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Prospective study of 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion and the risk of lung cancer

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Oxidative damage to DNA may be important in carcinogenesis and a possible risk factor for lung cancer. The urinary excretion of products of damaged nucleotides in cellular pools or in DNA may be important biomarkers of exposure to relevant carcinogens reflecting the rate of damage in steady state and may predict cancer risk. Oxidation of guanine in DNA or the nucleotide pool may give rise to 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) for urinary excretion. Oxoguanine glycosylase (OGG1) is the base excision enzyme repairing 8-oxodG in DNA by release of 8-oxoguanine. In a nested case–cohort design we examined associations between urinary excretion of 8-oxodG and risk of lung cancer as well as potential interaction with the *OGG1* Ser326Cys polymorphism in a population-based cohort of 25 717 men and 27 972 women aged 50–64 years with 3–7 years follow-up. We included 260 cases with lung cancer and a sub-cohort of 263 individuals matched on sex, age and smoking duration for comparison. Urine collected at entry was analysed for 8-oxodG by HPLC with electrochemical detection. The excretion of 8-oxodG was higher in current smokers, whereas *OGG1* genotype had no effect. Overall the incidence rate ratio (IRR) (95% confidence interval) of lung cancer was 0.99 (0.80–1.22) per doubling of 8-oxodG excretion and there was no interaction with *OGG1* genotype. However, among never-smokers (eight cases and eight sub-cohort members) the IRR was 11.8 (1.21–115) per doubling of 8-oxodG excretion. The association between 8-oxodG excretion and lung cancer risk among never-smokers suggests that oxidative damage to DNA nucleotides is important in this group.

Introduction

Lung cancer is one of the leading causes of death in the world and several factors play important causal roles, including active smoking, environmental tobacco smoke, various occupational exposures and probably ambient air pollution (1–4). Tobacco smoke contains more than 50 known carcinogens including polycyclic aromatic hydrocarbons (PAH), aromatic amines such as heterocyclic amines and nicotine-derived nitrosamines as well as large amounts of reactive oxygen species (ROS). Particles in tobacco smoke and polluted ambient air can induce oxidative stress and DNA damage from (i) direct generation of ROS from the surface of particles, (ii) soluble compounds such as transition metals or organic compounds, (iii) altered function of mitochondria or NADPH-oxidase and (iv) activation of inflammatory cells capable of generating reactive oxygen and nitrogen species (5–9).

The urinary excretion of products of damaged nucleotides in cellular pools or in DNA may be important biomarkers of exposure to relevant carcinogens and may predict cancer risk. It is important to recognize that in steady state the excretion reflects the rate of damage (10,11). Among the many oxidative DNA damage products 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is probably the most studied because of the relative ease of measurement and the mutagenic properties resulting in G→T transversion mutations upon replication of DNA (11,12). Oxidized guanine in DNA is mainly repaired by oxoguanine glycosylase (OGG1) resulting in release of 8-oxoguanine (13–15). This enzyme shows a common genetic polymorphism with a variant Ser326Cys, which in complementation assays *in vitro* appears to increase susceptibility to mutagenic properties of ROS considerably, whereas 8-oxodG levels and incision activity in leukocytes and some target tissues generally show no difference between the genotypes (16). In addition, repair of 8-oxodG may to some extent occur by nucleotide excision repair and transcription coupled mechanisms (11). A specialized enzyme (MTH1 or NUDT1) sanitizes the nucleotide pool by cleaving phosphates of 8-oxodGTP which if incorporated during DNA synthesis is highly mutagenic and mice deficient in that enzyme develop tumors (17). 8-OxodG from this process as well as from putative nucleotide excision repair and possibly mitochondrial turn-over is excreted unchanged into the urine and may serve as a biomarker of oxidative stress and oxidative damage to nucleotides and possibly DNA (10). The urinary excretion of 8-oxodG has consistently been found to be increased among smokers and with a number of occupational exposures, including air pollution among bus drivers (10,18). Moreover, some case–control studies have suggested that the urinary excretion of 8-oxodG or 8-oxoguanine is increased among cancer patients (19–22), although this could very well be a consequence of the disease with ongoing oxidative stress, inflammation and tissue turn-over (23).

So far, no published study has assessed the urinary excretion of oxidatively damaged nucleosides or corresponding bases as predictors of cancer in a prospective setting. In this study, we examined the association between the urinary excretion of 8-oxodG and risk for lung cancer as well as possible

Abbreviations: IRR, incidence rate ratio; MTH1, MutT homolog; OGG1, oxoguanine glycosylase; PAH, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

interactions with genetic variation in DNA repair in terms of the *OGG1* Ser326Cys polymorphism in a large population-based Danish cohort for which detailed information on smoking patterns and other lifestyle factors at enrolment is available.

Materials and methods

Study population

Diet, Cancer and Health is a Danish prospective follow-up study approved by the scientific ethics committees. Invited to participate were 160 725 individuals aged 50–64 years, of which 57 053 individuals with no previous cancer diagnosis were recruited (24). All participants were born in Denmark and virtually all were Caucasians (race was not registered). At enrolment (1993–1997), detailed information on diet, smoking habits, lifestyle, reproduction, medical treatment and other socio-economic characteristics and environmental exposures, including second hand smoke, were collected. Body weight and height were measured and spot urine samples were voided at the clinic and stored at −150°C.

Among the 57 053 persons recruited to the Diet Cancer and Health study, 542 were later registered in the Danish Cancer Register as having cancer diagnosed before the date of enrolment and were therefore excluded. Among the 56 511 individuals with no previous cancer diagnosis, we excluded 2291 individuals for whom information regarding smoking habits was incomplete, inconsistent or missing. Among the remaining 54 220 cohort members included in this study, 265 cases of lung cancer, diagnosed between 1994 and 2001, were identified in the files of the nationwide Danish Cancer Registry (25). Also from among the cohort members, a sub-cohort was selected. For this procedure, we divided the entire cohort (inclusive cases) in strata defined by sex, year of birth (5-year intervals) and duration of smoking (10-year intervals). For the sub-cohort, we sampled 272 persons (including 4 of the cases) such that the number of sub-cohort members in the different strata approximated the number of cases. We used a random procedure to select sub-cohort members within each stratum. Urine was available for 520 of the 533 selected individuals, and laboratory analyses of urinary biomarkers failed for one sample, leaving 519 persons [260 cases and 263 sub-cohort members (including 4 cases)] for analyses of lung cancer risk in association with the urinary biomarker. Blood samples were not available for further 11 individuals, leaving 508 individuals (251 cases and 261 sub-cohort members, including 4 cases) for analyses of lung cancer risk in association with combinations of the urinary biomarker and *OGG1* genotype. The DNA used for genotyping was isolated from buffy coat using standard phenol extraction procedures and the Ser326Cys *OGG1* genotype was determined as described elsewhere (26).

Determination of 8-oxodG

The urinary concentrations of 8-oxodG was determined by a recently developed high-performance liquid chromatography method based on anion exchange chromatography, precise fraction collection, reversed-phase chromatography and electrochemical detection as described elsewhere (27,28). The intrabatch and interbatch coefficients of variations were 2.2 and 2.7%, respectively. Twenty randomly selected urine samples were also assayed for 8-oxodG in another laboratory by an independent column-switching high-performance liquid chromatography method with electrochemical detection as described elsewhere (10). There was excellent agreement between the two methods ($r = 0.95$) and zero intercept on the regression line (not shown). In the second laboratory urine samples have been stored at −20°C and repeatedly measured for 8-oxodG during 15 years without decline in concentrations, whereas the 8-oxodG assay has shown excellent agreement ($r = 0.99$) with an HPLC-MS/MS-based assay from a third laboratory (29). The urinary concentration of creatinine was determined by a standard colorimetric method.

Statistical methods

The data were sampled according to the case–cohort design within the sampling strata (30). According to the sampling strategy, the unweighted case–cohort approach (31) was used to estimate incidence rate ratios (IRR) for lung cancer using a Cox proportional hazards model stratified according to the sampling strata. Age was the time axis, which ensured that the estimation procedure was based on comparisons of individuals of the same age. The analyses were corrected for delayed entry, such that persons were considered at risk only from the time of enrolment in the cohort. We calculated 95% confidence intervals (CI) and *P*-values based on Wald's test of the Cox regression parameter on the log rate ratio scale using robust estimates of the variance-covariance matrix (32). The urinary excretion of 8-oxodG was considered as a continuous variable and log-transformed before analysis such that an excessive influence of outliers in the right-skewed distribution was avoided. Log-2 was used for transformation, such that IRRs were estimated per doubling of the concentration per creatinine, assuming a linear relationship. We also calculated IRRs for high versus low excretion based on the median value among the cases. Differences in urinary excretion of 8-oxodG between sub-groups of sub-cohort members defined by gender, age, smoking and OGG1 genotype were tested by univariate and multiple linear regression analyses (PROC GLM, SAS version 8.2).

Results

The cases of lung cancer and the corresponding sub-cohort were older and showed a much larger proportion of smokers than the total cohort (Table I). Only eight never-smokers of whom six were women developed lung cancer during the follow-up period. There was no difference with respect to exposure to second hand smoke between the never-smoker cases and sub-cohort members. Among the cases and the sub-cohort 55 and 56% were men as compared with 48% in the total cohort, respectively.

The potential determinants of the excretion of 8-oxodG are summarized in Table II. Current smokers excreted more 8-oxodG than former smokers but no clear differences were evident between high and low smoking intensity among the current smokers. Women had higher levels of 8-oxodG corrected for creatinine than men did, and the levels were highest for the oldest age group (60–65 years). In multiple regression analysis the 8-oxodG per creatinine in urine was significantly associated with gender ($P < 0.0001$), smoking status ($P = 0.05$) and with age as a continuous variable ($P = 0.008$). There was no significant association ($P > 0.25$) between 8-oxodG excretion and total intake of fruit and vegetables, of energy or of alcohol with adjustment for gender, age and smoking (data not shown). The excretion of 8-oxodG was not associated with the *OGG1* genotype.

Overall there was no significant change in incidence of lung cancer with increased 8-oxodG excretion with or without full adjustment for smoking (Table III). The IRR for lung cancer associated with having a high excretion as compared with having a low excretion, using the median of the cases as cut-off, was 1.23 (0.85–1.77; 95% CI) in unadjusted analysis and

Table I. Age at enrolment and smoking characteristics of lung cancer cases, a matched sub-cohort and the total Diet, Cancer and Health cohort

	Cases ($N = 260$)		Sub-cohort ($N = 263$)		Total cohort ($N = 54\ 220$)	
	n	(%)	*n*	(%)	*n*	(%)
Age at inclusion in cohort						
50–54	63	24.2	63	24.0	23 071	42.6
55–59	75	28.9	76	28.9	16 780	30.9
60–65	122	46.9	124	47.2	14 369	26.5
Smoking at inclusion in cohort						
Never	8	3.1	8	3.0	19 762	36.4
Former	35	13.5	57	21.7	15 353	28.3
Current	217	83.5	198	75.3	19 105	35.2
Duration of smoking at inclusion in cohort (years)						
never-smokers	8	3.1	8	3.0	19 762	36.4
$0 \leq$ duration < 10	0	0.0	0	0.0	2920	5.4
$10 \leq$ duration < 20	6	2.3	6	2.3	4951	9.1
$20 \leq$ duration < 30	8	3.1	8	3.0	6224	11.5
$30 \leq$ duration < 40	84	32.3	87	33.1	12 637	23.3
$40 \leq$ duration < 50	146	56.2	144	54.8	7375	13.6
$50 \leq$ duration	8	3.1	10	3.8	351	0.6

Table II. Determinants of urinary excretion of 8-oxodG per creatinine at enrolment among cases of lung cancer and sub-cohort from the Danish Diet, Cancer and Health cohort

	N cases/ sub-cohort	8-OxodG (nmol/mmol)	
		Cases	Sub-cohort
All	260/263	1.94 (0.75–10.1)	1.81 (0.90–10.1)
Gender			
Male	144/147	1.54 (1.70–6.15)	1.60 (0.88–5.21)
Female	116/116	2.81 (1.04–11.5)	2.30 (0.95–13.6)[a]
Age			
50–54 years	63/63	1.74 (0.55–22.6)	1.68 (0.89–10.1)
55–59 years	75/76	2.00 (0.72–7.92)	1.65 (0.93–4.93)
60–65 years	122/124	2.02 (0.80–9.26)	2.05 (0.90–14.0)[b]
Smoking at enrolment			
Never-smokers	8/8	3.32 (1.61–9.26)	1.95 (0.88–5.21)
Former smokers	35/57	1.49 (0.54–22.6)	1.62 (0.68–5.25)
Current smokers	217/198	1.99 (0.77–10.5)	1.87 (0.92–13.6)[c]
Of $\leq$18 g tobacco per day	76/99	2.26 (0.73–9.63)	1.85 (0.86–14.0)
Of $>$18 g tobacco per day	135/89	1.86 (0.88–12.2)	1.90 (0.99–10.4)
Of unknown tobacco amount	6/10	2.32 (0.47–7.65)	2.31 (1.08–3.41)
OGG1 genotype			
wt/wt	144/154	1.92 (0.77–7.88)	1.81 (0.88–13.6)
wt/Cys326	93/88	2.00 (0.60–11.5)	1.76 (0.90–8.19)
Cys326/Cys326	14/19	1.61 (0.29–27.3)	2.18 (1.31–8.62)
Missing genotype	9/2	3.79 (0.25–22.8)	1.35 (1.27–2.42)

Wt, wild-type; values are medians with 5–95 percentiles.
GLM analysis results from sub-cohort:
[a]$P < 0.05$ versus male;
[b]$P < 0.05$ increasing value with age;
[c]$P < 0.05$ versus former smokers.

Table III. Incidence rate ratios and 95% confidence intervals for lung cancer according to urinary excretion of 8-oxodG stratified for smoking status at enrolment and oxoguanine glycosylase-1 (*OGG1*) genotype

	Cases/ sub-cohort	Per doubling of 8-oxodG/ creatinine (nmol/mmol)		
		IRR	95% CI	*P*-value
All (unadjusted[a])	260/263	1.03	0.84–1.25	0.78
All (adjusted[b])	254/253	0.96	0.78–1.19	0.71
Smoking stratified				0.10*
never-smokers	8/8	11.8	1.21–115	0.03
former smokers[a]	35/57	0.98	0.54–1.78	0.95
former smokers[b]	35/57	0.86	0.46–1.60	0.62
current smokers[a]	217/198	0.99	0.79–1.23	0.92
current smokers[b]	211/188	0.96	0.76–1.20	0.70
OGG1 genotype stratified				0.26*
wt/wt[c]	144/154	0.96	0.76–1.22	0.75
wt/Cys326[c]	93/88	1.07	0.74–1.54	0.73
Cys326/Cys326[c]	14/19	0.33	0.08–1.31	0.12

Wt, wild-type; *P-value for interaction.
[a]Unadjusted.
[b]Adjusted for smoking status, intensity and duration at entry; data was missing for 16 current smokers.
[c]Adjusted for smoking duration.

1.14 (0.77–1.68) with adjustment for smoking status, intensity and duration at entry. However, among the rather few never-smokers the IRR for lung cancer was significantly associated with 8-oxodG excretion [IRR: 11.8 (1.21–115) per doubling], whereas there was no significant association between the 8-oxodG excretion and IRR for lung cancer among either former or current smokers. A test for the interaction between smoking status and 8-oxodG excretion in relation to lung cancer risk was not statistically significant ($P = 0.10$). Stratification for *OGG1* genotype provided mixed and inconclusive results.

As reported elsewhere, the risk of lung cancer was not significantly associated with the *OGG1* genotype *per se* (26). The unadjusted odds ratio (OR) (95% CI) of lung cancer of subjects with two and one mutant alleles was 0.65 (0.30–1.41) and 1.09 (0.75–1.57) in comparison with subjects with two wild-type alleles.

Discussion

In this study, we examined the association between a biomarker of oxidative stress and damage to DNA in terms of 8-oxodG excretion and subsequent risk of lung cancer as well as potential interaction with the *OGG1* Ser326Cys polymorphism in a large population-based cohort study. Overall, the excretion of 8-oxodG was not significantly associated with the risk of lung cancer and there was no interaction with the *OGG1* genotype. However, among never-smokers a high excretion of 8-oxodG was significantly associated with an increased risk of developing lung cancer.

In the present material the urinary excretion of 8-oxodG was higher in current smokers than in former smokers among both cases and sub-cohort members, although there was no clear dose–response relationship with number of cigarettes smoked. The high urinary ratio of 8-oxodG to creatinine among never-smokers may be due to the fact that six out of eight were women, who had higher ratios than men did. The results confirm previous studies showing increased 8-oxodG excretion with smoking (10). Smokers usually excrete 10–50% more 8-oxodG than comparable non-smokers (33–35), and smoking cessation in a randomized intervention study was associated with a 21% decrease in excretion (36). However, the effect of smoking may also complicate the interpretation of associations between the urinary excretion of 8-oxodG and the risk of cancer and this relies on the understanding of possible causal pathways.

With the present matching procedure including smoking duration in 10 year strata for the selection of the comparison group and with or without further adjustment for smoking there was no sign of association between excretion of 8-oxodG and risk of lung cancer overall or among former or current smokers. The data do not support a cancer-predictive capacity of excretion of 8-oxodG among current and former smokers.

Among the few never-smokers from the present study a doubling of the excretion of 8-oxodG was significantly associated with an almost 12-fold higher risk of lung cancer, although with a wide confidence interval. This association is not likely to be caused by confounding by exposure to second hand smoke, which is a risk factor for lung cancer with a relative risk of <2 (37,38). Moreover, 8-oxodG excretion was not associated with exposure to second hand smoke in a study with substantial exposure (39). In the present study, there were no relevant differences between never-smoking cases and the corresponding subjects from the sub-cohort with respect to exposure to second hand smoke. There was a high proportion of women among the never-smoker cases of lung cancer.

However, gender was included in the matching of the cohort and is not likely to cause confounding. Accordingly, oxidative damage to DNA nucleotides may be important for development of lung cancer among never-smokers although the causative agents in this respect so far are unknown. 8-OxodG excreted into urine can originate from all cells in the body and represents in steady state an average rate of oxidative damage to guanine in dGTP and possibly DNA (10). The contribution from the lungs to urinary 8-oxodG is unknown and at present it can be considered as a biomarker of general oxidative stress. It would be of great interest to study the levels of 8-oxodG in cells, e.g. white blood cells, in relation to risk of cancer prospectively. However, due to spurious oxidation of DNA during storage this is not possible at present (40,41).

The present study was prospective and effects of cancer on the excretion of 8-oxodG can thus be excluded. A number of factors besides tobacco smoking are known to influence the 8-oxodG excretion, although none of these are likely to be particularly relevant in the present study. Heavy exposure to air pollution in occupational settings in terms of e.g. diesel exhaust, PAH and benzene has been associated with increased 8-oxodG excretion (18,42,43), whereas non-occupational exposure to ambient air pollution has not been significantly associated with urinary 8-oxodG (44). Exercise of very high intensity e.g. marathon running, or high altitude has been associated with increased 8-oxodG excretion (45–47). Cancer treatment with radiation and/or chemotherapy may also increase 8-oxodG excretion (48,49). The present lack of association between intake of fruit and vegetables and 8-oxodG excretion is in agreement with a number of intervention studies showing generally no effect or limited effect of diet and antioxidants on biomarkers of oxidative DNA damage, including 8-oxodG excretion (50,51).

If the major source of urinary 8-oxodG is nucleotide excision or transcription coupled repair as backup for the usual OGG1-mediated repair of 8-oxoguanine in DNA (11), a low activity of OGG1 would be expected to be associated with high urinary 8-oxodG excretion. However, excretion of 8-oxodG is similar in *ogg1*$^{-/-}$ knockout and wild-type mice (52) and we found no association with the Ser326Cys polymorphism in *OGG1* or sign of interaction or effect modification in the present study. If 8-oxodG excretion is considered as a biomarker of general exposure to oxidative stress a low level of OGG1 activity could also have been expected to enhance susceptibility related to a possible association with lung cancer risk. Thus, induction of oxidative stress is associated with a higher level of guanine oxidation in DNA and mutations in *ogg1*$^{-/-}$ knockout mice than in wild-type mice (53,54). On the other hand, the Ser326Cys polymorphism has not yet been convincingly shown to cause decreased OGG1 activity in humans (16), although a recent study found higher levels of oxidised guanine in human lymphocyte DNA from Ser326Cys homozygous subjects as compared with heterozygous or homozygous wild-type subjects after *ex vivo* treatment with sodium dichromate (55). There are also other base excision enzymes, which can excise 8-oxoguanine (56).

The Ser326Cys polymorphism has inconsistently been associated with risk of lung cancer as recently reviewed with ORs above unity for subjects with two mutant alleles in five studies, of which two were statistically significant, and two studies like the present showing an OR below unity (16). A new study included more than 2000 cases and showed an overall OR of 1.34 for the mutant genotype which was close to statistical significance (57), whereas no formal meta-analysis has been published.

In urine, 8-oxodG is extremely stable and storage at only −20°C has proved sufficient far beyond the storage time of the present study. Excellent interlaboratory agreement between different chromatographic methods and a very low analytical coefficient of variation indicate that measurement error is unlikely to be a cause of a lack of association between lung cancer risk and 8-oxodG among former and current smokers. However, only one spot sample of urine was collected at enrolment in the study and this may not be representative for the long period at risk. Nevertheless, the urinary excretion of 8-oxodG is relatively constant within an individual showing coefficients of variations $<20\%$ over prolonged periods of time (51,58). Urine was collected as spot samples in the present study and the concentrations of 8-oxodG had to be adjusted by the creatinine concentration. This is not ideal because creatinine excretion is affected by muscle mass and that may explain the higher levels of 8-oxodG per creatinine in women as compared with men and the higher levels with increasing age. With 24-h urinary collection, men usually excrete more 8-oxodG than women and there is a slow decrease in excretion with age (10,59). However, age and gender associated differences in the excretion of 8-oxodG are not likely to affect our results concerning risk of lung cancer because the sub-cohort for comparison with the cases was selected with match on sex and because age was used as the time axis in the statistical analyses.

In conclusion, this population-based cohort study indicates that the urinary excretion of the nucleotide damage product 8-oxodG is not significantly associated with subsequent risk of lung cancer among current or former smokers and the excretion is not affected by the *OGG1* Ser326Cys polymorphism. Among never-smokers a high excretion of 8-oxodG was significantly associated with an increased risk of developing lung cancer, suggesting that oxidative damage to DNA nucleotides is important in this group, although the numbers were small and the issue needs further study.

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