Urinary Biomarkers for Secondhand Smoke

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The use of a biomarker is mandatory for quantitative analysis of exposure to secondhand smoke (SHS). This article summarizes urinary biomarkers of smoke exposure which can be now quantified. The most reliable urinary biomarkers to assess the exposure to SHS are NNAL 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and NNAL-Glucuronides, which is metabolites of tobacco-specific nitrosamine. These substances were detected even in 50% of children who had undetectable level of cotinine (<0.5 ng/ml). Urinary cotinine, which is determined by a highly sensitive competing enzyme immunoassay, is also a useful biomarker. However, individual variability of CYP2A6 allele,

in which nicotine is catalyzed to cotinine, affects the level of urinary cotinine. Approximately 20% of Japanese subjects have homozygotes or heterozygotes of the CYP2A6*4 allele, which has impaired nicotine metabolism and subsequently may underestimate the actual exposure to SHS. In assessing the exposure to SHS, therefore, individual variability of CYP2A6 gene polymorphism should be taken into consideration. The combination of urinary cotinine measurement and self-report of parents' smoking seems to be accurate to assess the exposure to SHS in mass screening. *J. Clin. Lab. Anal.* 25:354–358, 2011. © 2011 Wiley-Liss, Inc.

Key words: smoking; biomarker; secondhand smoke; cotinine; cancer

INTRODUCTION

Smoking is a major risk factor of various types of cancer, respiratory diseases, such as chronic obstructive pulmonary disease and bronchial asthma, cardiovascular diseases, and is associated with a higher incidence of a majority of diseases occurring in humans. Therefore, previous studies have demonstrated that the average life span of an active smoker was shorter by approximately 10 years than that of a nonsmoker (1). Despite these well-known evidences, many smokers have difficulty to quit smoking because of biochemical action of nicotine in tobacco products. Nicotine combines $\alpha 4\beta 2$ nicotine acetylcholine receptor in the ventral tagment area and subsequently accelerates a release of a neurotransmitter of mesolimbic dopamine from the end of neuron in the brain reward system: a crucial element in the response to addictive substances. A lack of nicotine induces withdrawal symptoms, such as irritability, restless, anxiety, etc., the so-called nicotineism (2). On the other hand, exposure to second hand smoke (SHS) brings on the same diseases in a nonsmoker, although the relative risk is low (3).

In elucidating the relationship between the tobacco-induced disease and smoking, a self-report of yes or no of smoking and cigarette consumption is not always scien-

tific. There is some relation between total cigarette consumption, smoking duration, and the objective tobacco-induced disease. In exposure to SHS, however, these parameters remain to be methodologically questionable and inaccurate (4). Therefore, the use of a biomarker is mandatory for quantitative analysis of exposure to SHS. This article summarizes urinary biomarkers of smoke exposure, which can be now quantified.

CARCINOGEN IN CIGARETTE SMOKE AND URINARY BIOMARKERS

It is well known that tobacco smoke contains more than 4,000 chemicals and 43 of them are carcinogens (5). Table 1 shows a classification and concentration of main carcinogens contained in tobacco smoke. Hoffman et al. have stated in a report from International Agency for Research on Cancer that the carcinogenicity of these

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Received 3 April 2010; Accepted 14 July 2011

DOI 10.1002/jcla.20485

Published online in Wiley Online Library (wileyonlinelibrary.com).

TABLE 1. Classification and Concentration of Main Carcinogens Containing in Tobacco Smoke

Carcinogenic substances	Content per cigarette (ng/ml)
<i>Substances in gas component</i>	
Hydrazine	24–43 ng
Vinylchloride	1–16 ng
Urethane	10–35 ng
2-Nitropropane	0.73–120 µg
Quinoline	0.80–2.0 µg
Nitrosodimethylamine	4–180 ng
Nitrosoethylmethylamine	1–40 ng
Nitrosopyrrolidine	0–110 ng
<i>Tumor initiators</i>	
Benzo(a)pyrene	0.01–0.05 µg
Other PAH	0.3–0.4 µg
Dibenz(a,j)acridine	0.003–0.01 µg
Other Aza Azrenes	0.01–0.02 µg
Urethane	0.035 µg
<i>Carcinogen-promoting substances</i>	
Pyrene	0.05–0.2 µg
Other PAH	0.5–1.0 µg
1-Methylindoles	0.8 µg
9-Methylcarbazoles	0.14 µg
4,4-Dichlorostilbene	0.5–1.5 µg
Catechol	200–500 µg
Alkylcatechols	10–30 µg
<i>Organ-specific carcinogens</i>	
N'-Nitrosornornicotine	0.14–3.70 µg
4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone	0.11–0.42 µg
N'-Nitrosoanatabine	??
Polonium-210	0.03–0.07 pCi
Nickel compounds	0–5.8 µg
Cadmium compounds	0.01–0.07 µg
β-Naphthylamine	0.001–0.022 µg
4-Aminobiphenyl	0.001–0.022 µg

chemicals has been proved by lots of scientific evidences on human and animal studies (6).

Carcinogens in tobacco smoke are absorbed from the lung and then bind to the DNA in the target cell, and subsequently form DNA adducts. Carcinogenic DNA adducts can be usually removed by cellular repair mechanisms. But, if they persist, miscording may occur leading to permanent mutations. If the mutations occur in critical regions of genes involved in regulation of growth, such as oncogenes or tumor suppressor genes, normal cellular growth control mechanisms can be lost and ultimately cancer can develop. From these settings, the measurement of carcinogenic DNA adducts is considered to be theoretical and feasible in evaluating the relationship between cancer of target organ and tobacco exposure. On the other hand, carcinogens bind to protein, such as albumin or hemoglobin can sometimes be measured instead of DNA adducts, but it is not common. The most popular method is a measurement of carcinogen and its metabolites in urine.

Urine is simple to obtain in large quantities and compliance is almost never a problem.

Table 2 reveals literature assessment of urinary 18 biomarkers of tobacco exposure, which could have been measured until now (7). The right column in Table 2 shows overall estimation score percentage, and the higher the percentage number the more reliable the estimation. The majority of these biomarkers can be measured by mass analysis, such as gas and liquid chromatography. The biomarkers with more than 65% estimation are tt-MA, S-PMA and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)+NNAL-glucuronide (NNAL-Glu) (8). The sensitivities of tt-MA and S-PMA are enough to show the distinct tobacco exposure between smokers and nonsmokers, but not in exposure to SHS.

Among those, the most reliable urinary biomarkers to assess the exposure to SHS are NNAL {4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol} and NNAL-Glu. NNAL-Glu includes NNAL-O-Gluc {4-(methylnitrosamino)-1-(3-pyridyl)-1-(O-β-D-glucopyranuronosyl)butane} and NNAL-N-Gluc {4-(methylnitrosamino)-1-(3-pyridyl)-1-(N-β-D-glucopyranuronosyl)-1-butanolonium inner salt}. NNK, called tobacco-specific nitrosamine is a systemic lung carcinogen. NNAL and NNAL-Glu are exceptionally useful biomarkers because they are derived from a carcinogen-NNK that is specific to tobacco products. We discussed the reliability of these biomarkers in the Research Group of Japanese Ministry of Labor, Health and Welfare. In our opinions, the reliable and accurate quantification of these biomarkers will take several years until the establishment of the clinical use in Japan. Hecht et al. has previously reported lots of data regarding exposure to SHS by a measurement of NNAL+NNAL-Glu (9–11). In particular, the relationship between cotinine and NNAL+NNAL-Glu concentrations in urines of children gave us the impact. They demonstrated that 96% school children with more than 5 ng/ml of urinary cotinine concentration had a positive NNAL+NNAL-Glu, and even approximately 50% of them with less than 5 ng/ml of cotinine had the same. Tobacco-specific nitrosamine could be found even if urinary cotinine was not detected. This suggests that children exposed to SHS for a long time will have a significant risk of premature onset of lung cancer in the future.

COTININE AS A USE OF SUBJECTIVE ASSESSMENT FOR SHS

As described above, a highly sensitive biomarker is necessary to assess subjectively the exposure to SHS because less scanner levels of nicotine and carcinogens are exposed to SHS in nonsmoker than those in smoker. Until now, carbon monoxide, carboxyhemoglobin, nicotine, cotinine, tobacco-specific carcinogen, and carcino-

TABLE 2. Evaluation of Urinary Biomarkers of Tobacco Exposure

Biomarker	Well-established assay	Chemically specific	Distinguishes exposed and nonexposed				Specific to tobacco exposure	Overall score (%)
			Smokers	Smokeless tobacco	ETS-exposure			
tt-MA	○	△	○	NA	△		×	73*
s-PMA	△	○	○	NA	×		×	67*
Naphthols	△	○	○	NA	ND		×	60
Phenanthrene metabolites	△	○	△	NA	×		×	60
1-HOP	○	○	○	NA	×		×	73
Benzopyrene metabolites	×	○	ND	NA	ND		×	33
Aromatic amines	△	○	×	NA	×		×	53
Heterocyclic aromatic amines	△	○	×	NA	ND		×	47
N-Nitrosamines	○	○	△	△	ND		×	61
Volatile nitrosamines	○	○	×	ND	ND		×	44
NNAL + NNAL/Glu	○	○	○	○	○		○	100**
8-oxo-dG	○	×	△	ND	×		×	44
Thioethers	○	×	○	ND	×		×	50
Mercapturic acids	△	△	△	ND	△		×	50
3-ethyladenine	△	×	○	ND	×		×	44
3-Hydroxypropylmercapturic acid	△	×	△	ND	△		△	60

Rank of estimate: ○, high ranking; △, moderate; ×, poor; ND, no data; HA, not applicable; tt-MA, trans,trans-muconic acid; s-PMA, S-phenylmercapturic acid; 1-HOP, 1-hydroxypyrene; 8-oxo-dG, 8-oxodeoxyguanosine.

genic DNA adduct have been recommended as a biomarker of smoke exposure. Among those, cotinine is considered to be a reliable and established biomarker and has been extensively used in the clinical studies (12). The measurement of carbon monoxide by used Smokerlyzer is obligatory in computing medical expenses of nicotine dependence in Japan. This measurement seems to be appropriate to assess the tobacco exposure of active smoker, but not of SHS because of its scant concentration. On the other hand, cotinine has recently been measured upto 0.1 ng/ml by the enzyme-linked immunosorbent assay method, and therefore this is an appropriate biomarker for evaluating exposure to SHS. Cotinine is a major metabolite of nicotine and is oxidized in the liver and excreted to urine. Its characteristics are biological stability and long time half-life of 20–30 hr compared with a biological instability and short time half-life of 2 hr of nicotine. In assessing the exposure to SHS, therefore, cotinine is a more useful biomarker than nicotine. In addition, cotinine is excreted to not only urine, but also hair and saliva, and is measured in these specimens. However, cotinine and nicotine are not carcinogenic and, although cotinine measurement does provide a measure of nicotine exposure, they say little about potential carcinogenicity.

MEASUREMENT OF URINARY COTININE

Urinary cotinine can be determined by using high-sensitive competing enzyme linked immunesorbent assay method. The methods of measurement, which have been used to date, include gas chromatography, liquid chromatography, radioimmunoassay, and enzyme immunoassay. Chromatography is now considered as the most reliable and is gold standard method. But, its disadvantages are that it is expensive in a clinical setting and difficult in treating numerous specimens at the same time. On the other hand, the competing enzyme immunoassay method we have used is highly sensitive with a measurement limit of 0.1 ng/ml and is inexpensive, costing 700–800 Japanese yen per sample on the commercial base (vs. 5000–10,000 Japanese yen per sample in chromatography) (13). Therefore, the competing enzyme immunoassay method is suitable for mass screening in which lots of samples has to be simultaneously handled for a short term. As described later, it has been demonstrated that a cotinine concentration measured by the competing enzyme immunoassay does not reveal only an isolated cotinine, but total cotinine concentration consisting of cotinine plus its metabolites. The cotinine level determined by this method has been thought to result from a cross-reaction to other metabolites. Furthermore, an individual difference exists in enzymatic activity mediated among each metabolite.

In evaluating the exposure to SHS, a total cotinine concentration consisting of cotinine and its metabolites may be rather accurate.

INDIVIDUAL DIFFERENCES OF NICOTINE METABOLISM AND COTININE AS A BIOMARKER

Figure 1 shows a pathway of nicotine metabolism converted with a C-oxidation to cotinine and structures of their metabolites. Nicotine is absorbed in the lung by smoking and is catalyzed by cytochrome P4502A6 (CYP2A6). Subsequently, cotinine is hydroxylated to *trans*-3'-hydroxycotinine by CYP2A6. Nicotine, cotinine, and *trans*-3'-hydroxycotinine are glucuronidated to nicotine *N*-glucuronide, cotinine *N*-glucuronide, and *trans*-3'-hydroxycotinine *O*-glucuronide, respectively. Its glucuronidation is done by UDP-glucuronosyltransferase in liver microsomes. The competing enzyme immune assay method has a cross-reaction with *trans*-3'-hydroxycotinine, nicotine *N*-glucuronide, cotinine *N*-glucuronide, and *trans*-3'-hydroxycotinine *O*-glucuronide, as described above. Taking into consideration the individual differences of CYP2A6 and UDP-glucuronosyltransferase activities, a total cotinine concentration may be rather suitable for assessing the exposure to SHS.

Nakajima et al. have recently reported that there was a high incidence of individual variability in genetic polymorphism of CYP2A6 gene (14,15). This variability in genetic polymorphism could be an important determinant of difference of nicotine clearance. They have also reported that the cotinine/nicotine ratio in the plasma 2 hr after nicotine intake was very useful as a noninvasive and simple metabolic index to assess the enzymatic activity of CYP2A6. As shown in Figure 2, cotinine formation from nicotine is almost 0 in homozygotes of the CYP2A6*4 allele and is very low in heterozygotes of the CYP2A6*4 and CYP2A6*7.

Fig. 1. Metabolic pathways of nicotine and cotinine. Metabolites in gray is measured by enzyme immune assay as total cotinine.

More than 17 alleles of CYP 2A6 have been found until now and CYP2A6*1, CYP2A6*4, CYP2A6*7, CYP2A6*8, CYP2A6*9, CYP2A6*10, and CYP2A6*11 were identified in Japanese population. In particular, the allele frequencies of CYP2A6*4 and CYP2A6*9 are relatively high, up to 20%. Enzymatic activity is lacking in homozygotes of either CYP2A6*4, CYP2A6*7, CYP2A6*9, and CYP2A6*10 or decreased in heterozygotes of either allele. And it has been reported in the white American and European population that cotinine concentrations in the plasma of smokers who had a CYP2A6*2 allele tended to be low and in contrast, these of smokers who had a CYP2A6*1 allele was high compared with that in other smokers. However, the concentrations of carbon monoxide as an indicator of smoke exposure were low and high, respectively. These suggest that one should consider the possibility of effects of cigarette consumption and depth of smoke in evaluating the metabolic activity of nicotine.

Since 2007, we have performed a mass screening for SHS in 1,300–1,400 elementary school children of grade 4 in Kumagaya city every year. This mass screening program consisted of urinary cotinine measurement and questionnaire survey to their parents and was carried out at the expense of the public for all applicants. The details of this program are described in our previous reports (16–18). The level of urinary cotinine was positively correlated with parents' smoking, particularly mother's smoking and cigarette consumption and was several times higher in children whose parents or mother smoked than those in children whose father smoked or parents did not smoke (Fig. 3). This result indicated that exposure to SHS in children was strongly affected by parents' smoking, particular mother's. We classified these children into four groups according to the status of parent's smoking and compared the levels of urinary

Fig. 2. Cotinine/nitotine ratios in Japanese subjects who were genotyped for CYP2A6 alleles. *Indicates $P < 0.05$, compared with CYP2A6*1/CYP2A6*1.

Fig. 3. Mean concentration of urinary cotinine in school-children among 4 groups classified by parent's smoke each year.

Fig. 4. Ratio of cotinine positive/negative in each group.

cotinine among each group. Surprisingly, the urinary cotinine was not detected in 18.7% of children whose parents smoked and 38.5% of children whose mother smoked (Fig. 4). The reason was, even though the evidences remained uncertain, there has been thought of the daily contact and length of duration with parents. However, considering a high incidence of 20% of CYP2A6*4 allele, the reason why 18.7% of children whose parents smoked had undetectable level of cotinine could be attributed to individual variability of CYP2A6 gene polymorphism. This explanation is hypothetical and is not proved at present. Therefore, the individual variability of genetic polymorphism of CYP2A6 gene should be investigated in children whose parents smoke and urinary cotinine is not detectable. In the mass-screening setting, the investigation of genetic polymorphism may not be feasible in terms of cost benefit and time loss. The combination of urinary cotinine measurement and self-report of parents' smoking seems to be accurate to assess the SHS in children, although cotinine still remains to be a useful biomarker.

CONCLUSION

Cotinine, a metabolite converted from nicotine, is useful enough in the clinical setting among lots of urinary biomarker. However, individual variability in gene polymorphism of CYP2A6 at which nicotine is catalyzed to cotinine should be taken into consideration. In the recent future, a highly sensitive and specific determination of urinary tobacco-specific nitrosamine metabolites is mandatory to assess the relationship between exposure to SHS and carcinogenesis of organ.

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