RESEARCH ARTICLE

Levels of Tobacco-specific Metabolites among Non-smoking Lung Cancer Cases at Diagnosis: Case-control Findings

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Abstract

Background: Environmental tobacco smoking (ETS) significantly contributes to morbidity and mortality and is a known risk factor for lung cancer development in lifelong nonsmokers. The metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-Glucs) have now emerged as leading biomarkers for the study of carcinogen exposure in non-smokers exposed to ETS. Materials and Methods: We carried out our study on NNAL in the urine of non-smokers exposed to ETS and the association between ETS and lung cancer. Subjects were enrolled from 2008-2010. NNAL was analyzed for 74 non-smoking lung cancer and 85 healthy controls. The main objective of this study was to provide an estimate of the risk of lung cancer from exposure to ETS in the Korean population. Results: The mean NNAL concentration in urine was significantly lower in non-smoking patient groups (n=74) than in control groups (n=85) (4.7±15.0 pg/mg, 6.5±17.9 pg/mg, respectively, Mann-Whitney U test, p<0.001). Conclusions: The urine NNAL of non-smoking patients with lung cancer was not elevated with regard to the non-smoking control group. This may be due to life-style changes after diagnosis. A prospective study will be needed to evaluate the association of NNAL and non-smoking lung cancer.

Keywords: Tobacco smoke pollution - environmental tobacco smoking - NNAL

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Introduction

Though tobacco smoking is the primary risk factor for lung cancer (Park et al., 2008; Kamsa-Ard et al., 2013), a significant fraction of lung cancer deaths occur in lifetime non-smokers (Sisti and Boffetta, 2012). Environmental tobacco smoking (ETS) is defined as tobacco smoke produced by an active smoker both from the exhalation of smoked tobacco and by the burning end of the cigarette, which is inhaled by nonsmokers (Florescu et al., 2009). ETS significantly contributes to morbidity and mortality in non-smokers. Exposure to ETS is one important risk factors for lung cancer (Couraud et al., 2012). However, there were some debates about the association between the exposure to ETS and lung cancer development (Brennan et al., 2004). Non-smokers are exposed to tobacco smoke not only in their homes but also in schools, restaurants, cars, buses, and other public places. Tobacco use by a spouse was associated with a 30% excess risk of lung cancer development and the population attributable fractions (PAFs) for individual risk factors ranged from 0.4% to 19.9% (Sisti and Boffetta, 2012).

N'-nitrosonornicotine (NNN) and 4- (methylnitros amino) -1-(3-pyridyl)-1-butanone (NNK), now recognized as "carcinogenic to humans" by the International Agency for Research on Cancer (IARC), were present in significant quantities and were effective carcinogens in animal models (Hashimoto et al., 2004). The ability of NNK to induce lung tumors, mainly adenocarcinoma, in rats, at low doses and independent of the route of administration, was particularly notable (Parsons et al., 1998) because the lung carcinogen NNK was found only in tobacco products, and therefore its metabolites-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-Glucs), if detected in the urine of non-tobacco users, could have had only one source of ETS. The metabolites -NNAL and NNAL-Glucs-have now emerged as the leading biomarkers for the study of carcinogen exposure in non-smokers exposed to ETS (Hecht, 2006).

The measurement of NNAL and NNAL-Glucs in the urine of non-smokers exposed to ETS could provide evidence for the association of ETS and lung cancer. We hypothesized that the levels of NNAL among non-smoker patients with lung cancer would be elevated if ETS was associated with the development of lung cancer. Therefore,

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we carried out our study on NNAL in the urine of patients with never-smoke lung cancer and never-smoke controls. The main objective of this study was to provide an estimate of the risk of lung cancer development from exposure to ETS in the Korean population. The secondary objective of this study was to demonstrate NNAL as a new candidate of biomarker.

Materials and Methods

Never-smoke lung cancer patients were enrolled from 2008-2010 in one hospital-based population. The inclusion criteria for non-smoking lung cancer patients were: *i*) newly diagnosed non-small cell lung cancer; *ii*) non-smoker; and *iii*) over 18 years old. Normal subjects with other smoking-related diseases were excluded from the control group. Urine specimens were collected from the subjects. The urine volumes were noted and frozen at -20°C until analysis. The total NNAL was measured by liquid chromatography-mass spectrometry using modifications of methods described in a previous stuy (Jacob et al., 2008). Briefly, 50μL of 500pg/mL NNAL-d3 internal standard solution was added to 5 mL urine samples, standards and quality control materials in 50 mL of polypropylene centrifuge tubes followed by brief vortex mixing. A sodium potassium phosphate buffer (0.5 mL of 2 M, pH 7) was added followed by the addition of 25 μL of 50 mg/mL glucuronidase dissolved in 0.1 M phosphate buffer. The samples were then incubated for 20-24 hours at 37°C to convert the NNAL glucuronides to free NNAL. Potassium carbonate (0.5 mL of 50% w/v) was added, and the samples were extracted by vortex mixing for 5 min with 8 mL of toluene/1-butanol (70:30). The two phases were separated by centrifugation at 4000 x g for 5 minutes. After freezing the tubes in a dry ice-acetone bath, the organic upper layers were poured into 16 x 125 mm glass culture tubes containing 0.8 mL of 1 M H_2SO_4. The tubes were then vortexed 5 min and centrifuged as described above. The aqueous layers were frozen with a dry ice-acetone bath, and the organic layers were poured off and discarded. The acid layer was then washed with 5 mL of ethyl acetate/toluene (2:1) by vortex mixing for 5 min. The tubes were centrifuged, the aqueous acid layers were frozen in a dry ice-acetone bath, and the upper wash layer was discarded. The acid phases were made basic with 0.8 mL of 50% (w/v) K_2CO_3 and extracted by vortex mixing 5 min with 4 mL of ethyl acetate/toluene (1:2). After centrifugation and freezing the aqueous layers, the organic layers were transferred to 13 x 100 mm glass culture tubes. The organic extracts were then evaporated to dryness at 70°C for 20 minutes. Hexanoic anhydride (50 μL) and 10 μL of 50 mg/mL 4-(dimethylamino)pyridine (DMAP) in toluene was then added to the dry extracts, the tubes were then tightly capped and heated at 70°C for 15 minutes. Saturated aqueous sodium bicarbonate (0.5 mL) and 4 mL of 10% ethyl acetate in pentane were added, and the derivatives were extracted by vortex mixing, centrifugation, and freezing the aqueous layers. The organic layers were transferred to 13 x 100 mm tubes containing 0.5 mL of 1 M H_2SO_4 and the organic layers were discarded after vortexing, centrifuging and freezing. The acid layers were made basic with 0.5 mL of 50% (w/v) K_2CO_3 and then extracted with 4 ml of 10% ethyl acetate in pentane by vortex mixing, centrifugation, and freezing the aqueous layers. The organic layers were transferred to a new set of 13×100 mm tubes and evaporated to dryness at 50°C for 20 minutes. The samples were reconstituted in 125 μL of 10% methanol containing 12 mM HCl and transferred to 200 μL polypropylene insert vials for analysis. The extracts (50 μL) were chromatographed with an acetonitrile and water solvent system containing 10 mM ammonium formate at 0.4 mL/min using a linear gradient from 20-100% acetonitrile/water buffer over 6 minutes. The column was then held at 100% acetonitrile/water for 1 minute and then ramped back to the initial conditions over 2 minute. The re-equilibration time was 6 minutes for a total run time of 10 minutes per sample. The NNAL concentration was compared between the non-smoking lung cancer group and the control group.

Table 1. Urine NNAL Concentrations in Each Group

Group	n	NNAL (pg/mg)		Standard deviation
		Median	Mean	
Non-smoking lung cancer	74	1	4.7	15
Male	45	1.4	3.2	9
Female	29	0.8	7.1	21.2
Non-smoking control	85	2.4	6.5	17.9
Male	23	2.9	9.6	21.6
Female	62	2	5.3	16.3

Results

Among the 74 non-smoking patients, 45 patients were male and 29 patients were female. Mean age of the non-smoking patients (n=74) and control (n=85) groups were 64±10.3 and 55.5±7.2, respectively. Levels of total NNAL was summarized in table 1. Mean NNAL concentration was significantly lower in non-smoking patient groups than in control groups (4.7±15.0 pg/mg, 6.5±17.9 pg/mg, respectively, Mann-Whitney U test, $p<0.001$).

Discussion

In this study, the urine NNAL of non-smoking patients with lung cancer was significantly decreased as compared to that of the non-smoking control group. We speculated that the reason why the NNAL were relatively low in non-smoking patients with lung cancer might be due to life-style changes such as that patients intentionally avoided from ETS after lung cancer had been diagnosed.

The etiology of lung cancer in never smokers remains indefinite although many putative risk factors have been described including secondhand smoking, occupational exposures, pre-existing lung diseases, diet, estrogen exposure, among others(Yano, Haro, Shikada, Maruyama, and Maehara, 2011). IARC concluded that ETS causes lung cancer in humans. Since tobacco-specific N-nitrosamines are found only in tobacco products or related nicotine-containing materials, their adducts or metabolites should be specific biomarkers of tobacco exposure (Hecht, 2004). The total NNAL was found at measurable levels in the urine of 41% of nonsmokers and in 87.5% of those with

substantial secondhand-smoke exposure (Bernert et al., 2010). Several studies on the level of NNAL in the urine of non-smokers exposed to ETS demonstrated that an increase of NNAL in much of the epidemiologic studies has been performed demonstrating that their data clearly showed that women who lived with men who smoked had significantly higher levels of NNAL plus NNAL-Glucs (termed total NNAL), and in newborns of mothers who smoked cigarettes during pregnancy (Lackmann et al., 1999), in the urine of elementary school-aged children (Hecht et al., 2001). Recently, NNAL became a promising biomarker as a risk assessment for lung cancer development in smokers. Several studies measuring the total NNAL, a tobacco-specific carcinogen, to the risk of lung cancer in smokers demonstrated that the total NNAL was a useful biomarker significantly associated with risk (Church et al., 2009; Yuan et al., 2012). The risk of an ETS-exposed non-smoker to develop lung cancer is about 1-2% as great as that of a smoker, remarkably consistent with the total NNAL biomarker data (Anderson et al., 2001). In this pilot study, we measured the total NNAL in non-smoking patients with lung cancer and tried to measure the effect of ETS on lung cancer in a non-smoker. However, this case-control study could not demonstrate the NNAL to be a marker for assessing the risk of lung cancer among non-smokers although total NNAL could be measureable in non-smoking lung cancer. Further prospective studies that collect urine specimens for the measurement of NNAL should periodically be needed. In consideration of the features in never-smoking NSCLC or 'non-smoking-associated lung cancer', both the stratification and controlling confounders should be used to investigate the role of NNAL in association with the ETS of non-smoking associated lung cancer.

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