells) and presented as (PAS-1:2), or cells, normalized to results obtained with CAL27 (Fig. 3A) and CAL27 (B) cells were treated for 24 hours with vehicles, AhR antagonists: 10 μmol/L CH223191, 10 μmol/L CB799313 or AhR agonists: 10 μmol/L CH223191, 10 μmol/L CB799313, or AhR agonists: 0.5 μmol/L FICZ, 1 μmol/L DMBA, 10 μmol/L B(a)P, 1 μmol/L TCDD, particulate matter 2.5 μm (PM2.5), 10 μmol/L IS, 1 μmol/L B(a)P, P. aequorina supernatant (PAS-12), or P. gingivalis supernatant (PQS-110), with and without 10 μmol/L CH223191. Percent ALDHhigh cells were determined by a gate drawn in the absence or presence of DEAB, according to the manufacturer’s instructions. Data from 3 to 11 experiments were normalized to results obtained with naive cells (mean baseline = 0.91% ALDHhigh cells) and presented as mean fold change from naive + SE. Asterisks indicate a significant increase in the percentage of ALDHhigh cells; *, P < 0.05; **, P < 0.01; ***, P < 0.005. A cross indicates a significant decrease in ALDHhigh cells, +, P < 0.05; ++, P < 0.01; ++++, P < 0.005.

ALDH1, a marker associated with chemoresistance and C3Cs, is influenced by AhR activity

C3Cs contribute to tumor progression and recurrence in multiple cancer types, including OSCCs (15–22). Previous studies with normal tissue-specific stem cells suggest that the AhR contributed to properties of “stem-ness” (36, 37, 42, 43). One readily assessed marker of stem-ness is elevated expression of ALDH1, an enzyme implicated in chemoresistance (44) the activity of which can be quantified via flow cytometry (16, 20). In contrast to other tumor types, expression of surface C3Cs markers, such as CD44 and CD24, is highly variable and not reliable in OSCC (16, 20, 22). Therefore, a fluorescence-based ALDH1 enzyme activity assay (44) was used to quantify ALDH1 activity in HSC-3 and CAL27 cells in the absence or presence of diverse AhR ligands and/or AhR inhibitors.

As noted for baseline pGudLuc activity (Fig. 2A and B) and endogenous CYP1B1 mRNA levels (Fig. 2C), AhR inhibitors consistently reduced the baseline percentage of ALDH1high cells (Fig. 3A) and CAL27 (Fig. 3B) cells. Conversely, all of the AhR ligands significantly increased the percentage of ALDH1high cells in HSC-3 and/or CAL27 and this increase was inhibited by CH223191 for the vast majority of ligands in both cell lines. These data suggest that the AhR influences expression of this OSCC stem cell–associated marker.

AhR inhibition decreases chemoresistance

ALDH1 expression has been associated with chemoresistance (44, 45). As the AhR appears to influence ALDH expression (Fig. 3), we postulated that the AhR may confer relative chemoresistance to OSCC cells. To test this hypothesis, CAL27 cells were treated with titrated doses of a front-line chemotherapeutic, cisplatin, with or without CH223191 and cell viability was assayed 24 hours later. CH223191 treatment alone had no effect on viability (i.e., >95% viability in
untreated and CH223191-treated cultures, data not shown). However, CH223191 significantly \((P < 0.05–0.005)\) increased sensitivity to cisplatin in CAL27 cells over a dosing range of 1 to 500 \(\mu\text{mol/L}\) cisplatin. The EC50 of cisplatin alone was 232.5 \(\mu\text{mol/L}\) as compared with 84.2 \(\mu\text{mol/L}\) for cisplatin + CH223191 (Fig. 4). These results demonstrate that the AhR contributes to chemoresistance.

Tumorsphere formation, a functional readout of CSLCs, is influenced by AhR activity

Oral CSLC can form tumorspheres and produce progenitor cells in ultralow adherence conditions over several passages (21). To determine whether the AhR contributes to this functional readout of CSLCs, HSC-3 (Fig. 5A and B) and CAL27 (Fig. 5C and D) cells were cultured in Mammocult media under ultra-low adherence conditions and AhR activity was inhibited with CH223191 or CB7993113. Both the size and total number of HSC-3 or CAL27 tumorspheres were significantly reduced \((P < 0.05–0.01)\) by AhR antagonist treatment in primary and secondary cultures (Fig. 5). These results suggest that the AhR regulates tumorsphere formation by influencing: (i) the ability of CSLCs to differentiate into...
Figure 6.
AhR downregulation decreases migration of and AhR agonist treatment increases migration of HSC-3 cells. A, representative images of HSC-3 cell migration at 24 hours after cells were cultured to confluence, scratched, and treated with vehicle, 10 μmol/L CH223191 or 10 μmol/L CB7993113. Data are representative of six independent experiments. Vertical black lines indicate the borders of the original scratch at zero hours. B, HSC-3 cells were treated as in "A" and percent exposed area was quantified. Data from six experiments were normalized to results obtained with naive cells and presented as mean percent exposed area + SE. Crosses indicate a significant increase in exposed area, +++, P < 0.005. C, representative images of HSC-3 cell migration at 24 hours after cells were cultured to confluence, scratched, and treated with vehicle, 0.5 μmol/L FICZ, 10 μmol/L B(a)P, 1 nmol/L TCDD, 1 μmol/L PYO, with or without 10 μmol/L CH223191. Data are representative of 3 to 10 independent experiments. Vertical black lines indicate the borders of the original scratch at zero hours. D, HSC-3 cells were treated as in "A" and percent exposed area was quantified. Data from 3 to 10 experiments were normalized to results obtained with naive cells and presented as mean percent exposed area + SE. Crosses indicate a significant increase in exposed area, +, P < 0.05; +++, P < 0.005. Asterisks indicate a significant decrease in exposed area, *, P < 0.05; ***, P < 0.005.

progenitor cells, (ii) the ability of CS/Cs to (asymmetrically) divide, and/or, (iii) the ability of progenitor cells to divide. These functional data, together with the phenotypic data presented in Fig. 3, are consistent with a role for the AhR in CS/C development.

OSCC cell migration is altered by AhR activity
A key characteristic of invasive and malignant cancer cells is the ability to migrate. Here, the effects of AhR modulation on the ability of HSC-3 cells to migrate were determined in a scratch-wound assay. Wound repair, a measure of cell migration, was significantly inhibited by CH223191 or CB7993113 treatment (Fig. 6A and B; P < 0.005). Conversely, 0.5 μmol/L FICZ, 10 μmol/L B(a)P, 1 nmol/L TCDD or 1 μmol/L PYO accelerated cell migration (Fig. 6C and D). In all cases, AhR agonist-induced increases in migration rates were significantly inhibited by CH223191 treatment (P < 0.05–0.005). Combinations of AhR ligands similarly increased migration rates (Supplementary Fig. S2). These data suggest that environmental or bacterial-derived AhR ligands accelerate and AhR antagonists reduce OSCC cell migration and metastasis.

CB7993113, an AhR antagonist, decreases OSCC tumor formation in orthotopic xenografts
To further explore the role of the AhR in the OSCC, 3 × 10⁵ CAL27 cells were injected into tongues of 24 female, NCr nude mice. Twelve mice were lavaged daily with vehicle (sesame oil) alone (controls) and 12 mice were lavaged daily with CB7993113 (50 mg/kg/day). Tumor volume, animal weight, and survival were evaluated over the next 26 days. Tumor volumes in CB7993113-treated mice were significantly (P < 0.05–0.0005) smaller than in control mice beginning at day 5 and continuing until the time at which control mice had to be sacrificed (Fig. 7A). In parallel, the weights of control mice began to drop on day 9 and were significantly lower than those of CB7993113-treated mice by day 12, after which the first control mouse had to be sacrificed (Fig. 7B). Interestingly, the weights of CB7993113-treated mice that survived until day 26 were stable or slightly increased between day 15 and the termination of the experiment on day 26 (Fig. 7B and data not shown). These differences in tumor volume and mouse weights translated into significantly better survival of CB7993113-treated mice (83% survival at 26 days) as compared with control mice (25% survival at 26 days; Fig. 7C). These results are consistent with the findings from the in vitro
cells. Mice were treated daily with CB7993113 dissolved in sesame oil or sesame oil alone by oral lavage starting 24 hours before cell injection (12 mice/group). Mice were monitored daily and tongue measurements were taken as indicated.

CYP1B1 upregulated by the AhR has been indirectly linked to OSCC initiation. Notably, through the catabolic activity of AhR-regulated P450 oxygenases, the AhR can catalyze the generation of mutagenic compounds, including those found in cigarette smoke. A significant mechanism of carcinogenesis is the production of environmental AhR ligands, which may play an ongoing role in OSCC progression by driving the expression of several phenotypic and functional characteristics of advancing cancers. These studies also suggest the possibility that ongoing exposure to environmental AhR ligands accelerates still further the development of aggressive, difficult-to-treat OSCCs.

High-level AhR expression in primary cancers was demonstrated by immunofluorescence of primary tumor samples, computational analysis of mRNAs from 320 primary human cancers and 32 AE samples and Western blotting (data not shown). The findings that AhR was highly expressed and “constitutively” active, at least in representative OSCC cell lines, was supported by a decrease in baseline pGudLuc reporter activity, a decrease in CYP1B1 expression, a decrease in the percentage of ALDH<sup>high</sup> cells, a decrease in tumorigenesis in vivo following treatment with an AhR antagonist. Consistent with the hypothesis that environmental AhR ligands can exacerbate cancer progression, prototypic AhR ligands as well as environmental AhR ligands increased pGudLuc activity, CYP1B1 transcription, the percentage of ALDH<sup>high</sup> cells, and the rate of cell migration.

One of the outcomes mediated by the AhR in our studies was the acquisition or enhancement of cancer stem cell-like properties including increased percentages of ALDH<sup>high</sup> cells, resistance to cisplatin, and tumorigenesis in low adherence conditions. First described in 2004 (15), OSCC stem cells have an enhanced ability to form tumors in vivo and to resist chemotherapeutic- or radiation-induced cell death (19, 18, 21, 22). Cells isolated from patients on the basis of high ALDH expression exhibit an increased resistance to radiotherapy and increased tumorigenicity (21). ALDH<sup>high</sup> cells from OSCC lines exhibit increased invasiveness, quiescence, and epithelial-to-mesenchymal transition (EMT) as assayed by migration in a scratch-wound assay (22). In this context, the demonstration of a role for the AhR in the generation and maintenance of CSLCs takes on a particularly important connotation in that it suggests a treatment to reduce the number and/or activity of CSLCs, or to use in combination with traditional therapeutics to increase the sensitivity of CSLCs to traditional chemotherapeutics regimens. It has been recently reported that chemoresistance is directly linked to metastatic behavior (47), and our findings support the importance of increasing chemosensitivity in oral cancer treatment to prevent deleterious downstream outcomes.

Perhaps of special note is the ability of <i>P. aeruginosa</i> and <i>P. gingivalis</i> supernatants to activate the AhR, increase the percentage of ALDH<sup>high</sup> cells and accelerate cell migration. Our studies with <i>P. aeruginosa</i> confirm previous work (10) by demonstrating that <i>P. aeruginosa</i>–derived PYO is a potent AhR ligand and we extend previous studies by demonstrating that both <i>P. aeruginosa</i> supernatant and PYO exhibit AhR-dependent biologic activity in malignant cells (e.g., induction of ALDH activity, increased cell migration). With regard to <i>P. gingivalis</i>, this is the first demonstration of an AhR ligand being produced by this common oral pathogen, a finding that has significant implications for the possible role for <i>P. gingivalis</i> in OSCC. In this vein, it has recently been shown that infection of OSCC...
with \( P. \) gingivalis induces EMT and increases the percentage of \( \text{C}_{6}\text{C}_{6} \) in OSCC [17]. In those studies, it was suggested that these outcomes were due in part to increased IL8 expression, a cytokine regulated by the AhR in human macrophages [48, 49]. Current studies are underway to determine the nature of the AhR ligand produced by \( P. \) gingivalis and to determine whether AhR-dependent IL8 production leads to an increase in cells with stem cell-like properties.

We recently described a novel orthotopic xenograft model for human OSCC [32]. Here, this model was used to further explore the role of the AhR in OSCC and to begin to assess whether AhR inhibition represents a viable, targeted approach to OSCC therapy. Indeed, daily oral lavage with CB7993113, a nontoxic competitive AhR inhibitor [33], significantly reduced tumor load and weight loss and increased overall survival. The lack of weight loss observed in CB7993113-treated mice over the course of the experiment was consistent with the lack of toxicity seen with this potential therapeutic in vitro [33]. Of note, studies from other laboratories demonstrate that AhR downregulation blocks B(a)P-induced OSCC development by blocking DNA adduct formation, likely through inhibition of AhR-dependent CYP1A1 and CYP1B1 induction [50, 51]. Therefore, CB7993113 could fairly be viewed as a potential inhibitor of tumor initiation as well as a potential therapeutic.

Collectively, the data presented here strongly suggest that OSCC is characterized by relatively high AhR expression and constitutive activity. AhR inhibition in OSCC cell lines altered population dynamics specifically by reducing the percentage of \( \text{ALHD}^{\text{high}} \) cells and the ability of CSC to form tumorspheres. Relevant to environmental chemical exposures, including exposure to several AhR ligands in cigarette smoke, prototypic and environmental PAHs as well as a halogenated hydrocarbon upregulated phenotypic and functional markers of cancer stem cells and increased cell migration, all of which increases concern over the possible contribution of environmental chemical exposures to OSCC progression. Furthermore, the demonstration that \( P. \) gingivalis produces an AhR ligand(s), suggests that this bacterium may play a role in oral cavity carcinogenesis in the absence of or perhaps in combination with smoke-derived carcinogens. Finally, the results suggest an opportunity for targeted therapy for otherwise refractory OSCCs.

**Disclosure of Potential Conflicts of Interest**

C.A. Genco is a consultant/advisory board member for WilmerHale (Boston, MA). No potential conflicts of interest were disclosed by the other authors.

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**Acknowledgments**

The authors thank Bonnie Campbell and Elizabeth Lindsay for their assistance as lab technicians in the D.H. Sherr’s Laboratory, and Connie Slocum and Mary Zabinski for their assistance in \( P. \) gingivalis and \( P. \) aeruginosa supernatant collection.

**Grant Support**

This work was supported by Boston University Oral Cancer Research Initiative, Art beCAUSE Breast Cancer Foundation, Avon Foundation for Women, Superfund (5 P42 ES007381-18; to D.H. Sherr). 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 February 29, 2016; revised April 6, 2016; accepted April 13, 2016; published OnlineFirst April 29, 2016.

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