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ORIGINAL ARTICLE

# Effect of Areca Nut Consumption on Hypoxia-inducible Factor-1 Alfa Expression in Patients with Oral Squamous Cell Carcinoma

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## ABSTRACT

**Introduction:** Oral squamous cell carcinoma (OSCC) is a major health problem in Southeast Asia, including India. Areca nut chewing is a major health hazard in India, which has been implicated in the etiology of OSCC. Hypoxia-inducible factor-1 (HIF-1) is a major transcription factor involved in adaptation under hypoxic condition, a common finding in solid tumors. The present study was conducted to evaluate the effect of different habits including areca nut chewing on HIF-1 expression in patients with OSCC.

**Materials and methods:** It was a hospital-based observational case-control study. The study comprised 50 histologically proven cases of OSCC and 50 healthy controls. The HIF-1$\alpha$ level was measured by commercially available enzyme-linked immunosorbent assay (ELISA) in the blood samples. The data were analyzed using Statistical Package for the Social Sciences (SPSS) software version 20.

**Results:** The HIF-1$\alpha$ levels were found significantly higher in the patients with areca nut consumption in addition to other addictive habits. Isolated influence could not be discerned as there was only one patient who gave history of only areca nut chewing.

**Conclusion:** Our findings prove that HIF-1$\alpha$ expression is upregulated by areca nut chewing, which leads to worse prognosis. This calls for widespread awareness programs regarding the deleterious effects of areca nut chewing among the general population.

**Keywords:** Arecoline, Hypoxia-inducible factor-1 alfa, Oral squamous cell carcinoma.

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## INTRODUCTION

Oral squamous cell carcinoma is a common malignancy affecting Asians.[1] Among the various known risk factors for OSCC, areca nut chewing is a common occurrence in countries, such as India.[2] The increasing incidence of consumption of areca nut has nullified the awareness programs that have only targeted tobacco as a major risk factor. The primary constituent of areca nut—alkaloid arecoline—has been identified as a group I carcinogen. Arecoline has genotoxic, cytotoxic, and mutagenic effect on various cells.[3] Studies have implicated areca nut chewing as a cause for submucous fibrosis, oral cancer, hepatocellular carcinoma, carcinoma of cervix, lung, and stomach.[4] Areca nut is either consumed in isolation or taken in the form of betel quid along with tobacco, catechu, lime, and sweeteners. This augments the carcinogenicity due to the effect of lime and betel leaf component—safrole.[5]

Hypoxia-inducible factor-1 alfa is a key transcription factor induced by hypoxia and activates a transcription program that promotes the survival of cells in a hypoxic state. Studies have indicated a plausible role of arecoline in HIF expression through reactive oxygen species (ROS) pathway.[6]

The present study was planned to evaluate the effect of areca nut consumption on HIF-1$\alpha$ levels. Our hypothesis is that arecoline leads to stabilization of HIF-1$\alpha$ even under normoxic conditions, thereby leading to more aggressive tumors in areca nut chewers.

## MATERIALS AND METHODS

The study was conducted in the Department of Biochemistry, in collaboration with the Departments of Pathology and Dental and Oro-maxillofacial Surgery, Lady Hardinge Medical College and Associated SSKH. The study was commenced after prior approval from the Institutional Ethical Committee. Fifty histologically confirmed cases of squamous cell oral carcinoma attending the outpatient and inpatient Department of Dental and Oro-maxillofacial Surgery in SSKH were enrolled as cases during the study period. The controls were recruited from the Departments of Dental and Oro-maxillofacial Surgery and Biochemistry, fulfilling the inclusion criteria.

### Inclusion Criteria

#### *Cases*

Fifty patients with histologically confirmed OSCC.

#### *Controls*

Fifty healthy age- and sex-matched healthy volunteers from the Department of Biochemistry, Pathology and Dental and Oro-maxillofacial Surgery fulfilling the following criteria:

- No past history of any malignancy
- Apparently healthy oral healthy cavity on inspection
- No history of acute/chronic illness

### Exclusion Criteria

#### *Cases*

- Past history of any ischemic disease; e.g., cerebral ischemia, myocardial infarction, etc.
- Initiation of chemotherapy or radiotherapy for oral cancer

### *Sample Collection and Analysis*

A total of 10 mL of venous blood sample was collected from the subjects under sterile conditions. The blood samples were processed immediately for separation of serum, which was subsequently aliquoted and stored at −40° C for further analysis. Repeated freeze–thaw cycles were avoided.

All routine blood investigations were carried out on fully automated analyzers using standard reagents. The HIF-1α levels were estimated by commercially available ELISA kit supplied by QayeeBio (People's Republic of China).

### Statistical Methods

All analyses were performed with the SPSS software program version 20. For comparison of variables with a normal distribution, unpaired two-tailed Student's t-test was used; $p \leq 0.05$ was considered statistically significant.

## RESULTS

Table 1 depicts the demographic characteristics of the study population. The cases and controls were age and sex-matched. A high incidence of the risk factors for oral cancer (intake of alcohol, khaini, tobacco, sharp tooth, regurgitation of food) is seen in the cases with oral cancer. The majority of the patients presented at a later stage and with moderately differentiated tumors as depicted in Tables 2 and 3. The majority of the cancers afflicted the buccal area (Graph 1). Tables 4 and 5 give a detailed description of the stage and grade of tumors in patients with different addictive habits. Graph 2 is an illustration of HIF levels in cases and controls. The HIF levels were higher in patients with areca nut chewing habits as depicted in Table 6.

## DISCUSSION

Areca nut is the fruit of the oriental palm *Areca catechu*. It is used extensively in Asia in various forms. In India, it

**Table 1:** Demographic characteristics of study population

| | *Cases (n = 50)* | *Controls (n = 50)* |
|---|---|---|
| Age (years) (mean ± SD) | 50.32 ± 8.04 | 50.15 ± 8.03 |
| Sex: M/F | 41/9 | 41/9 |
| Tobacco chewing | 40 | 3 |
| Smoking | 30 | 12 |
| Alcohol | 24 | 10 |
| Khaini | 11 | 0 |
| H/O hypertension | 9 | 2 |
| H/O diabetes mellitus | 1 | 0 |
| H/O hot spicy food | 8 | 5 |
| H/O sharp tooth | 3 | 0 |
| H/O regurgitation of food | 12 | 5 |
| Family h/o cancer | 1 | 0 |

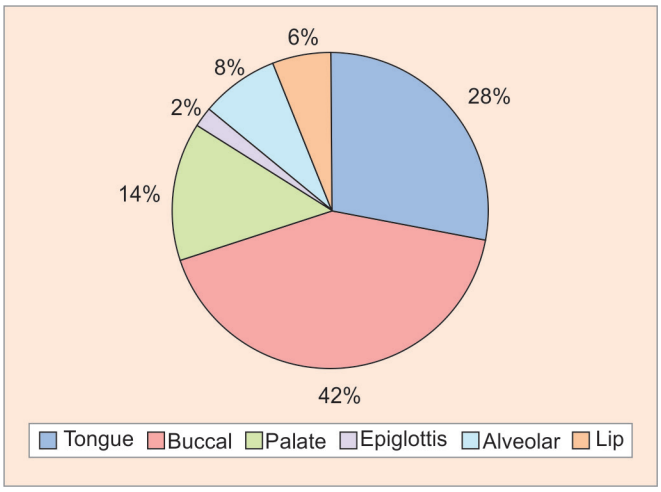
SD: Standard deviation; H/o: History of

**Table 2:** Stage of OSCC of the cases at the time of presentation

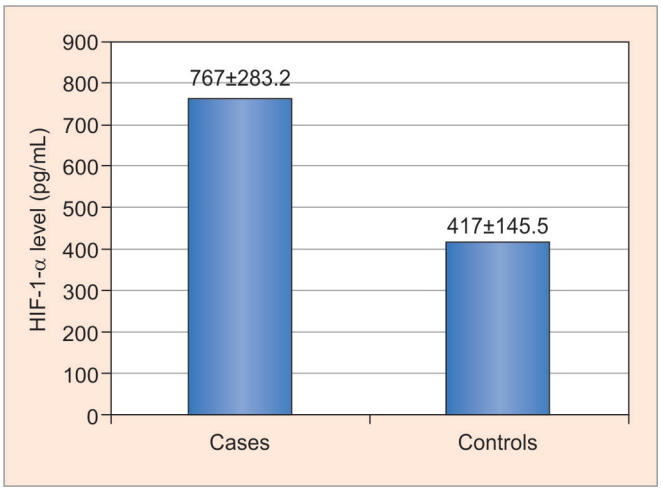
| *Stages* | *Cases (n = 50)* | *Percentage* |
|---|---|---|
| I | Nil | Nil |
| II | 4 | 8 |
| III | 43 | 86 |
| IV | 3 | 6 |

**Table 3:** Histological grade of OSCC of the cases at the time of presentation

| *Histopathological grading* | *Cases (n = 50)* | *%* |
|---|---|---|
| Well differentiated | 10 | 20 |
| Moderately differentiated | 26 | 52 |
| Poorly differentiated | 14 | 28 |




**Graph 1:** Site preference




**Graph 2:** Representation of the HIF-1α levels in the cases as compared with controls. The levels were significantly higher in the cases as compared with the controls

**Table 4:** Stage of presentation in patients with different risk factors

| *Risk factors* | *Number of cases* | *Stage I* | *Stage II* | *Stage III* | *Stage IV* |
|---|---|---|---|---|---|
| Smoking only | 7 | 0 | 2 | 5 | 0 |
| Alcohol only | 3 | 0 | 0 | 3 | 0 |
| Tobacco consumption only | 2 | 0 | 0 | 2 | 0 |
| Areca nut consumption (betel quid and paan masala) only | 1 | 0 | 0 | 1 | 0 |
| Tobacco + alcohol | 5 | 0 | 2 | 3 | 0 |
| Tobacco + smoking | 9 | 0 | 1 | 7 | 1 |
| Tobacco + areca nut (betel quid and paan masala) | 3 | 0 | 0 | 3 | 0 |
| Alcohol + smoking | 2 | 0 | 0 | 2 | 0 |
| Tobacco + alcohol + smoking | 11 | 0 | 2 | 7 | 2 |
| Smoking + alcohol + areca nut (betel quid and paan masala) | 2 | 0 | 0 | 2 | 0 |
| Tobacco + smoking + areca nut (betel quid and paan masala) | 3 | 0 | 1 | 2 | 0 |
| Smoking only | 2 | 0 | 0 | 1 | 1 |

**Table 5:** Histological grade of presentation in patients with different risk factors

| *Risk factors* | *Number of cases* | *Poorly differentiated* | *Moderately differentiated* | *Well-differentiated* |
|---|---|---|---|---|
| Smoking only | 3 | 1 | 1 | 1 |
| Alcohol only | 2 | Nil | 2 | Nil |
| Tobacco consumption only | 7 | 3 | 3 | 1 |
| Areca nut consumption (betel quid and paan masala) only | 1 | Nil | 1 | Nil |
| Tobacco + alcohol | 5 | 2 | 3 | Nil |
| Tobacco + smoking | 9 | 2 | 5 | 2 |
| Tobacco + areca nut (betel quid and paan masala) | 4 | 1 | 2 | 1 |
| Alcohol + smoking | 2 | Nil | 2 | Nil |
| Tobacco + alcohol + smoking | 11 | 3 | 5 | 3 |
| Smoking + alcohol + areca nut (betel quid and paan masala) | 3 | 1 | 1 | 1 |
| Tobacco + smoking + areca nut (betel quid and paan masala) | 2 | 1 | 1 | Nil |

is consumed raw, in betel quid, or as paan masala. Paan masala is a dry commercial preparation containing areca nut, catechu, lime, unspecified spices, and flavoring agents. The same mixture with tobacco is called gutka. Due to extensive publicity and affordability, these products are very popular among minors. It is a major risk factor for oral submucous fibrosis and OSCC.[7]

In our study, most of the cases presented to the hospital for initial evaluation during stage III. This can be attributed to ignorance regarding oral lesions. We also

**Table 6:** HIF-1α levels in patients with different predisposing habits

| *Habit* | *HIF-1α level (pg/mL)* |
|---|---|
| Smoking only | 720 ± 87.6 |
| Alcohol only | 220 ± 67.8 |
| Tobacco consumption only | 715 ± 162.5 |
| Areca nut consumption (betel quid and paan masala) only | 896 |
| Tobacco + alcohol | 907 ± 433.1 |
| Tobacco + smoking | 760 ± 331.6 |
| Tobacco + areca nut (betel quid and paan masala) | 740.3 ± 210.3 |
| Alcohol + smoking | 701 ± 279.2 |
| Tobacco + alcohol + smoking | 722 ± 36.6 |
| Smoking + alcohol + areca nut (betel quid and paan masala) | 992 ± 372.6 |
| Tobacco + smoking + areca nut (betel quid and paan masala) | 948 ± 328.8 |
| Tobacco + alcohol + smoking + areca nut (betel quid and paan masala) | 983 ± 210.2 |

tried to evaluate the effect of habits on the stage and histological grade of the tumors. Most of the patients gave a history of more than one habit, and hence, assessment of a single factor on the histological features is not possible. Nonetheless, we observed that areca nut chewing had a synergistic effect with alcohol and smoking with increased incidence of late stage and poor differentiation. This is congruent with many studies conducted in the recent past.[8] The problem is more acute in Asian countries due to the age-old custom of betel quid chewing.

The areca nut and/or its constituents (polyphenols and tannins) undergo metabolic activation to produce the active carcinogenic derivatives. Areca nut derivatives cause deoxyribonucleic acid (DNA) strand breaks, mutations, and trigger biochemical changes that promote carcinogenesis.[8] Mutations of tumor suppressor genes, such as TP53, BRCA1, and BRCA2 have also been reported in areca nut chewers which adversely affects their tumor suppressive properties.[9] Studies have highlighted the role of areca nut extract in induction of free radical generation and oxidative stress.[5]

The expression of mitogen-activated protein kinases, phosphatidylinositol 3-phosphate kinase/Akt, and nuclear factor κB is also upregulated by arecoline.[10] It has also been shown that areca nut reduces the glutathione synthetase content in cells, leading to increased oxidative stress.[11] Lee et al[12] corroborated that Snail overexpression mediated by ROS generation in areca quid chewing-associated OSCC can be correlated with tumors differentiation and lymph node metastasis. Areca nut chewing also promotes tumor progression by inducing matrix metalloproteinase-2 and -9 secretions.[13]

Areca nut also leads to microabrasions in the oral cavity due to its particulate nature.[14] Studies have also demonstrated that collagen homeostasis is disturbed leading to cross-links and initiation of submucous fibrosis.[7] Arecoline facilitates premature transition from G1 to S leading to error-prone DNA replication. The BRCA1 and BRCA2 gene expression is reduced and human telomerase reverse transcriptase is overexpressed.[15] Arecoline also promotes keratinocyte migration and facilitates invasion by upregulation of αvβ6 expression.[16] The high copper content in areca nut stimulates tumor angiogenesis activating several angiogenic factors, such as vascular endothelial growth factor, tumor necrosis factor alfa. Chewing areca nut increases copper levels, which in turn stimulates fibrogenesis through upregulation of lysyl oxidase activity.[17,18]

Hypoxia-inducible factor-1α is a transcriptional factor, which is activated due to intratumoral hypoxia and plays a significant role in cancer progression and metastasis.[19] We studied the serum HIF-1 α levels in patients with histologically confirmed OSCC. The levels were significantly higher in the cases as compared with the healthy controls. Further, on comparison of HIF levels in patients classified according to their addictive habits, we found significantly higher levels among subgroups, who indulged in areca nut chewing along with smoking/alcohol, etc. Our study group had only one patient with isolated areca nut chewing, so the effect on HIF-1α expression cannot be ratified with statistical significance. Nonetheless, the highest concentration was noted in patients with simultaneous smoking, alcohol, and areca nut consumption.

Lee et al[20] conducted a study involving 25 oral biopsy samples with areca quid chewing-associated OSCC and 10 normal oral tissue biopsy samples without areca quid chewing, which were analyzed by immunohistochemistry. They proved that HIF-1α expression is significantly upregulated in areca quid chewing-associated OSCC in a dose-dependent manner. Chang et al[21] demonstrated that arecoline renders human buccal mucosal fibroblasts more vulnerable to other reactive agents in cigarettes via glutathione S transferase reduction in a dose-dependent manner. They went on to conclude that compounds of tobacco products may act synergistically with arecoline in the pathogenesis of oral mucosal lesions in areca quid chewers. Zhang et al[22] conducted a study on nonsmall cell lung cancer (NSCLC) cell lines and concluded that nicotine stimulates HIF-1α protein accumulation in NSCLC cells. They also demonstrated that HIF-1α contributes to nicotine-promoted cell migration, invasion, and tumor angiogenesis by lung cancer cells.

We may conclude that arecoline is a major carcinogen implicated in oral cancer. Potential interplay between nicotine and arecoline has been proved by various studies. The upregulation of HIF-1α by arecoline may be based on

the biochemical aggressive tumors in areca nut chewers. Our study highlights the need to pursue awareness programs more aggressively with equal importance to areca nut chewing besides tobacco, which has been in the forefront for OSCC prevention strategies.

Our study has several limitations inherent to its small sample size and lack of follow-up data. Areca nut chewing, smoking, alcohol history was self-reported and therefore, was subject to recall bias. A large-scale prospective trial comprising subjects with addictive habits besides healthy controls and confirmed cases of OSCC with follow-up is necessary to substantiate the findings with reasonable statistical accuracy and clinical relevance.

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