ORIGINAL ARTICLE

Urinary Nitrosamines: An Underestimated Biomarker in Laryngeal Cancer

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Abstract

Laryngeal cancer is the ninth and the seventh most common cause of cancer in males in Asia and India, respectively, and enlisted as one of the tobacco-related cancers. The carcinogen in tobacco is the tobacco-specific nitrosamines (TSNAs). Both secondary and tertiary amines can react with nitrite yielding nitrosamines which are excreted in urine. This study aims at quantifying urinary nitrosamines.

Aims and objectives:

- To quantify urinary nitrosamines in smokers and subjects consuming chewable forms of tobacco.
- Correlation of urinary nitrosamine levels with the development of laryngeal cancer.

Materials and methods: This study was a retrospective cross-sectional study conducted in adult patients presenting at the outpatient department of ENT in a tertiary care hospital over a period of 2 years. One hundred twenty-six cases were studied in detail. The urine of all adult patients presenting with malignant or premalignant lesions of larynx was analyzed and the sample was then subjected to liquid chromatography followed by mass spectrometry and the final amount of urinary nitrosamines was obtained in picograms/nanoliter (pg/nL).

Results: Out of 126 laryngeal lesions that presented at the outpatient department, 107 cases were malignant and 19 cases had premalignant lesions. The mean of quantity of urinary nitrosamines was found to be the highest 843pg/nL among the subjects practicing combined modality (smoke + smokeless) of tobacco consumption. The mean of urinary nitrosamines was significantly higher 778.23 pg/nL in smokers as compared with tobacco chewers 613.45 pg/nL. Out of the 107 patients of carcinoma larynx (Ca larynx), 78 cases were smokers suggesting smoking that has a stronger association in the development of carcinoma larynx.

Conclusion: The amount of urinary nitrosamines was higher in smokers, high in cases when more than one modality of tobacco was consumed and was more in cases of malignancy of larynx as compared to benign lesions.

Keywords: Combined modality of tobacco consumption, Laryngeal cancer, 4(Methylnitrosoamino)-1-(3-pyridyl)-1butanone, 4-Methyl nitrosoamino-1-3-pyridyl-1 butanol, Urinary nitrosamines.

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Introduction

Laryngeal cancer is the ninth and the seventh most common cause of cancer in males in Asia and India, respectively.[1,2] In 2012, 25,446 new cases were diagnosed and 17,560 Indians lost their lives from cancer larynx.[1] Incidence reported = 1.26–8.18/100,000 population.[2] Carcinoma larynx attributes to 3–6% of all cancers in males and only 0.2–1% of all cancers in females.

The most important risk factor for cancer larynx appears to be the use of tobacco. Carcinoma larynx is enlisted as one of the tobacco-related cancers. International Agency for Cancer Research Monograph classifies tobacco use as a carcinogenic to humans.[3,6,27] In India, tobacco is both chewed and smoked in several forms. A common form of smoking tobacco is *beedi* containing 0.2–0.3 g of tobacco rolled in a dried leaf.[4,28] Rao et al. found *beedi* and cigarette to be associated with cancer of larynx; however, studies show that the risk of cancer larynx is much more for *beedi* than for cigarette. The main reason for continued use of tobacco is dependence on nicotine. It is well established that both secondary and tertiary amines can react with nitrite, yielding nitrosoamine which are excreted in urine. These TSNAs are the most abundant and strong carcinogens in the smoke of tobacco (procarcinogens).[5,18,26]

Of the seven TSNAs that have been identified prominent being N'nitrosonornicotine, 4(methylnitrosoamino)-1-(3-pyridyl)-1butanone (NNK) and 4-methyl nitrosoamino-1-3-pyridyl-1 butanol (NNAL) are the most carcinogenic.[6,19–21] TSNA are procarcinogens, agents that require metabolic activation. The active forms of the carcinogenic TSNAs react with cellular components, including DNA, and with hemoglobin.[26] These DNA adducts produce changes in the cells which turn on their mitotic activity. The measurements of these DNA adducts can be used as biomarkers. Since ages, there have been studies regarding *beedi*, cigarette, and other smokeless forms of tobacco. This study aims at quantifying urinary nitrosoamines.

Aims and Objectives

- To quantify the urinary nitrosamines in smokers and subjects consuming chewable forms of tobacco.

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- Correlation of urinary nitrosamine levels with the development of laryngeal cancer.

Selection of Subjects

All adult patients attending the outpatient department of ENT with a premalignant or malignant lesion of the larynx.

Inclusion Criteria

- Patients willing to participate in this study.
- Patients who are currently smoking or chewing tobacco.
- Patients having signs and symptoms of laryngeal pathology.

Exclusion Criteria

- Patients not willing to participate in this study.
- Patients taking only alcohol and no tobacco.
- Patients who have stopped smoking more than 5 years ago.

Materials and Methods

The present study was prospectively conducted at the Department of Otorhinolaryngology at a tertiary hospital during the period of 1st October 2014–30th September 2016.

A careful thorough history and clinical examination of the oral cavity, oropharynx, hypopharynx, and nasal cavity was carried out for any abnormality in the mucosa or mucosal changes or growth or mass. Patients with a specific mucosal change in the oral cavity or larynx, mass in the neck or growth in the larynx, or mucosal changes in the larynx were studied in detail.

In all these cases, the patients were subjected to cytological and histopathological examination. One hundred twenty-six cases were studied in detail.

The urine of smokers in the present and the past was analyzed at the Department of Epidemiology, Regional Medical Research Centre, Bhubaneswar. The urine samples were thawed, vortexed, and then heated to 37°C for 20 minutes to release possible NNAL from the precipitate, followed by centrifugation at 5,000*g* for 5 minutes. For free NNAL determination, 50 µL of urine was spiked with 50 µL solution containing 0.5 ng each of d3-NNK and d3-NNAL as internal standards in the deionized water. For total NNAL (free NNAL plus NNAL-Gluc) determination, the isotope-spiked urine samples (100 µL) were hydrolyzed with 50 µL of β-glucuronidase (2,000 U/mL) in 75 mM of phosphate buffer (pH 6.8) at 37°C for 24 h in the dark to convert NNAL-Gluc to NNAL. After automatic sample cleanup, the urine was subjected to liquid chromatography followed by mass spectrometry and the final amount of urinary nitrosoamines was obtained for each sample in picogram/nanoliter of urine.

Observation and Results

Table 1 depicts the distribution of study subjects by the status of malignancy wherein among the total 126 laryngeal lesions cases that presented at the outdoor department, 107 cases were found to be malignant and 19 cases were found to be premalignant. This suggested the late-stage presentation of the disease as well as a rapidly growing course of the disease after clinical manifestation.

Table 1: Distribution of study subjects by status of malignancy

Lesions	*Malignant*	*Premalignant*	*Total*
	107	19	126

Table 2 shows the association of different forms of tobacco intake with the amount of urinary nitrosamines. The mean of quantity of urinary nitrosamines was found to be the highest (843 pg/nL) among the subjects taking smoked and smokeless form together followed by the subjects taking smoked form alone (792 pg/nL). The subjects taking more than 1 modalities of smokeless forms and smokeless forms alone had a considerably lower amount of nitrosamines in their urine, respectively, being 713 pg/nL and 684 pg/nL, which indicated higher association of malignancy with cases taking smoked form of tobacco ($p = 0.000$).

Table 3 illustrates the association of the quantity of urinary nitrosamines in study subjects with laryngeal carcinoma exposed to smoke and smokeless forms of tobacco. When a comparison was drawn among the 107 malignant cases to find out the difference of the mean of urinary nitrosoamines among the subjects taking smoked form of tobacco and the subjects taking smokeless forms of tobacco, the value was considerably much higher in subjects of smoked form being 778.23 pg/nL as compared to the subjects taking smokeless form which was 613.45 pg/nL. This evidence took us even closer to the association of urinary nitrosamines with smoked form and in turn malignancy. Table 4 illustrates the association of the development of lesions with different modalities of tobacco use.

In the present study, out of the 107 patients CA larynx, only 28 persons chewed tobacco in comparison to 78 persons who smoked indicating that smoking has a stronger association in the development of CA larynx.

Discussion

Carcinoma larynx is the seventh most common cancer in India and is considered a multifactorial disease.[1] Malignant laryngeal tumors could cause progressive destruction of the voice box with the loss of its functions such as voice and breathing. It is, however, known that laryngeal squamous cell carcinoma (SCC) is a predominantly male disease, possibly because of the fact that men smoke more tobacco than women, as is found in other parts of the world.[3,7] In summary, our data clearly show that TSNA levels in cigarette smoke are more as compared to smokeless forms. The analytical findings agree with other studies that tobacco products, including chewed forms of tobacco, contain carcinogenic compounds, which are of concern in the formation of cancer in users of these products. Most importantly, the growing body of scientific evidence in the tobacco research field has advanced our understanding of the process of tobacco-associated carcinogenesis, and such findings will help provide needed information to researchers in their quest for effective prevention of cancer from the use of tobacco products.

In our study, out of the total laryngeal lesions that presented at our outpatient department, 72% of the cases were malignancy suggesting the late stage of presentation of the patients to the hospital and a rapidly growing course of the disease. This is in consistence with Fasunla et al. study.[8]

The quantity of urinary nitrosamines in smokers alone when compared with that of combined modality use of tobacco was found to be nearly equal without much significant difference. This has been demonstrated by Hoffmann and Stephen in their study which showed a high incidence of nitrosamines in the urine of smokers.[5,11,13] However, the amount of nitrosamines found in subjects taking smokeless forms alone or in combination with other

Table 2: Association of different forms of tobacco with amount of nitrosamines

Modality of use	*Smoke + smokeless*	*Smoke*	*More than 1 modalities of smokeless forms*	*Smokeless forms*	*t value 16.857*
Mean of quantity of urinary nitrosamines (pg/nL)	843	792	713	684	*p* value 0.000

Table 3: Association of quantity of urinary nitrosamines with smoke and smokeless forms in patients with laryngeal carcinoma

Modality	*Smoke form*	*Smokeless form*
Mean of quantity of urinary nitrosamines (pg/nL)	778.23	613.45

Table 4: Association of development of lesions with different modalities of tobacco use

Modality of use	*Smoke form*	*Smokeless form*	*Nonsmokers*	*Total*	$X^2 = 29.372$
Malignant	78	28	1	107	$p = 0.001$
Premalignant	16	3	0	19	

modalities was significant too, but not at par with smoked form. This showed a significant association of smokers with the accumulation of nitrosamines (carcinogens) in the body and so considered to have a higher chance of developing malignancy.

In our study, we came across 107 cases of carcinoma larynx, out of which, 78 subjects smoked, 28 used smokeless modality of use, and there was a lone nonsmoker which depicts the strong association of smoking with the development of malignancy. Along with this, there is also a very strong message regarding the use of smokeless forms like tobacco chewers who though have less predisposition but certainly can go on to develop the disease in future. The lone nonsmoker and nontobacco chewer developing malignancy show the multifactorial association of carcinoma larynx such as human papilloma virus.[9,10] Alcohol drinking and tobacco smoking are known to be major risk factors of laryngeal cancer (Maier and Tisch, 1997; Elwood et al., 1984).[12,22,24] Smoking and chewing of betel leaf with tobacco were found to increase the risk of laryngeal cancer 4.54- and 2.37-fold, respectively. An earlier study conducted in India reported the relative risk of laryngeal cancer among tobacco chewers vs nonchewers as 1.8 times higher (Notani and Jayant, 1987).

To the best of our knowledge, ours is the first paper which finds an association of nitrosamines in laryngeal cancer patients and also quantifies the amount of nitrosamines in patients using different modalities of tobacco use.

Conclusion

The amount of urinary nitrosamines is found to be higher in smokers compared to subjects who used smokeless forms of tobacco in patients with carcinoma larynx. The amount of urinary nitrosamines is found to be higher in cases where more than one modality of tobacco is consumed. The amount of urinary nitrosamines is found to be more in cases of malignancy of larynx in comparison to benign lesions.

Clinical Significance

The quantitation of nicotine-derived nitrosamine-macromolecule adducts in smokers or chewers is a potentially exciting area of research because levels of these adducts could provide an index of an individual's capacity to metabolically activate these carcinogens. Such data might eventually lead to an estimate of susceptibility to tobacco-related cancer. Nicotine-derived nitrosamines are better candidates for this experimental approach than are other carcinogens such as benzo(*a*)pyrene, because they occur only in tobacco and tobacco smoke. Ultrasensitive methods using either immunochemical techniques or postlabeling with 32P are presently available for the measurement of carcinogen–DNA adducts and, in some cases, have already been applied to humans.[14–17] A sensitive biotin–avidin enzyme-linked immunosorbent assay for the measurement of O6-methyldeoxyguanosine in DNA has been developed in our laboratory and will be applied to human tissues exposed to NNK.[18,23–25] The detection of hemoglobin adducts of NNK or NNN is also under investigation. This approach has been used previously for monitoring human exposure to ethylene oxide and for experiments with methylating agents. An advantage of this strategy is the relative stability of circulating hemoglobin in humans (120 days) which can provide a cumulative index of exposure and activation.

Quitting smoking and avoiding exposure to environmental tobacco smoke are the only established means to significantly reduce the risk associated with exposure to TSNAs and other harmful chemicals in tobacco smoke. The surgeon General's health warnings should be included on the *beedi* packaging to help dissuade the misconception that *beedi* cigarettes are somehow more 'natural' products and, therefore, less hazardous. Additional action is needed to counter the popularity of *beedi* cigarette smoking, especially among young people and others who may experiment with *beedis*. Additional research, consumer education, and public advocacy are critically needed to help reduce the health consequences of *beedi* smoking.

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