

Evaluation of aryl hydrocarbon receptor expression in oral squamous cell carcinoma and normal oral mucosa using western blot

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Abstract

Background: Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that acts as a binding site for toxic chemicals, particularly the dioxin group of chemicals. Elevated levels of AHR have been observed in various human cancers, including lung carcinomas, hepatic carcinomas and in mammary tumors. However, the expression of AHR in oral squamous cell carcinoma (OSCC) patients who are tobacco users are less explored.

Aims and Objectives: The aim of the present study is to evaluate and compare AHR levels in OSSC patients and in normals using Western blot technique in an attempt to explore the possible role of AHR in oral carcinogenesis.

Materials and Methods: The study sample consisted of ten oral squamous cell carcinoma cases which were diagnosed clinically and confirmed histopathologically as OSCC and four samples of the normal oral mucosa. AHR protein expression was evaluated using Western blot technique and chemiluminescence detection kit. The densitometry was performed on a Microtek scan maker MSP flatbed scanner and quantified using Image J software. Mean AHR protein levels were calculated and compared between OSCC and normal oral mucosa using Student's *t*-test.

Results: The mean AHR protein level in OSCC samples ($n = 10$) was 2878.90 ± 1231.27 and 975.75 ± 227.27 in the normal oral mucosa ($n = 4$). The OSCC samples showed significantly higher levels of AHR protein compared to the normal oral mucosa ($P = 0.008$).

Conclusion: The study showed a significantly higher expression of AHR in oral squamous cell carcinoma samples when compared to the normal oral mucosa, suggesting a possible role of AHR in the initiation, promotion and progression of oral squamous cell carcinoma.

Keywords: Aryl hydrocarbon receptor, oral squamous cell carcinoma, Western blot

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INTRODUCTION

Oral cancer is a serious growing problem in many parts of the globe and is estimated by the WHO as the eighth-most common cancer worldwide. The annual estimated incidence is around 275,000 for oral cancers, two-thirds of these cases occurring in developing countries.^[1] Oral squamous cell carcinoma (OSCC) encompasses at least 95% of all oral malignancies. OSCC is an epithelial neoplasm generally beginning as a focal clonal overgrowth of altered stem cells near the basement membrane, expanding upward and laterally, replacing the normal epithelium. The neoplastic process is a result of normal epithelium progressing through hyperplasia to dysplasia to carcinoma *in situ* and invasive carcinoma.^[2]

Oral squamous carcinogenesis is a multistep process in which multiple genetic events occur that alter the normal functions of oncogenes and tumor suppressor genes. This can result in increased production of growth factors or numbers of cell surface receptors, enhanced intracellular messenger signaling and/or increased production of transcription factors. All these along with a combination of loss of tumor suppressor activity, leads to a cell phenotype capable of increased cell proliferation, with loss of cell cohesion and the ability to infiltrate local tissue and spread to distant sites.^[3]

In response to external stimuli, the extracellular and intracellular receptors of cells sense and transduce the received signals into changes in gene expression. Most of the cellular responses to environmental and developmental stimuli are mediated by PAS (per-ARNT-Sim) proteins. A member of this family of proteins is the aryl hydrocarbon receptor (AHR), a ligand-activated basic helix-loop-helix transcription factor and E3 ubiquitin ligase that is expressed in all cells. AHR was first discovered as a binding site for planar, nonhalogenated and halogenated xenobiotics, in particular the highly toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Nearly all toxic and carcinogenic effects of these anthropogenically produced compounds are mediated by the AHR.^[4]

With the identification of naturally-occurring compounds as AHR ligands,^[5] its role in the physiological process has been revealed apart from its role as a sensor for xenobiotics and a mediator of toxicity and carcinogenesis. Studies on AHR-deficient mice, however, revealed that AHR signaling is essential for normal physiological cell signaling.^[6] In a number of different tissues, including the immune system, the liver, the gastrointestinal tract, the reproductive organs and the skin, the AHR regulates proliferation and

differentiation processes, thereby maintaining homeostasis. As a transcription factor and ubiquitin ligase, the AHR can modulate the expression levels of proteins involved in mediating important normal cellular functions.^[7,8]

Approximately 70%–90% of OSCC cases are linked to tobacco use, with a linear relationship between the number of smoking years and OSCC risk.^[9] More than 60 carcinogens have been identified in the tobacco products, including several AHR ligands such as benzopyrene and 2,3,7,8-TCDD.^[10] Benzopyrene has been implicated in OSCC through the activation of the AHR, subsequent induction of AHR-regulated P450 enzymes (CYP1A1, CYP1B1) and P450-dependent production of DNA-reactive metabolic intermediates.^[11] Similarly, TCDD, a high affinity and poorly metabolized AHR ligand, likely contributes to carcinogenesis by chronically activating the AHR signaling pathway.^[12] Furthermore, AHR activation in other cell types has been implicated in cancer progression, even in the absence of environmental ligands.^[13] 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, i.e., the AHR, regardless of disease etiology.

The present preliminary study focuses on a comparative analysis of the expression of AHR protein in OSCC and in normal oral mucosa using the Western blot technique.

MATERIALS AND METHODS

The study sample consists of ten histopathologically confirmed cases of oral squamous cell carcinoma and four normal buccal mucosa specimen obtained during surgical removal of impacted third molars. Informed consent was taken from all the participants and the study was approved by the Institutional Review Board of Rajah Muthiah Dental College and Hospital, Annamalai University. A small bit of postoperative tissue specimen was collected in an Eppendorf tube containing protein preservative solution and kept overnight in refrigerator (4°C). The collected specimens were then transferred to deep freezer (–80°C) and stored until the tissues are taken for homogenization.

Tissue lysate preparation

The tissue stored in preservative solution was rinsed with phosphate-buffered saline, minced into several small pieces and placed in separate homogenizers. The tissues were homogenized in freshly prepared radioimmunoprecipitation assay buffer. The homogenate was transferred to centrifuge tubes, and the homogenized lysate was kept in a rotating shaker at 4°C for 2 h. The contents were spinned at 12,000

rpm for 15 min at 4°C, and the supernatant was transferred to a prechilled tube.

Electrophoresis and immunoblotting of tissue lysate

OSCC tissue lysates were fractionated on 4%–10% sodium deoxycholate-polyacrylamide (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked using 3% bovine serum albumin and incubated with rabbit anti-human AHR primary antibody (Santa Cruz, Texas, USA) for overnight at 4°C. Protein-specific detection was carried out with horseradish peroxidase-labeled anti-rabbit secondary antibodies (Santa Cruz, Texas, USA) and chemiluminescence detection kit. Densitometry was performed on a Microtek scan maker MSP flatbed scanner and quantified using Image J software.

The mean AHR protein levels were calculated for OSCC and normal oral mucosa samples and compared using Student’s *t*-test. All the data were analyzed using the Statistical Package for the Social Sciences software version 10.0.2, and *P* < 0.05 was considered to be statistically significant.

RESULTS

The clinical details of patients with OSCC are given in Table 1. The age of the patients in the study group ranged between 44 and 68 years and between 45 and 55 years in the control group. All the participants of this study were males. Seven out of ten patients with OSCC had the habit of smoking either cigarettes or beedi and all the ten patients had the habit of tobacco chewing, while in the control group, none of the people had the habit of smoking or chewing tobacco. In all the ten patients with OSCC, the lesion was clinically noticed on the buccal mucosa.

In the present study, the level of AHR protein was analyzed in ten samples of OSCC and compared with that of normal oral mucosa using the Western blot

Table 1: Clinical details of patients with oral squamous cell carcinoma

Age	Sex	Tobacco chewing		Smoking	
		Frequency (times/day)	Duration (in years)	Frequency (number/day)	Duration (in years)
44	Male	5–6	10	5–10 beedi	15
53	Male	5–6	22	10–15 beedi	22
51	Male	10–15	20	2 cigarettes	10
55	Male	7–8	30	Nil	Nil
55	Male	5–6	25	5–7 beedi	5
52	Male	3–4	10	3–4 beedi	9
65	Male	2–3	20	Nil	Nil
45	Male	3–5	10	2–3 beedi	20
68	Male	3–5	30	Nil	Nil
65	Male	8–10	30	4–5 cigarettes	30

method. [Figure 1] shows the bands obtained on the PVDF membrane in OSCC samples. The intensity of the bands correlates to the expression level of AHR protein and was analyzed using Image J software. The values obtained in the form of the number of pixels correspond to the mean AHR protein level and are represented in [Figures 2 and 3].

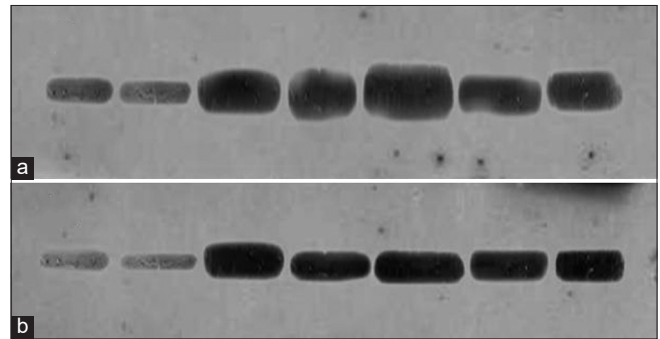


Figure 1: (a and b) Expression of aryl hydrocarbon receptor protein in oral squamous cell carcinoma samples using immunoblotting technique

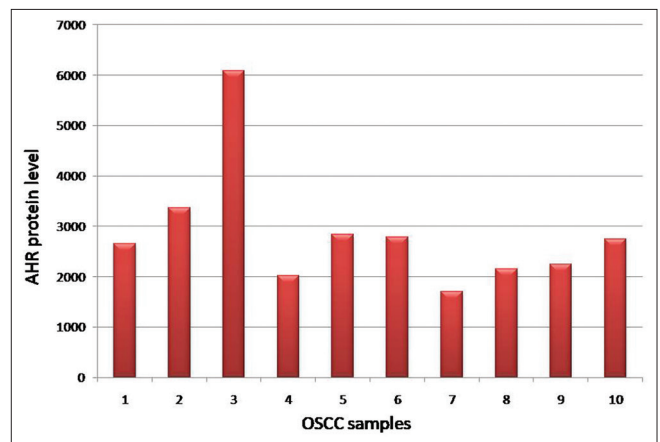


Figure 2: Graph showing aryl hydrocarbon receptor protein levels in 10 samples of oral squamous cell carcinoma

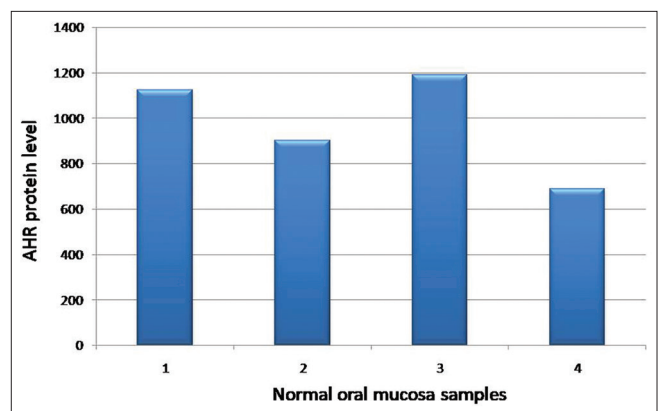


Figure 3: Graph showing aryl hydrocarbon receptor protein levels in 4 samples of normal oral mucosa

The mean AHR protein level in OSCC samples ($n = 10$) was 2878.90 ± 1231.27 and 975.75 ± 227.27 in the normal oral mucosa ($n = 4$). The OSCC samples showed significantly higher levels of AHR protein compared to the normal oral mucosa ($P = 0.008$).

DISCUSSION

Among the various carcinogenic chemicals present in tobacco, which is one of the main causative agents for OSCC, the lipophilic polycyclic aromatic hydrocarbons (PAHs) need special mention. On cellular entry, PAH activates the cytosolic protein AHR. Activated AHR then translocates to the nucleus and binds to aryl hydrocarbon receptor nuclear translocator (ARNT). This AHR-ARNT complex, in turn, binds to xenobiotic response element (XRE) and upregulates a battery of AHR-regulated genes which encode both Phase I and Phase II xenobiotic-metabolizing enzymes. The Phase I detoxification system is composed mainly of cytochrome p450 supergene family of enzymes and are indeed the first enzymatic defense against foreign compounds. Phase I enzymes convert inert carcinogens to active genotoxins, and hence, the AHR plays a key role in tumor initiation. As a consequence of this step in detoxification, reactive molecules, which may be more toxic than the parent molecule, are produced. If these reactive molecules are not further metabolized by Phase II enzymes, they may cause damage to DNA, RNA and proteins.^[14,15]

Reduced AHR levels are shown to decrease the toxicity of induced benzopyrene, which is a PAH, in studies done on mouse hepatoma cells by Schreck *et al.*^[16] Studies by Shimizu *et al.*^[17] showed that carcinogenicity of PAH is lost in AHR knockout mice, and thus, it is generally accepted that this “canonical” AHR-dependent pathway is required for tumor initiation by PAHs in animals and human beings. Besides this classical route, the AHR mediates tumor promotion, and evidence suggests that AHR also plays a role in tumor progression.^[18] It has been observed that AHR expression is higher in invasive than in noninvasive tumor cells and tissues.^[19] Chang *et al.* showed that downregulating the AHR function in lung adenocarcinoma cells diminishes anchorage-independent growth *in vitro*.^[20] Upregulation of nuclear AHR expression in human urothelial tumors is associated with increased invasion and poor prognosis, as proved by Ishida *et al.*^[21] All these studies proved that AHR is found to be one of the major signaling molecules in tobacco-induced carcinogenesis, and overexpression of AHR is considered as a characteristic feature of various tumors.

Proliferation rates are decreased in AHR-deficient cells, as stated by Ma and Whitlock, suggesting a pivotal role of the AHR in the cell cycle regulation.^[22] AHR appears to interact with various components of the cell cycle machinery to promote cell cycle progression. AHR-p65 interactions allow quiescent cells to enter into the cell cycle by inducing c-myc gene expression.^[23] Association of AHR with CDK4, CCND1 and Rb-E2F sequesters Rb to CDK4, thereby allowing its phosphorylation and the release of E2F, which promotes the cell cycle progression to the S phase.^[24]

Earlier studies have identified a higher level of expression of AHR and ARNT in tumor tissue and also in tissues that are exposed to ligands.^[25,26] Western immunoblotting studies by Trombino *et al.*^[25] clearly demonstrated a profound increase in AHR expression in DMBA, a prototypic PAH and AHR ligand-induced rat mammary tumors. This showed that the level of AHR expression was directly proportional to its ligand exposure. AHR over expression in pancreatic cancer was demonstrated by Koliopoulos *et al.*^[27] and in lung adenocarcinoma by Lin *et al.*^[28]

In another study by Ma *et al.*^[29] the AHR expression was remarkably elevated in gastric cancer in comparison to their noncancerous counterparts. Studies by Chang *et al.*^[20] showed that smokers have a high level of AHR and CYP1A1. AHR and CYP1B1 over expression might be involved in the development of mixed bronchioloalveolar carcinoma, and lung adenocarcinoma and AHR agonists present in cigarette smoke condensate are capable of inhibiting culture-induced senescence of human oral keratinocytes.^[30]

It has been established by Kim *et al.*^[31] that the level of AHR and ARNT are regulated by dose and duration of exposure to ligands such as PAH. Studies by Pollenz *et al.*^[32] and Franc *et al.*^[33] in rodents showed a dose-dependent variation in AHR proteins when exposed to different doses of TCDD. As the dose increased, a corresponding increase in AHR levels was noticed. The increased expression of AHR observed in our study might be due to exposure to tobacco carcinogens as all the patients with OSCC had the habit of chewing and/or smoking tobacco.

Our preliminary study to analyze the expression of AHR in normal and OSCC patients using immunoblotting method showed significantly higher levels of AHR protein in OSCC samples compared to the normal oral mucosa. However, we could not find out the extent of carcinogen exposure in OSCC patients, as the habits varied from patient to patient. The frequency and duration of carcinogen exposure, site-specific difference in epithelial

lining within the oral cavity, which affects the penetration rate of carcinogens into bloodstream and the diverse systemic and local factors among the patients also hold back our way to find the extent of carcinogen exposure in these patients.

conclusion

Various experimental studies revealed the fact that AHR-dependent pathway is required for tumor initiation by PAHs, and the same pathway is needed for tumor promotion by inducing a release from contact inhibition and also by inhibiting apoptosis. Considering the role of AHR in diverse studies, we can conclude by saying that the increased expression of AHR, as observed in our study, might play a role in the initiation, promotion and progression of OSCC. However, further studies with an increased sample size should be attempted to address the level of expression of AHR in oral squamous cell carcinoma and to assess the exact role of AHR in the initiation, promotion and progression of oral squamous cell carcinoma.

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Conflicts of interest

There are no conflicts of interest.

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