The effect of hOGG1 and glutathione peroxidase I genotypes and 3p chromosomal loss on 8-hydroxydeoxyguanosine levels in lung cancer


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Polymorphic genes for the peroxide scavenger glutathione peroxidase I (GPX1) and 8-hydroxydeoxyguanosine (8-OHdG) DNA glycosylase/apurinic (AP) lyase (hOGG1) map to loci on chromosome 3p which are subject to frequent loss of heterozygosity (LOH) in lung tumours. Levels of the pro-mutagenic, oxidative DNA lesion 8-OHdG, were measured in 37 paired normal and tumorous lung specimens using HPLC with electrochemical detection. Lung tumours were also analysed for 3p LOH by fluorescent PCR with Genescan analysis. No significant difference was observed between 8-OHdG levels in tumour (7.7 ± 6.7 mean ± SE) 8-OHdG/10^6 2’-deoxyguanosine (dG)] and normal (8.1 ± 8.8 8-OHdG/10^6 dG) lung tissue. Adduct levels in normal lung tissue DNA were not associated with constitutive hOGG1 genotype although there was a trend towards lower 8-OHdG levels in individuals possessing the ALA6 GPX1 polymorphism. Lung tumours exhibiting 3p LOH (40%) contained higher levels of 8-OHdG adducts (10.9 ± 2.6 8-OHdG/10^6 dG) (P = 0.05) and lower GPX1 enzyme activity [45.5 nmol glutathione (GSH)/min/mg] (P = 0.09) when compared with tumours without LOH at these sites (5.55 ± 0.87 8-OHdG/10^6 dG and 63.6 nmol GSH/min/mg, respectively). In conclusion, tumours with 3p LOH at loci associated with hOGG1 and GPX1 appear to have compromised oxidative defence mechanisms as measured by reduced GPX1 enzyme activity and elevated 8-OHdG levels and this may affect the prognosis of lung cancer patients.

Introduction

Lung cancer accounts for 18% of the global cancer burden in men and over 1 000 000 new cases of this disease were reported in 1990 (1). Cigarette smoke is implicated in the development of 90% of these tumours (1), through exposure to carcinogens such as benzo[a]pyrene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and reactive oxygen species (ROS) generated by compounds including catechol and hydroquinone (2). At least 35 different oxidative base modifications have been reported when ROS react with DNA (3–7). One of the most common lesions formed is the pro-mutagenic DNA lesion, 8-hydroxydeoxyguanosine (8-OHdG) (6,8). Levels of this adduct are reported to be raised in the peripheral leukocytes and lung tissue of smokers compared with non-smokers (2,9). Several in vivo models have demonstrated that 8-OHdG mispairs preferentially with adenine during replication, inducing GC→TA transversions (5,8,10).

In view of its abundance and mutagenicity, a number of defence mechanisms operate to minimize 8-OHdG accumulation within the genome. Primary defence mechanisms include antioxidants and enzymes such as glutathione peroxidase (8,11). Glutathione peroxidases reduce organic peroxides and hydrogen peroxides through the coupled oxidation of reduced glutathione (GSH). Glutathione peroxidase I (GPX1) is the major cytosolic form of this enzyme, but other isozymes are found in the plasma and phospholipid membranes (12).

Once formed, 8-OHdG lesions are subject to DNA repair primarily through the base excision repair pathway (13). A key component of this pathway is a specific DNA glycosylase/apurinic (AP) lyase which catalyses the release of 8-OHdG:dC and is the cleavage of DNA at the AP site (14). Inactivation of this 8-OHdG glycosylase generates a mutator phenotype characterized by GC→TA transversions in Escherichia coli and yeast (15,16). Recently the human homologue of this gene, hOGG1 has been identified (17–19). The gene product constitutes an 8-OHdG DNA glycosylase/AP lyase which exhibits greatest specificity and activity for 8-OHdG:dC and is completely inactive against 8-oxodeoxyguanosine:dA (17,19). hOGG1 has also been shown to excise 2,6-diamino-4-hydroxy-5-formamidopyrimidine residues in a similar manner to its yeast homologue (20–22).

The level of 8-OHdG measured in a tissue at any one time is an integration of a number of parameters including the level of ROS, tissue redox status, cellular antioxidant defence mechanisms and DNA repair systems. The effectiveness of these latter systems may be subject to modulation by gene polymorphisms and gene dosage effects. A number of hOGG1 polymorphisms have been described in Japanese populations, and a Ser/Cys (0.57/0.43) substitution in exon 7 is highly prevalent (23–25). Preliminary evidence from an E.coli complementation assay suggests that the hOGG1-Cys isoform exhibits reduced 8-OHdG repair activity (23) and may play a role in increasing susceptibility to squamous cell carcinoma of the lung (25). Polymorphisms in GPX1 are characterized by a variable polyanalane repeat and the six-alanine repeat form (ALA6) also contains a proline→sleucine substitution at codon 198 towards the C-terminus (12). Thus, constitutive genotype may play a significant role in determining 8-OHdG levels within tissue DNA.

Finally, both GPX1 and hOGG1 locate to regions of chromosome 3p (3p21 and 3p25/26, respectively) which are subject to frequent and early loss of heterozygosity (LOH) during lung cancer development (12,18,26). Deletions at these sites may compromise antioxidant and 8-OHdG repair mechanisms via gene dosage effects, resulting in the accumulation of 8-OHdG within dysplastic and neoplastic tissue.

Abbreviations: AP, apurinic; dG, 2’-deoxyguanosine; GPX1, glutathione peroxidase I; GSH, glutathione; HPLC-ECD, high pressure liquid chromatography with electrochemical detection; 8-OHdG, 8-hydroxydeoxyguanosine; LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; ROS, reactive oxygen species.
The aims of this study were to examine the effect of GPX1 and hOGG1 genotype on lung 8-OHdG levels and to establish whether 3p LOH events modulate 8-OHdG levels in lung tumour tissue.

Materials and methods

Tissue collection

Resected lobectomy and pneumonectomy specimens were collected on ice at surgery from untreated primary non-small cell lung cancer patients (n = 37) at Leeds General Infirmary (LGI). Paired tumour and uninvolved peripheral lung tissue (designated normal), which were surplus to diagnostic requirements, were dissected by a consultant pathologist. Specimens were cut into pieces (200–400 mg) and fixed either in formalin for histology or frozen immediately at −80°C for molecular and biochemical analyses. Smoking status from patients was limited to self-reported information, but all patients were either current or former smokers. Most squamous cell carcinoma samples (82%) collected were from male patients, as opposed to adenocarcinomas, which were predominantly (75%) obtained from female patients.

Histology

Histopathological diagnosis was performed by hospital pathologists according to the WHO lung carcinoma classification. To verify that tumorous specimens contained >50% tumour cells and that uninvolved tissue contained <10% containing tumour material, paraffin-embedded sections (5 μm) from lung specimens were stained with haematoxylin and eosin and examined by light microscopy. Normal and tumorous lung tissue was microdissected where appropriate from sequential tissue sections (5 μm) and genomic DNA extracted using proteinase K digestion followed by phenol/chloroform extraction as described previously (27).

Genotyping and LOH analysis

DNA was subject to PCR using fluorescent primers directed against the following chromosome 3p markers: GPX1, FAM 5’-GAACTGCT-GTGGCCAAGTGACC-3’ and 5’-CGAGAAGGCTACAACGGCTGGGC-3’; hOGG1, FAM 5’-ACTAATGCTACCCAGCTGGAC-3’ and 5’-TGGCCCTTGAGAATGTACGAC-3’; D3S1259, FAM 5’-GCTGACATATTATGTGA-AATCT-3’ and 5’-TTTACGAGGCAAGATACTGCTG-3’; D3S1351, TET 5’-ACAGAACCAGACGAAATGG-3’ and 5’-AGCCCTATAACCTGCATGAA-3’.

Aliquots of 0.5 μl of template DNA were added to a PCR mix containing 0.5 U AmpliTaq Gold (Perkin-Elmer Cetus, Norwalk, CT), 10 pmol each primer, 2.5 μl 10× Taq buffer, 200 μM dNTPs, 1.5 mM MgCl2 and water to a final volume of 25 μl. Cycling conditions for GPX1, D3S1259 and DS1351 were: 95°C for 15 min; 30 cycles of 1 min at 95°C, 1 min at 56°C, 1 min at 72°C followed by an extension step for 10 min at 72°C. The annealing temperature was modified to 50°C for the hOGG1 PCR. For every PCR experiment, water served as a negative control template.

PCR products were separated on a 6% denaturing acrylamide gel and analysed using a 373A automated DNA sequencer with Genescan analysis software. Glutathione peroxidase activity was measured according to the method of Paglia and Valentine (28) where hydrogen peroxide was used as substrate at a final concentration of 0.25 mM. The reaction mix (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1.5 mM sodium azide, 1 mM GSH, 0.16 mM NADPH, 0.33 U/ml glutathione reductase and 200 μl of cytosolic fraction. After a 5 min preincubation, the reaction was initiated by the addition of hydrogen peroxide. The rate of reaction was determined at 20°C by measuring the decrease in absorbance at 340 nm. Cytosolic protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK). Specific activity is given as nmol GSH oxidized/min/mg cytosolic protein.

Statistics

Statistical analyses were performed using the Mann–Whitney U-test and Kruskall–Wallis non-parametric ANOVA.

Results

The mean 8-OHdG levels measured in normal and tumorous lung tissue from 20 adenocarcinoma and 16 squamous cell carcinoma cases are shown in Figure 1. Adduct levels ranged from 0.5 to 47 8-OHdG/108 dG in normal and 0.3 to 34 8-OHdG/108 dG in tumour DNA in this study. The majority (80%) of normal and tumour samples contained <10 8-OHdG/108 dG. There were no significant differences observed in 8-OHdG levels either between normal and tumorous tissue or across different tumour types (Figure 1). Furthermore, nodal involvement was not related to tumour 8-OHdG levels. In view of this, data from adenocarcinoma and squamous cell carcinoma 8-OHdG levels were pooled for further analysis. Only limited quantitative information was available on the smoking status of these lung cancer cases, except that all cases were known to be current or former smokers. Therefore adduct levels could not be correlated quantitatively to smoking status.

The relationship between 8-OHdG level and possession of the GPX1 A1A6 allele is shown in Figure 2. Although there was a trend towards reduced 8-OHdG levels in the presence
Chromosome 3p and 8-OHdG levels in lung cancer

**Fig. 1.** The relationship between tumour histopathology and 8-OHdG levels in normal and matched tumorous lung specimens. Data presented are mean 8-OHdG values/10^6 dG bases ± SE.

**Fig. 2.** The relationship between possession of the GPX1 ALA6 allele and 8-OHdG levels in normal lung tissue. Data presented are mean 8-OHdG values/10^6 dG bases ± SE.

of the ALA6 allele this result was not statistically significant (P = 0.421).

We also examined the association between hOGG1 genotype and adduct level. A typical hOGG1 restriction fragment length polymorphism (RFLP) result is shown in Figure 3A. It was noted that the hOGG-Ser allele was more prevalent (0.76) in this control Caucasian population compared with previous reports on Japanese populations (0.59) (23,25). Furthermore, hOGG1 allele frequencies (Ser/Ser:Cys/Ser:Cys/Cys) did not vary significantly between the lung cancer cases (0.53:0.41:0.06) and the general Caucasian population (0.56:0.40:0.04) in this study, indicative that a strong hOGG1 allele selection pressure was not operating in the lung cancer cases. This is consistent with the fact that levels of 8-OHdG in normal lung DNA did not vary with hOGG1 genotype (P = 0.472) (Figure 3B).

LOH at one or more markers on chromosome 3p was observed in 40% of lung tumours. The mean 8-OHdG level in these tumours was double that observed in tumours which showed no evidence of LOH (P = 0.05) (Figure 4A). Furthermore, the GPX activity of these tumours was reduced by one third when compared with tumours exhibiting no 3p LOH (Figure 4B), although this was not significant (P = 0.09).

**Fig. 3.** (A) RFLP analysis of the codon 326 hOGG1 Cys/Ser polymorphism. The primary amplification generates a 293 bp amplicon (lane 2). Fnu4HI specifically cuts cysteine alleles generating 123/124 and 170/169 bp products. Lane 1, 50 bp ladder; lane 2, primary amplification product; lane 3, serine homozygote control digest; lane 4, cysteine/serine heterozygote control digest; lane 5, cysteine homozygote control digest; lane 6, serine homozygote; lane 7, cysteine/serine heterozygote lung specimens. (B) The relationship between hOGG1 genotype and 8-OHdG levels in normal lung tissue. Data presented are mean 8-OHdG values/10^6 dG bases ± SE.

**Discussion**

A role for specific gene polymorphisms in relation to susceptibility to lung cancer has been highlighted for a number of genes, including myeloperoxidase (29) and glutathione S-transferases (30,31). However, markers of biological effects on the disease pathway are rarely used to demonstrate a mechanistic link between genotype and disease. Such a link would contribute to establishing a causal association between the two. In this study, the significance of GPX1 and hOGG1 polymorphisms in protecting against oxidative stress and potentially in the aetiopathogenesis of lung cancer was addressed by examining their effect on levels of the promutagenic lesion 8-OHdG in lung tissue.

Since guanine is the most readily oxidized DNA base, the use of 8-OHdG as a dosimeter for oxidative stress is the subject of debate in the light of susceptibility to artefactual formation during sample processing and measurement (32–34). In particular, phenol, light, heat, methanol and silylation steps in GC-EIMS have all been highlighted as potential sources of artefactual 8-OHdG formation. Thus, rigorous procedures were employed in our study to minimize and
control for sources of experimental error, which included: (i) the transit time between surgery and sample storage was minimized; (ii) tissue dissection was performed by a consultant pathologist in order to optimize and standardize the quality of tissue specimens collected; (iii) all tissue was processed using a standardized laboratory protocol with carefully timed tissue homogenizations, an additional factor which we found could lead to an increase in adduct levels (see Materials and methods).

Levels of adduct which were measured in this study are consistent with those reported by Inoue et al. (35), who described a mean 8-OHdG level of 5.2 8-OHdG/10^6 dG in the peripheral lung of lung cancer cases compared with 8.1 8-OHdG/10^6 dG in the current study. There was somewhat more inter-individual variability observed in the present study, but 80% of samples fell within the range (i.e. <10 adducts/10^6 dG) reported by Inoue et al. (35). Neither study found a difference in 8-OHdG levels between non-tumorous lung tissue from adenocarcinoma and squamous cell carcinoma patients. These findings also concur with an earlier study (2) on the central lung, where 8-OHdG levels did not differ between normal and tumorous tissue nor across different lung tumour types.

Preliminary findings presented here indicate a trend towards lower 8-OHdG levels in normal lung tissue possessing the ALA6 allele of the GPX1 gene, implying a possible functional change in enzyme activity as a result of sequence characteristics. However, further studies are required to define the role of this polymorphism in relation to the function of GPX1 and its role in protection against oxidative DNA damage.

The hOGG1-Ser and -Cys polymorphisms were initially identified by Kohno et al. (23). Based upon in vitro evidence utilizing an E.coli complementation assay, the hOGG1-Cys allele appeared to exhibit a poorer 8-OHdG repair activity compared with the hOGG1-Ser isoform. In the current study, we were unable to find evidence to support a role for the hOGG1 genotype in determining 8-OHdG levels in non-tumorous tissue from a series of 34 lung specimens. This suggests that differences in 8-OHdG glycosylase activity within hOGG1 polymorphic variants are insufficient to impact upon tissue 8-OHdG levels. Alternatively, it may reflect a lack of resolution regarding adduct analysis in that current methodologies are limited to measurements in total genomic DNA and cannot be targeted to specific regions, e.g. transcriptionally active sites. In addition, repair of 8-OHdG may not be wholly effected by hOGG1. Monden et al. (36) confirmed that OGG1 (type 1a isoform) constitutes the major 8-OHdG glycosylase activity detectable in human cells. However, other repair enzymes also demonstrate some 8-OHdG glycosylase activity in vitro and could contribute to the persistence of 8-OHdG within tissues (37,38). Perhaps of most significance, Hazra et al. (38) recently identified OGG2, which preferentially excises 8-OHdG when paired with G or A. In light of the substrate specificities outlined for OGG1 and OGG2, the effects of hOGG1 polymorphisms may be more subtle and perhaps require discrimination within a base pair context. Furthermore, chromatin structure and sequence context are likely to be important factors not only in determining the site of 8-OHdG adduct formation, but may also affect efficiency of DNA repair and adduct persistence. This information could be particularly pertinent in relation to oxidative DNA damage within target genes where mutation is key to disease initiation and/or progression.

In principle, one of the limitations in the type of study we describe here is that tissue specimens were collected from people undergoing surgery and hence with prevalent disease. Additionally, it is often a fact that there is incomplete quantitative data available on other environmental exposures, e.g. smoking, as in this study. Thus, both the presence of disease and these latter factors could act as confounders of the relationship between adduct level and genotype. Therefore, for a polymorphism to cause detectable change in 8-OHdG levels, the association would have to be relatively strong.

Notwithstanding the caveats outlined above, a global elevation in 8-OHdG levels was observed in tumor tissue which exhibited LOH within GPX1 or at loci close to hOGG1. This appears to indicate that oxidative defences are indeed compromised in lung tumours which exhibit 3p LOH at these sites and is further supported by the finding that GPX1 enzyme activity was reduced in these tumours. To date, most studies on the lung have compared GPX1 activities across normal and tumorous tissue with somewhat inconclusive results (39,40). That GPX1 activity is reduced in some but not all tumours relative to normal tissue may in part reflect 3p LOH events in these tumours. In the current study, reduced GPX1 enzyme activity does not appear to completely account for the elevated 8-OHdG levels observed in tumours with 3p LOH, because the correlation between GPX1 enzyme activity and 8-OHdG levels in tumours at the individual level was poor (data not shown). Future studies will address whether hOGG1 activity is also compromised in tumours exhibiting 3p LOH, although the involvement of other genes which locate to these regions of chromosome 3p cannot be precluded.

In conclusion, preliminary results indicate that endogenous 8-OHdG levels in the lung may be lower in individuals possessing the GPX1 ALA6 polymorphism but are not affected by the hOGG1 genotypes examined. 3p LOH at chromosomal

Fig. 4. (A) The association between 3p chromosomal loss and 8-OHdG levels in lung tumours. Data presented are mean 8-OHdG values/10^6 dG bases ± SE. (B) The effect of LOH on chromosome 3p on lung tumour glutathione peroxidase activity. Data presented are mean nmol GSH oxidized/min/mg cytosolic protein ± SE.

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In conclusion, preliminary results indicate that endogenous 8-OHdG levels in the lung may be lower in individuals possessing the GPX1 ALA6 polymorphism but are not affected by the hOGG1 genotypes examined. 3p LOH at chromosomal
sites associated with lung cancer development appear to compromise antioxidant defences, including GPX1 enzyme activity, leading to elevations in 8-OHdG levels within genomic DNA. It is interesting to speculate that as a consequence of elevated 8-OHdG levels, tumours with 3p LOH will have a higher mutation frequency. This may result in heightened tumour aggressiveness but may also affect responsiveness to therapeutic agents when compared with tumours which have not lost these sites on chromosome 3p, as many of these agents, e.g. γ-irradiation, induce oxidative DNA damage.

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