International Journal of Research and Review
Vol. 9; Issue: 2; February 2022
Website: www.ijrrjournal.com
Original Research Article *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

Clinical Relevance of Cotinine and Nicotine Levels as Tobacco Exposure Biomarkers in Oral Cancer Patients

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DOI: https://doi.org/10.52403/ijrr.20220234

ABSTRACT

Introduction: Oral cancer is cause mainly due to habits of tobacco consumption and it is a major health hazard across the world. The death rate due to the disease is also very high. However, the association of tobacco exposure with stage of the disease and treatment outcome is not reported. Therefore, we assessed correlation between levels of tobacco exposure with the disease status and treatment outcome in oral cancer patients.

Materials and Methods: Urine samples were collected from enrolled pre-therapeutic oral cancer patients (N=96), healthy individuals with tobacco habits (N=19) and healthy individuals without tobacco habits (N=14). Urinary nicotine and cotinine levels were analyzed as indicators of tobacco exposure by HPLC methods. Data was statistically analyzed using the SPSS statistics version 20.0.

Results: Present study revealed that tobacco exposure levels were significantly higher in oral cancer patients and healthy individuals with tobacco habits as compared to healthy individuals without tobacco habits. Receiver Operating Characteristics (ROC) curve analysis revealed that tobacco exposure levels have a good discriminatory efficacy between healthy individuals without habit of tobacco and oral cancer patients as well as between healthy individuals without habit of tobacco and healthy individuals with habits of tobacco. Kaplan Meier survival curve analysis also revealed that patients who were having higher tobacco exposure levels at the time of diagnosis showed worse survival than patients who were having lower tobacco exposure levels.

Conclusion: Tobacco exposure levels could be a simple, non-invasive and cost effective prognostic biomarker for management of oral cancer.

Keywords: Tobacco exposure, Cotinine, Nicotine, HPLC, ELISA, Oral Cancer, Urinary biomarker

INTRODUCTION

Oral cancer is one of the major causes of mortality across the globe. As documented by GLOBOCAN 2020, incidence of oral cavity cancer across the world was 3,77,713 (2.0%) and mortality was 1, 77,757 (1.8%). [1] Moreover, its incidence rate is higher in developing countries compared to the developed countries. One third of the oral cancer cases of the world are found in India. According to the GLOBOCAN 2018, 1,19,992 (10.4%) new cases and 72,616 (10.16%) deaths of lip and oral cavity cancer is reported in India. [1]

Globally, India is the second largest consumer of tobacco. In India, generally oral cancer affects lower socioeconomic groups, because of their higher use of tobacco products. [2] Tobacco smokes and chews contain more than 60 different carcinogenic compounds including tobacco specific nitrosamines, polycyclic aromatic

hydrocarbons and others. [3,4] Almost all tobacco products hold nicotine in higher concentration. A major metabolite of nicotine and cotinine can be simply detected in various body fluids like blood, urine and saliva. [5] Techniques like High Performance Liquid Chromatography (HPLC) and Enzyme- linked Immune Sorbent Assay (ELISA) are generally used to estimate the concentration of nicotine and cotinine. Besides, the *in vivo* effect of tobacco exposure on therapeutics response sustains nicotine as an important component of tobacco for decreasing the effectiveness of the cancer treatments.

Oral cancer is considered as a grave problem across the India and surgery is the ultimate treatment given to these patients. Though there is massive improvement in treatment protocols including chemotherapy, radiotherapy and targeted therapy in current decade, the prognosis of oral cancer still remains deprived due to aggressive local invasion and metastasis leading to recurrence. This might be addressed with a simple, non-invasive and cost-effective prognostic biomarker. [6] There are no reports on evaluation of tobacco exposure as a simple routine test. In light of this, the aim of the study was to evaluate the role of tobacco exposure in oral cancer patients as a simple urinary prognostic biomarker.

MATERIALS AND METHODS

The study was conducted at The Gujarat Cancer and Research Institute. The study was approved by the institutional ethics committee and informed consent was taken from all the enrolled subjects. The demographics details were collected from enrolled 96 pre-therapeutic oral cancer patients, 19 healthy controls with tobacco habits and 14 healthy controls without tobacco habits using an interview questionnaire. The information ascertained included details of age, sex, tobacco habits, clinic-pathological characteristics.

Urine samples were collected from oral cancer patients prior to initiation of any anticancer therapy and stored at -20˚C until analyzed. Urinary cotinine and nicotine were extracted in chloroform under alkaline conditions. [7] Further, cotinine and nicotine levels were analyzed by modified HPLC method using a UV detector. [8,9] Urinary cotinine levels among healthy individuals without habits of tobacco, healthy individuals with habit of tobacco and oral cancer patients were also carried out by ELISA.

Statistical Methods:

Data were statistically analyzed using the SPSS statistics version 20.0. The results were presented as Mean ±Standard Error (SE) of cotinine and nicotine values. The independent t-test was performed to determine whether there is a statistically significant difference between the means in two unrelated groups such as healthy controls and pre-therapeutic oral cancer patients. Discretionary efficacy of tobacco exposure levels was determined by Receiver Operative Characteristic (ROC) curve. The discriminatory efficacy was compared between healthy individuals without habit of tobacco and oral cancer patients as well as healthy individuals without habit of tobacco and healthy individuals with habit of tobacco.

RESULTS

In the 96 pre-therapeutic oral cancer patients, the age range was observed to be from 24 to 70 years (median: 42). Among these, 84 (87.5%) were males and 12 (12.5%) were females with the majority of the patients having carcinoma of tongue (n=49; 51%) followed by carcinoma of buccal mucosa (38; 39.6%) and remaining with other oral malignancies. The patients had different tobacco consuming habits mainly tobacco chewing (n=50; 52.1%) and smoking (n=24; 25%). Mainstream of the patients had stage IV disease (n=38; 39.6%) and moderately differentiated tumors (n=54; 56.3%) with no lymph node involvement (n=44; 61.4%) which is mention in Table-1.

Table: 1. Patients' Characteristics

PATIENTS' CHARACTERISTICS	N(N=96), PERCENTAGE (100%)	PATIENTS' CHARACTERISTICS	N(N=96), PERCENTAGE (100%)
AGE (years)		CLINICAL STAGES	
<44	48(50%)	Stage-I	11(11.5%)
>44	48(50%)	Stage-II	15(15.6%)
GENDER		Stage-III	14(14.6%)
Male	84(87.5%)	Stage-IV	38(39.6%)
Female	12(12.5%)	Undetermined	18(18.8%)
DIAGNOSTICS		HISTOPATHOLOGY REPORT	
ca Buccal Mucosa	38(39.6%)	Poorly Differentiated	5(5.2%)
ca Tongue	49(51%)	Moderately Differentiated	54(56.3%)
Others	9(9.4%)	Well Differentiated	25(26%)
HABITS		Undetermined	12(12.5%)
Tobacco Chewing	50(52.1%)	TUMOR SIZE	
Smoking	24(25%)	T1	14(14.6%)
Tobacco Chewing+ Smoking	9(9.4%)	T2	25(26%)
Smoking + Alcohol	3(3.1%)	T3	18(18.8%)
Tobacco Chewing+ Alcohol	4(4.2%)	T4	23(24%)
Tobacco Chewing+ Smoking + Alcohol	2(2.1%)	Undetermined	16(16.6%)
Passive Exposure	1(1%)	LYMPH NODE	
No Habits	3(3.1%)	Presence	37(38.5%)
		Absence	44(61.4%)

Figure 1 shows mean urinary cotinine and nicotine levels estimated in healthy individuals without tobacco habits, healthy individuals with tobacco habits and pre-therapeutic oral cancer patients. Mean cotinine levels were higher in healthy individuals with habits of tobacco and oral cancer patients as compared to healthy individuals without tobacco habits. Mean nicotine levels were significantly higher in healthy individuals with habits of tobacco and oral cancer patients as compared to healthy individuals without tobacco habits.

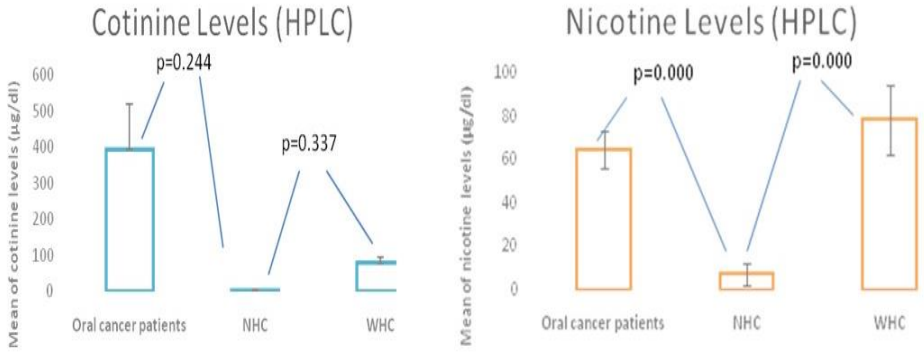

Figure: 1 Urinary Nicotine and Cotinine Levels (by HPLC) in healthy individuals without habit of tobacco, healthy individuals with habit of tobacco and oral cancer patients

In present study, 49 urinary samples of pre-treated oral cancer patients, 6 urinary samples of healthy individuals with tobacco habits and 6 urinary samples of healthy individuals without tobacco habits were taken for estimation of urinary cotinine levels by ELISA method. Figure 2 mean urinary cotinine levels were higher in healthy individuals with tobacco habits and pre-therapeutic oral cancer patients as compared to healthy individuals without tobacco habits. Here, similar results were also observed by HPLC method.

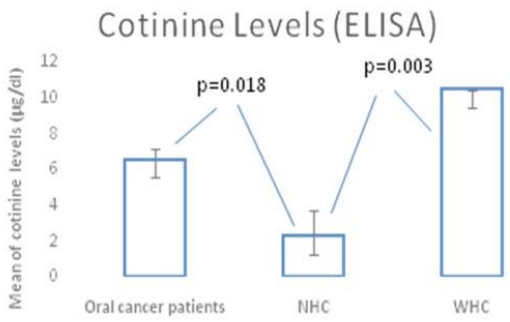

Figure: 2 Urinary Cotinine Levels (by ELISA) in healthy individuals without habit of tobacco, healthy individuals with habit of tobacco and oral cancer patients

Further, the present study also assessed the sensitivity and specificity of tobacco exposure markers by ROC curve. Figure 3 depicts ROC curves for comparison of nicotine and cotinine levels, which can be considered as tobacco exposure markers as they have good prejudiced efficacy between healthy individuals without tobacco habits and oral cancer patients as well as between healthy individuals without tobacco habits and healthy individuals with tobacco habits.

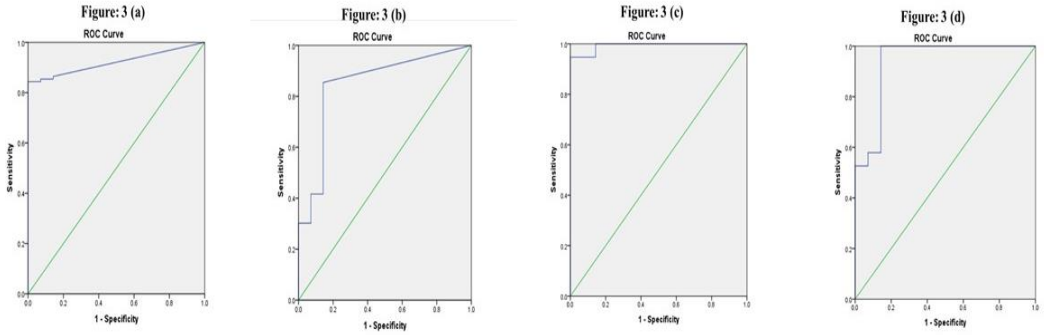

Figure Titles	Area Under Curve	Standard Error	Significance
3 (a): Cotinine Levels Oral Cancer Patients vs NHC	0.920	0.026	0.000
3 (b): Nicotine Levels Oral Cancer Patients vs NHC	0.846	0.059	0.000
3 (c): Cotinine Levels WHC vs NHC	0.992	0.010	0.000
3 (d): Nicotine Levels WHC vs NHC	0.936	0.046	0.000

Figure: 3 ROC Curves for Comparison of Cotinine and Nicotine levels between Controls and Oral Cancer Patients

The current study also evaluated the correlation of urinary cotinine and nicotine levels with clinic-pathological parameters in oral cancer patients, which revealed no significant association shown in Table 2

Table:2 Correlation of Cotinine Levels and Nicotine Levels with Clinico-pathological Parameters

		Cotinine	Nicotine
		Mean ± S.E.	**Mean ± S.E.**
Tumor Size	T1+T2	237.74±37.61	61.21±12.46
	T3+T4	470.11±265.30	56.47±11.02
LN status	Negative	254.77±49.42	74.32±15.17
	Positive	420.21±232.53	42.21±5.63
Histopathology Report	Well	533.05±276.51	52.37±12.65
	Moderate	219.35±37.95	57.31±9.65
	Poor	108.25±115.97	110.13±60.14
Clinical Stage	Early	251.32±46.27	75.38±16.18
	Advanced	393.16±202.75	49.35±8.62

All patients received uniform treatment of surgery followed by adjuvant radiotherapy or chemotherapy. The present study also showed correlation of tobacco exposure with survival curve. In the study, positive and negative values were defined based on cut off value of healthy individuals without habits of tobacco. (Mean± S.E.) Kaplan Meier survival curve analysis also revealed that patients who were having higher cotinine and nicotine levels at the time of diagnosis showed worse survival than patients who were having lower cotinine and nicotine levels shown in Figure 4

Figure: 4(a) Cotinine

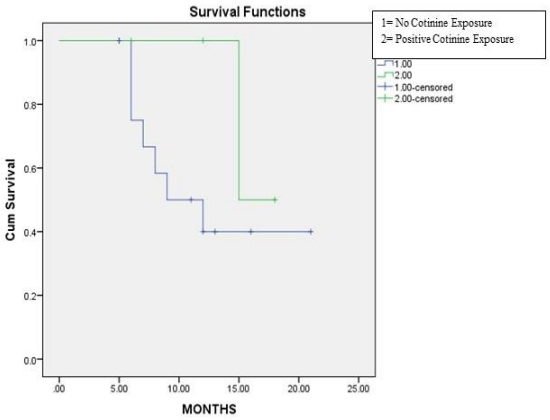

Figure: 4(b) Nicotine

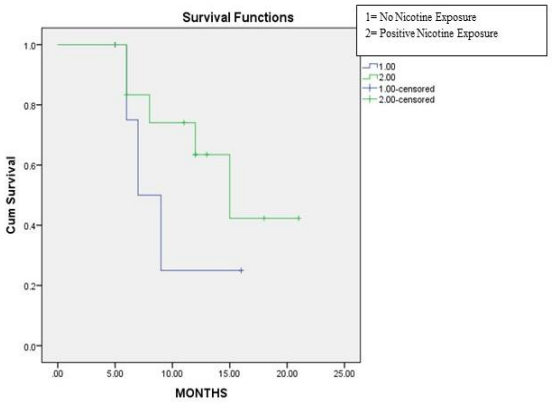

Figure: 4 Survival Curves of cotinine and nicotine biomarkers in oral cancer patients

DISCUSSION

Oral cancer is one of the major ailments across the globe with high mortality rate. In India, high prevalence of oral cancer has been associated with habits of chewing smokeless tobacco and smoking. Some studies have suggested the association of high risk of oral cancer with high amount of tobacco used and prolonged duration of use while the reduction in the risk of oral cancer was associated with tobacco cessation. [10]

Virtually all tobacco products contain nicotine in substantial concentration. [11] Cotinine, a major metabolite of nicotine, can be easily detected in various body fluids like blood, urine and saliva. [12,13] It is most commonly used marker to distinguish between tobacco users and non-tobacco users because of its greater sensitivity and specificity than other biochemical tests. [14,15,16] There is a high correlation between blood and saliva cotinine concentrations. A widely used biomarker is urine cotinine level since cotinine concentrations are four to six times higher in urine than in blood or saliva. [14] This makes quantitative methods like gas chromatography/mass spectrometry or high-performance liquid chromatography, colorimetric assays and immunoassay which measure urine cotinine more valid and reliable. [13]

In present study, higher urinary nicotine and cotinine levels were found in healthy individuals with habits of tobacco and oral cancer patients as compared to healthy individuals without habits of tobacco. HPLC method may be helpful for screening of tobacco exposure in large studies. Some studies reported that urinary nicotine and cotinine can be used as the biomarkers to environmental tobacco smoke. [17] The nicotine and cotinine in urine appears to be most specific and sensitive biomarker to estimate environmental tobacco smoke. [18] Kulza et. al. has also reported that the concentration of salivary cotinine can be detected using high performance liquid chromatography. [13] It was suggested that salivary cotinine is useful in the assessment of tobacco. Whereas, Murphy et. al. has observed low level of nicotine and cotinine in serum after addition of nicotine to the drinking water. [19] In a previous study, it was reported that mean urinary cotinine expression were higher in passive smokers than the unexposed individuals. [20] This study on a concordance with another study showed, healthy controls and oral cancer patients having tobacco chewing and smoking habits were also obtained higher levels of urinary nicotine and cotinine in comparison with non-habituated healthy controls. [9] Besides these, cotinine levels were also correlated with rate of tobacco products consumption. [21]

It is the established fact the early detection of oral cancer results in better long

term survival as well as improved prognosis, while making health care less expensive. Reduced overall survival and specific disease survival after cancer detection have been found to be higher among current smokers compared to the patients with the habit of smokeless tobacco and non-smokers, though the present treatment protocol improves the quality of life of oral cancer patients, but the overall survival rate of five years has not improved in the past decades. [20]

More studies are required on health effects of nicotine exposure in humans, based on *in vitro* and *in vivo* effects of nicotine products during cancer treatment unless it is needed temporarily to stop tobacco smoking. [20] Warren et. al. studied role of nicotine on response to radiotherapy and chemotherapy *in vivo* which suggested that nicotine exposure specifically during treatment is a critical determinant of therapeutic outcome. [22] Sanner T and Grimsrud TK, also suggested that nicotine may promote cancer progression independent of the combustion products of tobacco smoke. [20] The authors also reported that people who have used tobacco after a cancer diagnosis and during cancer treatment have also had a history of tobacco consumption prior to their diagnosis. However, in these studies the tobacco exposure was not reported with treatment outcome. It is reported that tobacco cessation during treatment for cancer does in general result in better response and increased survival. In the present study, tobacco exposure was associated with survival rate of oral cancer depicting that the tobacco consumers had a worse prognosis even post therapy even though, the patients received same treatment.

CONCLUSION

Early diagnosis and treatment of oral cancer can be established only after having a complete perceptive regarding molecular basis of carcinogenesis. Thus, outcome of the present study would be useful to monitoring tobacco exposure and screen risk population susceptible for this disease and may play a noteworthy role for enhancement of prognosis of oral cancer. A study on a larger cohort may aid in understanding the significant role of tobacco exposure markers in oral carcinogenesis. In future, study of tobacco exposure status as estimation of nicotine and cotinine with survival of oral cancer patients would be given better outcome for prognosis of oral cancer.

Acknowledgement: None

Conflict of Interest: None

Source of Funding: None

Ethical Approval: Approved

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How to cite this article: Jethva DD, Rathod BS, Thobias AR et.al. Clinical relevance of cotinine and nicotine levels as tobacco exposure biomarkers in oral cancer patients. *International Journal of Research and Review*. 2022; 9(2): 247-253. DOI: *https://doi.org/10.52403/ijrr.20220234*

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