Microsomal epoxide hydrolase gene polymorphism and susceptibility to colon cancer

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Summary We examined polymorphisms in exons 3 and 4 of microsomal epoxide hydrolase in 101 patients with colon cancer and compared the results with 203 control samples. The frequency of the exon 3 T to C mutation was higher in cancer patients than in controls (odds ratio 3.8; 95% confidence intervals 1.8–8.0). This sequence alteration changes tyrosine residue 113 to histidine and is associated with lower enzyme activity when expressed in vitro. This suggests that putative slow epoxide hydrolase activity may be a risk factor for colon cancer. This appears to be true for both right- and left-sided tumours, but was more apparent for tumours arising distally (odds ratio 4.1; 95% confidence limits 1.9–9.2). By contrast, there was no difference in prevalence of exon 4 A to G transition mutation in cancer vs controls. This mutation changes histidine residue 139 to arginine and produces increased enzyme activity. There was no association between epoxide hydrolase genotype and abnormalities of *p53* or Ki-*Ras*.

Keywords: epoxide hydrolase; colon cancer; polymorphism; cancer risk

The colon is subject to oxidative and free radical damage by both products of endogenous metabolism and bacterial fermentation within the gut lumen. These injurious stimuli may cause cell damage or cell death or even lead to mutations, resulting in tumour initiation and progression. Colon cancer is a common disease, with the highest incidence in developed countries. The aetiology is unknown, but in addition to diet and cigarette smoking (Giovannucci and Willett, 1994) there is a complex polygenic background that determines individual susceptibility to disease. There is currently much interest in the roles of oncogenes, tumour-suppressor genes and mismatch repair enzymes in colon cancer (Tomlinson et al, 1997). In addition, interindividual variation in the ability to dispose of reactive xenobiotics catalysed by glutathione S-transferases GST-M1 and GST-T1 has been investigated (Lang et al, 1986; Strange et al, 1991; Zhong et al, 1993; Chenevix-Trench et al, 1995). However, results from a number of studies show only weak and inconsistent associations with disease susceptibility. *N*-acetyltransferase 2 (NAT-2) polymorphism may be implicated in susceptibility to colon cancer (Lang et al, 1986; Wohlleb et al, 1990; Illett et al, 1994; Probst-Hensch et al, 1995), but there is evidence that its relationship may be by linkage with other genes rather than causally (Hubbard et al, 1997).

We have used a polymerase chain reaction (PCR) strategy to investigate whether polymorphisms in the microsomal epoxide hydrolase gene (mEPHX) (Hassett et al, 1994) have any relationship to colon cancer. The enzyme is expressed in many tissues, including colon and liver. Polymorphisms of mEPHX may have functional significance. There is variation in exon 3, where a T to C alteration changes tyrosine residue 113 to histidine and is associated with lower enzyme activity when expressed in vitro. By contrast, A to G transition in exon 4 changes histidine residue 139

to arginine and produces increased enzyme activity. The effect of combining the alleles has not been established. The activity of mEPHX varies more than 50-fold in Caucasians (Omiecinski et al, 1993). This variation of activity is, thus, due to a combination of genetic polymorphism, transcriptional and post-transcriptional control of gene expression.

MATERIALS AND METHODS

Controls and cancer cases

Control blood samples ($n = 203$) were obtained anonymously from the Scottish National Blood Transfusion Service. These were Caucasian individuals aged between 18 and 65 years with equal sex distribution. This group has been previously described (Cantlay et al, 1994) and was drawn from the same geographical area as the cancer study group. The presence of colorectal neoplasia was not specifically excluded, but all patients were healthy. Peripheral blood from patients with colorectal cancer was collected from a consecutive series of operable colorectal cancer cases after surgery in four local hospitals between 1988 and 1993 ($n = 101$).

Cancer patient data

Cancer diagnosis was confirmed histopathologically and cases were classified according to Dukes' stages (A, B, C), and according to position of cancer in the colon as either right (caecum, transverse or ascending) or left (sigmoid, descending or rectum) sides. All samples were Caucasian in origin. DNA was extracted as previously described (Cantlay et al, 1995). In addition, the frequency of immunodetectable stabilization of *p53* was recorded using antibody DO7 (Dako) on formalin-fixed, paraffin-embedded tissue ($n = 92$). Loss of heterozygosity at the *p53* locus on chromosome 17 ($n = 93$) was determined as previously reported (Cripps, 1994). The presence of codon 12 mutations in Ki-*ras* oncogene was determined using allele-specific PCR ($n = 81$) as described previously (Kotsinas et al, 1993).

Table 1 Distribution of microsomal epoxide hydrolase genotypes in control and disease populations

	Exon 3 polymorphism						Exon 4 polymorphism				
	Tyr (%)	Tyr/His (%)	His (%)	χ^2 (*P*-value)	OR (95% CI)	MAF	His (%)	His/Arg (%)	Arg (%)	χ^2 (*P*-value)	MAF
Controls (*n* = 203)	91 (45)	99 (49)	13 (6)	–	–	0.31	147 (72)	53 (26)	3 (2)	–	0.15
Colorectal cancer (*n* = 101)	42 (50)	38 (40)	21 (10)	14.5 0.007	3.84 (1.83–8.04)	0.40	76 (75)	21 (21)	4 (4)	2.66 >0.10	0.15
Right-sided (*n* = 33)	11 (33)	16 (49)	6 (18)	5.7 >0.10	3.25 (1.14–9.27)	0.43	21 (64)	9 (27)	3 (9)	6.79 >0.10	0.23
Left-sided (*n* = 68)	31 (46)	22 (32)	15 (22)	15.2 0.002	4.10 (1.87–9.15)	0.38	55 (81)	12 (18)	1 (1)	2.0 >0.10	0.10

For exon 3 genotype, the designation 'Tyr' represents an individual carrying homozygous Tyr113 mEPHX sequence, 'His' represents a homozygous His-113 and 'Tyr/His' a heterozygote. For exon 4 genotype, the designation 'His' represents an individual carrying homozygous His-139 mEPHX sequence, 'Arg' represents a homozygous Arg-139 and 'His/Arg' a heterozygote. *P*-values for χ^2 for right- and left-sided compared with controls are given as Bonferroni corrected. Odds ratios (OR) and 95% confidence intervals (CI), relative risk of the presence of two His-113 exon 3 alleles in disease population vs controls. MAF = 'mutant' allele frequency.
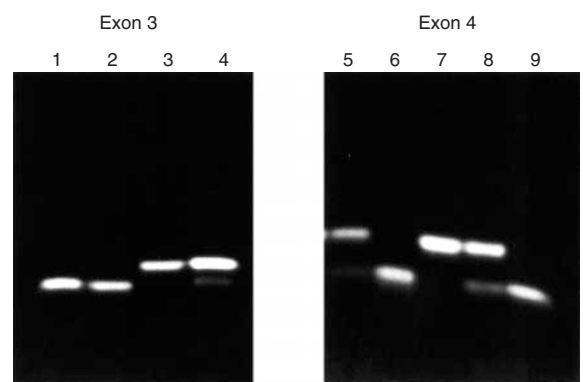
Table 2 Distribution of mEPHX exon 3 genotypes and *p53* immunodetection, *p53* loss of heterozygosity and Ki-*ras* codon 12 mutation

	Exon 3 polymorphism			
	Tyr (%)	Tyr/His (%)	His (%)	*P*-value
p53 immunodetection (*n* = 92)				
Positive	17 (18)	13 (14)	9 (10)	>0.10
Negative	21 (23)	22 (24)	10 (11)	
p53 loss of heterozygosity (*n* = 93)				
Loss	8 (9)	9 (10)	3 (3)	>0.10
Retained	11 (12)	8 (9)	4 (4)	
Non-informative	21 (22)	17 (18)	12 (13)	
Ki-*Ras* mutation at codon 12 (*n* = 81)				
Wild type	7 (9)	6 (7)	5 (6)	>0.10
Mutated	28 (34)	24 (30)	11 (14)	

Nomenclature of mEPHX exon 3 alleles as described in Table 1. *P*-value calculated from χ^2 comparing exon 3 allele frequency within each group, for example p53 positive vs p53 negative.

Figure 1 Epoxide hydrolase

mEPHX PCR analysis

Two separate PCR assays were used to detect the two mutations in mEPHX. The assay for the exon 3 T to C variant, changing tyrosine 113 to histidine, uses the primer pair E1 5′-GATCGATAAGTTC-CGTTTCACC [starting at bp 321 in mEPHX cDNA] and E2 5′-ATCCTTAGTCTTGAAGTGAGGaT (starting at bp 461). The downstream primer abuts directly onto the mutation site and an engineered base change (shown in lower case: an A for a G) produces an *Eco*RV restriction enzyme site (GATATC) in the wild type only. The exon 4 A to G transition produces an *Rsa*I restriction fragment length polymorphism (ATAC to GTAC). The primer pair E3 5′-ACATCCACTTCATCCACGT (bp 494) and E4 5′-ATGC-CTCTGAGAAGCCAT (bp 685) is used to assay this polymorphism. Figure 1 shows a typical result of the genotyping assays. Polymorphisms were detected using restriction enzymes *Eco*RV (exon 3) and *Rsa*I (exon 4). The polymerase chain reaction was performed on a Hybaid Omnigene thermal cycler using 200 ng of genomic DNA, 200 ng of primers E1/E2 or E3/E4, 200 mM dNTPs (Pharmacia, UK), ×1 polymerase buffer (Promega, UK), 1.5 mM

magnesium chloride, 4% DMSO and 2 u of *Taq* Polymerase (Promega, UK) in a total volume of 50 µl. Twenty microlitres of each PCR reaction was digested with 5 u of the appropriate restriction enzyme (Gibco BRL, UK). Digested PCR products were separated by size on 1.8% Metaphor (Hoeffer Scientific) agarose gel. Bands were visualized by ethidium bromide staining and ultraviolet illumination. Main cycling parameters were: 38 cycles of 94°C for 30 s, 55°C for 25 s and 72°C for 20 s.

Statistical analysis

Associations between disease groups and specific genotypes and phenotypes were analysed for significance by the two-tailed chi-squared test and *P*-values were Bonferroni corrected. Odds ratios and 95% confidence intervals were calculated to assess relative risk of disease conferred by a particular allele or genotype.

RESULTS

mEPHX gene analysis

Analysis of the control group showed that the exon 3 histidine 113 ('slow') form was threefold more common than the exon 4 arginine 139 ('fast') form in the Caucasian population; 13 (6%) out of

203 individuals were homozygous for histidine 113 (exon 3) and 49% heterozygous. In comparison, arginine 139 (exon 4) was only detected in 28% of individuals, with only three homozygous subjects in total (Table 1). These results are similar to previous studies (Smith and Harrison, 1997).

mEPHX polymorphisms in disease groups

The distribution of mEPHX genotypes are shown in Table 1, and the comparison of exon 3 alleles with *p53* and Ki-*ras* mutations shown in Table 2. In the group of colon cancer patients (Table 1), there was a significant increase in the proportion of individuals homozygous for the histidine 113 (exon 3) [$P = 0.007$; odds ratio (OR) = 3.84]. Twenty-one out of 101 patients (21%) were homozygous for the histidine 113 (exon 3, putative 'slow' activity) versus only 6% of controls. There was no significant difference in the distribution of arginine 139 (exon 4, putative 'fast' activity) between control and cancer groups. Cancers were divided into right- and left-sided groups and the association with histidine 113 polymorphism was recalculated. For right-sided tumours, there was a trend for the putative 'slow' allele to be more common in the cancer group, but this was not statistically significant when the Bonferroni correction was applied. By contrast, left-sided colon cancers showed a highly significant increase in histidine 113 'slow' allele compared with controls ($P = 0.002$; OR = 4.1). No association with sex, age or Dukes' stage was found for either allele (data not shown). No association between arginine 139 polymorphism and right- or left-sided tumours was identified. When compared with the frequency of immunodetectable stabilization of *p53*, loss of heterozygosity at the *p53* locus, or codon 12 mutation of Ki-*Ras*, no association was noted with polymorphisms of mEPHX at either exon 3 or exon 4 (Table 2). Comparisons of the observed distributions of mEPHX genotypes and those predicted by allele frequencies by chi-squared analysis showed that the populations studied were in Hardy–Weinberg equilibrium, indicating that the control and study groups were sufficiently random and representative (data not shown).

DISCUSSION

The demonstration that genetically defined polymorphisms in mEPHX, predicted to affect enzyme activity at least in part, are associated with an increased incidence of colorectal cancer suggests that reactive epoxide intermediate metabolites may play a role in the development of colon cancer. Individuals with histidine 113 instead of tyrosine 113 (exon 3) had more than a threefold relative risk of having colorectal cancer. This is particularly true for cancers arising in the left side of the colorectum, i.e. descending and sigmoid colon and rectum, in which the relative risk increased to more than 4. The absence of a correlation with *p53* or Ki-*ras* mutations is unsurprising. mEPHX is a protective enzyme involved in general oxidative defence, rather than in specific protection of individual genes. Previously studies describing weak association between glutathione-dependent enzymes and colorectal cancer have found that the risk is more consistently with tumours originating in the left side (Zhong et al, 1993). This site may be more at risk from oxidative stress, or may be more likely to have high exposure to oxidants because of the higher transit time for faecal material at this site. Epoxides may be present in diet, or generated from a number of sources, including benzpyrene (which is present in cigarette smoke), dietary polycyclic aromatic hydrocarbons and

nitrosamines (Craft et al, 1988; Yang et al, 1988). Some further evidence suggests that mEPHX may be involved in steroidogenesis reactions which may explain preliminary observations that 'slow' genotype may be protective for ovarian cancer (Lancaster et al, 1996) and is not involved in lung (Smith et al, 1997) or bladder (Brockmoller et al, 1996) cancer risk.

The present study has examined genotype and not phenotype. There is clear evidence that the presence of a 'slow' exon 3 allele does confer lower enzyme activity, but genotype alone is insufficient to explain the variation of microsomal epoxide hydrolase enzyme activity seen in population studies (Hassett et al, 1997). In particular, the effect of carrying both exon 3 and exon 4 polymorphisms is undetermined and, thus, assumptions concerning enzyme activity from our study should be necessarily guarded. It is still possible that the described mutations are of themselves not causally related to colon cancer, but rather are in linkage with other, as yet unidentified, factors. However, the clear association does indicate that this enzyme is an important candidate to relate diet with susceptibility of the colorectal mucosa to injury. Evidence that dietary supplements, particularly fish oils which are thought to be chemopreventative for colon cancer, can induce microsomal epoxide hydrolase (Yang et al, 1993) and, thus, increase enzyme activity further strengthens this association and indicates the need for further investigation.

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