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Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer

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Two variant glutathione S-transferase cDNAs have been described at the GSTP1 locus, which differ by a single base pair (A−G) substitution at nucleotide 313 of the GSTP1 cDNA. This results in an amino acid substitution which alters the function of the enzyme. In this study, a novel PCR assay has been developed which demonstrates that these two variant cDNAs represent distinct *GSTP1* alleles (*GSTP1a* and *GSTP1b*). In a study of individuals with different forms of cancer, the *GSTP1b* allele is found to be strongly associated with bladder cancer and testicular cancer. In controls 6.5% of individuals were homozygous for the *GSTP1b* allele. In bladder cancer cases, this rose to 19.7% [*n* = 71, odds ratio 3.6 (1.4–9.2), *P* = 0.006] and in testicular cancer to 18.7% [*n* = 155, odds ratio 3.3 (1.5–7.7), *P* = 0.002]. In addition, in prostate cancer a highly significant decrease in the frequency of the GSTP1a homozygotes was observed [control 51.0% versus 27.8% cancer cases, *n* = 36, odds ratio 0.4 (0.02–3.3), *P* = 0.008]. Increases in the frequency of GSTP1b homozygotes was also observed in lung cancer and chronic obstructive pulmonary disease. However, these were not statistically significant. No change in breast or colon cancer allele frequencies was observed.

Introduction

The glutathione S-transferase supergene family consists of four gene subfamilies (GSTA, GSTM, GSTT and GSTP) which play a central role in the inactivation of toxic and carcinogenic electrophiles (1). Certain genes within the GSTM and GSTT (GSTM1 and GSTT1) subfamilies exhibit deletion polymorphisms (2,3) which have been associated with cancer susceptibility (2,4,5). The risk to the individual carrying a variant of one of these genes is estimated to be low, but the high frequency in the population of some of these variants suggests that the population attributable risk may be very high. The GSTP subfamily comprises a single gene (*GSTP1*) which is over-expressed in both preneoplastic and neoplastic lesions in both rat and man (6–8). In addition, the GSTP1 gene is inactivated by hypermethylation in the early stages of prostatic carcinogenesis (9) and is over-expressed in tumour cells made resistant to anticancer drugs (10). The almost ubiquitous presence of disturbances in the regular pattern of expression of GSTP1 in tumour samples (8) suggests that this enzyme may play an important role in the aetiology of malignant disease. However, unlike the GSTM1 and GSTT1 genes, genetic polymorphisms at the GSTP1 locus have not been described. Two variant cDNAs have been reported (11,12), which differ at base pair 313 A versus G. This results in a single amino acid difference, valine versus isoleucine at codon 105 (Figure 1). This residue lies in close proximity to the hydrophobic binding site for electrophilic substrates (13) and it has been demonstrated that the val variant has altered specific activity and affinity for electrophilic substrates (14). In order to establish whether these sequences represent alleles at the GSTP1 locus, we designed a PCR assay to rapidly and simply detect their presence in the population. Since GSTP1 is a major enzyme involved in the inactivation of cigarette smoke carcinogens such as benzo[*a*]-pyrene diol epoxide and also other toxic cigarette smoke constituents such as acrolein (1), we were interested to determine whether this polymorphism was related to diseases associated with cigarette smoking such as certain types of cancer and chronic obstructive pulmonary disease (COPD*).

***Abbreviations:** COPD, chronic obstructive pulmonary disease; OR, odds ratio.

Materials and methods

Patient materials

Populations analysed for GSTP1 genotype were 155 control individuals from the Edinburgh area. These random samples were obtained from the Clinical Biochemistry Department at Edinburgh Royal Infirmary; they were all Caucasians. Other samples tested were 79 COPD cases; 155 lung cancer cases [mean age 67.2 years (49–87 years), 97% smokers or ex-smokers, 69% males]; 71 bladder cancer cases [mean age 72.2 years (54–91 years), 86% smokers or ex-smokers, 68% males]; 90 teratoma cases [mean age 40.5 years (22–56 years), 70% smokers or ex-smokers]; 65 seminoma cases [mean age 45.6 years (30–64 years), 50% smokers or ex-smokers]; 36 prostatic cancer cases [mean age 70.4 years (62–88 years), 90% smokers or ex-smokers]; 62 breast cancer cases [mean age 60.3 years (37–82 years), 60% smokers or ex-smokers] and 100 colon cancer cases were studied. All samples from cancer patients were taken from individuals attending chemotherapy, radiotherapy or surgical clinics at hospitals in Edinburgh over a 3-year period. In most cases, samples were supplied as EDTA treated blood. The colon cancer samples were supplied as DNA. Patient anonymity was maintained. Investigations were performed with ethical agreement, and informed consent was obtained from each patient.

Determination of genotype at the GSTP1 locus

A crude lysate was made from 100–200 ml whole blood (15) and 5–10 µl (corresponding to ~50 ng DNA) was used as a template in a PCR reaction with 200 ng each of primers P105F (5′ ACC CCA GGG CTC TAT GGG AA 3′) and P105R (5′ TGA GGG CAC AAG AAG CCC CT 3′), 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Promega, Southampton, UK) in a total volume of 40 µl. Initial denaturation was carried out at 95°C for 5 min. Cycling conditions were: primer annealing at 55°C for 30 s, polymerization at 72°C for 30 s and strand separation at 94°C for 30 s. Thirty cycles were carried out. A final polymerization step of 72°C for 5 min was carried out to complete the elongation processes. The PCR product (20 µl) was then digested with 5 U *Alw*261 (New England Biolabs, Hertfordshire, UK) in a total volume of 25 µl, and the products separated on a 3.5% agarose gel and subsequently stained with ethidium bromide (10 mg/ml) to visualize the bands.

Direct sequencing of PCR products

Approximately 50 µl of PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) prior to the sequencing reactions. The concentration of the PCR product was estimated by quantification on a

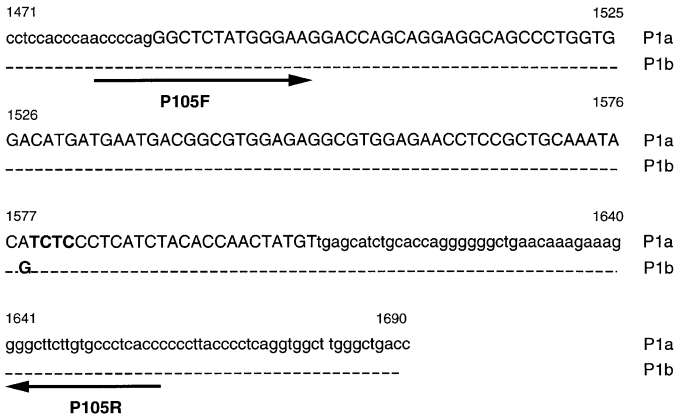

Fig. 1. Comparison of variant genomic DNA sequences at the GSTP1 locus. The upper sequence refers to the putative *GSTP1a* allele and the lower sequence refers to the putative *GSTP1b* allele. The sequence and numbering for GSTP1a is taken from Cowell *et al.* (17). The variant GSTP1b sequence was adapted from the sequence of Cowell *et al.* (17) by reference to the sequence of Ahmad *et al.* (11). The positions of the intron sequences are marked in lower case lettering, and the position of the ile105val change at codon 105 is noted. The *Alw*261 site created by the A−G mutation at position 1578 is represented in bold type. The position and sequences of PCR primers P105F and P105R are marked with arrows.

2% agarose gel. Approximately 250 ng of this product was used as a template in a double-stranded cycle sequencing reaction using the ds-DNA cycle sequencing system (Gibco-BRL, Paisley, UK) labelled with [γ^{32}P]dATP and primer P105F. All reactions were carried out in an Omnigene thermal cycler (Hybaid, Middlesex, UK). Cycling conditions included an initial denaturation at 94°C for 5 min followed by 20 cycles of denaturation at 94°C for 30 s, primer annealing at 47°C for 60 s and polymerization at 72°C for 60 s. The reaction was completed by 10 cycles of denaturation at 94°C for 30 s and polymerization at 72°C for 60 s. Sequencing products were visualized on an 8% polyacrylamide sequencing gel with the inclusion of 40% formamide to enhance resolution, followed by autoradiography at −70°C for ~14 h.

Statistics

Odds ratios (OR) for the effect of genotype at the GSTP1 locus were calculated according to the equation OR = P1P4/P2P3 where P1 = GSTP1b homozygotes in the cancer cohort, P2 = GSTP1a/GSTP1b or GSTP1a/GSTP1a genotypes in the cancer cohort, P3 = GSTP1b homozygotes in the control cohort and P4 = GSTP1a/GSTP1b or GSTP1a/GSTP1a genotypes in control cohort. Frequencies for GSTP1b/GSTP1b homozygotes in cancer and control cohorts were compared using χ^2 analysis for one degree of freedom or Fisher's exact test where cell numbers were <5.

Results and discussion

In order to establish whether the published GSTP1 cDNA sequences represent alleles of GSTP1, we designed a PCR assay to rapidly and simply detect their presence in the population. Gene specific PCR primers P105F and P105R (Figure 1) were designed from the published sequence of GSTP1 (16,17) to amplify a 176 bp region of the gene. The A−G base pair substitution present in the *GSTP1b* allele allows each genotype to be identified with the diagnostic restriction enzyme *Alw*261 (Figure 2). Analysis of a random population did indeed demonstrate that GSTP1 was polymorphic at amino acid 105. The polymorphism was confirmed by direct sequence analysis of individuals predicted to be GSTP1a/GSTP1a, GSTP1a/GSTP1b and GSTP1b/GSTP1b from the assay results (Figures 2 and 3).

In a population of 155 healthy volunteers from the Edinburgh area, the frequencies of GSTP1a and GSTP1b were 0.72 and 0.28, respectively. A total of 6.5% of individuals were homozygous for the low activity allele *GSTP1b*.

In order to establish whether the *GSTP1* alleles were

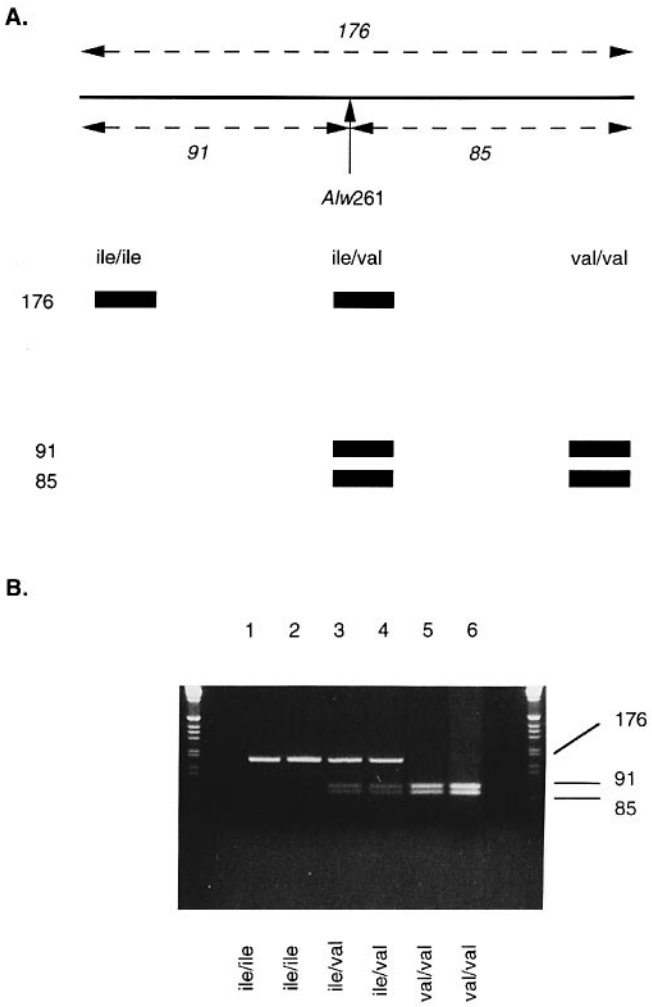

Fig. 2. A novel PCR assay to detect genetic polymorphism at the GSTP1 locus. (**A**) Diagrammatic representation of the assay design. A 176 bp region of genomic DNA flanking exon 5 was amplified using primers P105F (5′ ACC CCA GGG CTC TAT GGG AA 3′) and P105R (5′ TGA GGG CAC AAG AAG CCC CT 3′). The PCR product was then digested with *Alw*261. As a result of the A−G difference at position 1578, an *Alw*261 is created in the *GSTP1b* allele. The consensus sequence corresponding to GSTP1a will not be cut, but the val105 sequence corresponding to GSTP1b will cleave to yield two fragments of 91 bp and 85 bp. The predicted pattern of bands is shown. (**B**) Identification of individuals homozygous for GSTP1a (ile105/ile105), heterozygous for GSTP1a/GSTP1b (ile105/val105) or homozygous for GSTP1b (val105/val105). The validity of the assay for the determination of these genotypes was determined by direct sequencing of PCR products as in Figure 3.

associated with cancer susceptibility we studied the frequency of each genotype in different cancer groups. In a cohort of bladder cancer patients a highly significant increase in the frequency of the GSTP1b/GSTP1b genotype was observed, with an OR of 3.6 (95% CI = 1.4–9.2, $P = 0.006$). We should like to emphasize the need for a larger study of bladder cancer patients in order to substantiate these preliminary results. Interestingly, an increase was also observed in the proportion of individuals with this haplotype in lung cancer patients with an OR of 1.9 (95% CI = 0.7–4.8). However, this did not reach statistical significance ($P = 0.233$). In a population with COPD (18), an increase in the proportion of individuals homozygous for the low activity *GSTP1b* allele was also observed. This finding is also interesting (although the *P* value of 0.174 was not significant) in view of the known activity of

GSTP1 in the inactivation of certain toxic components of cigarette smoke such as acrolein (1).

In further studies on other cancer types a highly significant increase in the proportion of individuals homozygous for the *GSTP1b* allele was found in both teratoma and seminoma cancer samples with ORs of 3.4 (95% CI = 1.4–8.4, P = 0.005) and 3.3 (95% CI = 1.2–8.8, P = 0.014), respectively, and an OR of 3.3 (95% CI = 1.5–7.7, P = 0.002) when the two tumour types are combined (Table I). As the age of the control group was not determined, the change in *GSTP1* allele frequency in testicular cancer could theoretically be explained by an age difference between the control and testicular cancer groups, and a link between the *GSTP1b* allele and longevity. However, this is unlikely since there is no evidence to suggest that GSTP1 alleles affect longevity (R.C.Strange, personal communication). The causative agents for these diseases are as yet unclear. To date, only cryptoorchidism, physical trauma, testicular atrophy and exposure to sexually transmitted diseases have been associated with increased risk of testicular cancers. Other factors such as occupation, smoking, alcohol intake or personal characteristics such as handedness have shown no such association (19). As smoking has not been linked with the risk of developing these tumours, further work will be required to understand the molecular basis of our observation.

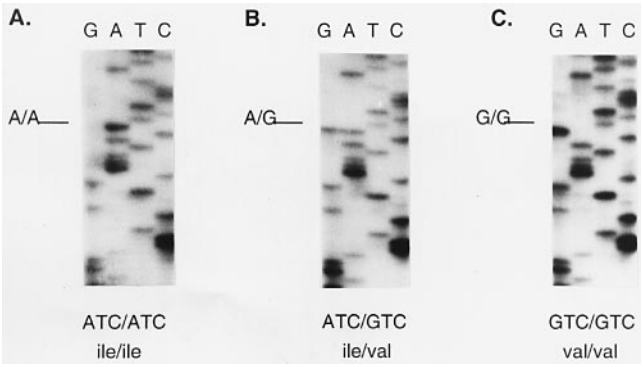

Fig. 3. Determination of GSTP1 genotype by sequence analysis. The presence of A or G at nucleotide 1578 was determined by direct sequencing of the PCR product derived from individuals predicted to be (**A**) GSTP1a/GSTP1a, (**B**) GSTP1a/GSTP1b or (**C**) GSTP1b/GSTP1b by the banding patterns produced from the PCR-RFLP assay. In all cases the predicted genotype was confirmed. The presence of two bands corresponding to A and G at nucleotide 1578 can clearly be seen in the GSTP1a/GSTP1b heterozygous individual (B).

A study of breast cancer and colon cancer did not show a significant increase in susceptibility associated with the *GSTP1b* allele compared to controls (P = 0.77 and 0.82, respectively).

In prostatic cancer, although the numbers are small (n = 36), there was a marked reduction in the frequency of GSTP1b homozygotes, although this was not statistically significant (P = 0.693). However, there was a highly significant reduction in individuals homozygous for the *GSTP1a* alleles (P = 0.008) and a significant increase in the proportion of heterozygotes (P = 0.003). This is interesting because in almost all other cancers the expression of GSTP1 in the tumour tissue is increased, whereas in prostatic cancer this enzyme appears to be inactivated (9). The aetiological factors implicated in prostatic carcinogenesis are also unclear, but hormone levels, a high dietary fat intake, exposure to sexually transmitted diseases and exposure to cadmium have all been suggested as risk factors (20). No association has been reported between either tobacco or alcohol intake and prostatic cancer (20). It is likely that genetic factors are involved, given the dramatic fluctuations in risk of this particular cancer in different ethnic groups. In particular, the risk of prostatic cancer is universally high in black individuals originating from a wide variety of geographic locations (20).

The fact that seven independent correlations have been made between *GSTP1* genotype and cancer susceptibility raises the possibility that the correlations observed were due to chance. However, correcting the P values for this by multiplying by a factor of seven still results in highly significant results for bladder and testicular cancer (P = 0.0036 and 0.014, respectively).

In summary, the above observations indicate that polymorphism at the GSTP1 locus may be an important factor in susceptibility to different types of cancer. The possession of a lower activity form of GSTP1—the *GSTP1b* allele— may be predicted to have severe consequences for an individual's cancer risk since GSTP1 is well known to have an important role in the detoxification of carcinogenic compounds such as benzo[*a*]pyrene diol epoxide. Combined with extensive data showing aberrant expression of GSTP1 in various types of tumours—again suggesting a detoxification role for GSTP1 in human malignancy, it would be expected that a GSTP1 isozyme with lower detoxification capacity would predispose an individual to cancer. Association of the *GSTP1b* allele with

Table I. Frequency of GSTP1 genotypes in cancer and control populations

Sample group	Sample no.	GSTP1a/1a	GSTP1a/1b	GSTP1b/1b	Odds ratio (95% CI)	*P* value
Random controls	155	51.0	42.5	6.5	N/A	N/A
COPD	79	43.0	44.3	12.7	2.1 (0.8–5.8)	0.174
Lung cancer	115	43.5	45.2	11.3	1.9 (0.7–4.8)	0.233
Seminoma	65	37.0	44.5	18.5	3.3 (1.2–8.8)	0.014
Teratoma	90	40.0	41.1	18.9	3.4 (1.4–8.4)	0.005*
Total testicular cancer	155	38.7	42.6	18.7	3.3 (1.5–7.7)	0.002*
Bladder cancer	71	35.2	45.1	19.7	3.6 (1.4–9.2)	0.006*
Prostatic cancer	36	27.8	69.4	2.8	0.4 (0.02–3.3)	0.693
Breast cancer	62	40.3	51.6	8.1	1.3 (0.4–4.3)	0.77
Colon cancer	100	37.0	55.0	8.0	1.3 (0.4–3.6)	0.825

Odds ratios were calculated by comparison of random controls and cancer groups homozygous for the *GSTP1b* allele by methods described in the text.
Estimates of statistical significance were calculated by standard Chi-squared analysis for one degree of freedom, or by Fisher's exact test where cell numbers were <5. Statistically significant results are marked with *.
N/A, odds ratios, chi-squared and *P* values were not applicable to these samples.
COPD, chronic obstructive pulmonary disease.

cancer would be expected since it has a lower activity due to the conversion of the amino acid at codon 105 from isoleucine to valine. The amino acid at this site has been predicted to lie in the hydrophobic binding site for electrophile substrates from molecular modelling and thus to affect substrate binding (14).

It is important to note that these data are initial observations and need to be substantiated by more detailed case-control studies for each cancer population. There is an increasing body of evidence suggesting that polymorphism in glutathione S-transferases are associated with cancer susceptibility and the polymorphism at the GSTP1 locus may be of particular importance, especially in view of the almost ubiquitous expression of this protein in a wide range of different cell types including lung, bladder, colon, testicular and prostate tissue. In addition, this protein is often over-expressed in tumour cells made resistant to anticancer drugs. This raises the possibility that the polymorphism may also be important in the outcome of anticancer drug therapy.

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