Overexpression of CYP2A6 in human colorectal tumors

Yoko Matsuda,[1] Kousuke Saoo,[1] Keiko Yamakawa,[1] Masanao Yokohira,[1] Satoshi Suzuki,[1] Toshiya Kuno,[1] Tetsuya Kamataki[2] and Katsumi Imaida[1,3]

[1]Onco-Pathology, Department of Pathology and Host-Defense, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793; [2]Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

(Received March 08, 2007/Revised May 23, 2007/2nd Revised June 05, 2007/Accepted June 10, 2007/Online publication August 7, 2007)

CYP2A6 metabolizes various nitrosamines, such as those in the diet and in tobacco smoke, which have been implicated as risk factors for colorectal tumors. To determine whether changes in expression levels could contribute to their progression, we carried out immunohistochemistry for CYP2A6 in human colon tumors. Colon specimens (n = 53) were diagnosed as adenoma (n = 16), adenocarcinoma (n = 30) or carcinoma in or with adenoma (n = 7). Colon tumor cells showed cytoplasmic granular immunoreactivity for CYP2A6. Adenocarcinomas and adjacent mucosa showed similar highly elevated degrees of CYP2A6 expression, whereas carcinomas in or with adenoma and adenomas showed lesser increases. To further determine whether CYP2A6 mRNA was expressed at the same level as the CYP2A6 protein, we carried out *in situ* hybridization of CYP2A6 in two cases of adenocarcinoma. *In situ* hybridization for CYP2A6 revealed mRNA expression in adenocarcinoma cells. The data indicate that CYP2A6 may have important roles in human colorectal tumorigenesis and progression, so that it could be a candidate therapeutic and chemopreventive target for colorectal cancers. (*Cancer Sci* 2007; 98: 1582–1585)

Colorectal tumors arise as a result of the accumulation of genomic alterations.[1] Most environmental chemical carcinogens are metabolized by phase I and phase II enzymes to exert their biological effects through the formation of DNA adducts.[2] Epidemiological studies have shown that one of the major risk factors for colorectal tumors is consumption of meat containing dietary carcinogens such as nitrosamine compounds.[3–6]

Cytochrome P450 (CYP), a major family of phase I enzymes, is responsible for metabolic activation of carcinogens. In human colorectal epithelium, expression of CYP1A1, 1A2, 2C, 2E1, 3A4 and 3A5 at the mRNA or protein levels has been detected.[7–9] It has been reported that human colon tumor tissues express CYP1B1 or 2C, and cultured colon cell lines express CYP2A6.[10–13] Recent studies have shown that in normal colon mucosa of patients with adenomas, protein concentrations of CYP2C8, 3A4 and 3A5 were lower than in disease-free controls, and several CYP might contribute to the development of neoplasia in the colon.[14] Colorectal cancer risk is related to genetic polymorphisms of CYP1A1, 2E1, 2C9 and 7A1, but there is some disagreement concerning which genotypes actually impact on colorectal tumorigenesis and how to interpret the roles of genetic polymorphisms.[15–19]

CYP2A6 is well known to metabolize tobacco-specific nitrosamines, as well as their counterparts in the diet.[20–22] Exposure to such nitrosamines in both the diet and tobacco smoke has been implicated as a risk factor for colorectal tumors.[3–6,17,23] Several polymorphisms of the CYP2A6 gene have been identified to date[20,24] and shown to have associations with lung cancer risk.[25] Previously, we revealed that human lung carcinomas express CYP2A6 and that upregulation may occur in cases with high malignant potential.[26] Recent studies have shown a strong relationship between colorectal cancer risk and CYP2A6 activity, as assessed by the urinary caffeine metabolite ratio.[27,28] In an *in vivo* study, CYP2A6 expression in colon cancer cells augmented cellular sensitivity to tegafur.[11] Therefore, CYP2A6 in colon tissues might have important roles in detoxification of xenobiotics, metabolism of chemotherapeutic drugs and colorectal tumorigenesis. However, there have been no reports on whether this enzyme is actually expressed and activated in human colorectal epithelium or tumor cells. In the present study, we carried out immunohistochemistry for CYP2A6 in human colon tumors in comparison with adjacent mucosa.

Materials and Methods

Tissue samples. A summary of all cases is shown in Table 1. Colon tissue samples were obtained from polypectomies (n = 21) and surgical resections (n = 32) between 2003 and 2004. The study protocol followed the ethical guidelines of Kagawa University. Informed consent was obtained from all subjects.

All of the excised tissues were fixed in 10% neutral formalin and processed routinely for embedding in paraffin. Sections (4 μm) were stained with hematoxylin and eosin for histological diagnosis. Colon specimens were diagnosed as adenoma (n = 16), adenocarcinoma (n = 30) or carcinoma in or with adenoma (n = 7) based on the criteria of the World Health Organization.[29] Adenomas were subdivided into low-grade dysplasia (n = 12) and high-grade dysplasia (n = 4). Colorectal adenocarcinomas were subdivided into well (n = 10), moderately (n = 19) and poorly differentiated (n = 1) groups. Furthermore, adenocarcinomas were subdivided into Tis (n = 7), T1 (n = 5), T2 (n = 4) and T3 (n = 14) based on the criteria of the World Health Organization.[29]

Immunohistochemistry. A total of 53 colorectal sections were used for immunohistochemistry. CYP2A6 immunoreactivity was identified with rabbit polyclonal antiserum to human cytochrome P450 2A6 (Biomol International, Plymouth Meeting, PA, USA). This antibody crossreacts with human CYP2B6 with which it shares a similar C-terminal sequence. The primary antibody (CYP2A6) was applied for 60 min at room temperature at 200-fold dilution. Enzyme-labeled biotin–streptavidin techniques were applied throughout with the VENTANA HX automated immunohistochemistry slide staining system (Ventana Japan, Yokohama, Japan). To assess CYP2A6 immunoreactivity, the percentage of positive cells and cytoplasmic staining intensity were determined for the tumor lesions (carcinomas or adenomas), adjacent mucosa and normal mucosa (Fig. 1). A tumor lesion was defined as an adenoma or adenocarcinoma cell component. Adjacent mucosa was defined as non-neoplastic epithelium adjacent to tumors. Normal mucosa was defined as non-neoplastic epithelium at least 1 cm distant from tumors in the same slide. A total of seven cases of carcinoma in adenoma were included. At a magnification of ×100, 10 randomized microscope images from

[3]To whom correspondence should be addressed. E-mail: imaida@med.kagawa-u.ac.jp

doi: 10.1111/j.1349-7006.2007.00572.x

Table 1. Summary of all cases

Variable	*n*
Age 68.66 ± 1.64 years	53
Sex	
Male	14
Female	39
Location	
Cecum	3
Ascending colon	10
Transverse colon	5
Descending colon	4
Sigmoid colon	11
Retum	10
No information	10
Histology	
Adenoma	16
Carcinoma	30
Carcinoma in or with adenoma	7
Adenoma	
Low-grade dysplasia	12
High-grade dysplasia	4
Carcinoma	
Differentiation	
Well	10
Moderate	19
Poor	1
Tumor stage	
Tis	7
T1	5
T2	4
T3	14
Lymph node metastasis	
Negative	22
Positive	4

colon tissue areas in each case were analyzed microscopically. The degree of positive staining for CYP2A6 was evaluated by scoring on a scale of 0–4 for percentage of positive cells (1+, <25% of the epithelial cells positive; 2+, 25–50% positive; 3+, 50–75% stain positive; 4+, >75% positive) and on a scale of 0–3 for strength of intensity of staining (0, no staining of epithelial cells; 1+, mild staining; 2+, moderate staining and 3+, intense staining). The total score was generated by adding the two scores. The average value obtained for the 10 images was calculated and was regarded as the CYP2A6 index. We assessed CYP2A6 indices for each area of tumor lesion, adjacent mucosa and normal mucosa.

***In situ* hybridization of CYP2A6.** To further determine CYP2A6 expression at the mRNA level we carried out *in situ* hybridization (ISH) for two cases of human adenocarcinoma. Total RNA was extracted from human liver using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and cDNA was prepared using the RNA PCR Kit (AMV) Ver. 3.0 with Random 9-mer primers (Takara Bio, Ohtsu, Japan), using the first and second polymerase chain reaction (PCR) primers (Table 2) added to SP6 or T7 promoters. The first PCR cycle conditions were: 94°C for 2 min followed by 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The second PCR cycle conditions were 25 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 1 min. After purification using a Pure Link PCR Purification Kit (Invitrogen, Carlsbad, CA, USA), 100 ng PCR product was used for cRNA sense and antisense probe synthesis. The probes were labeled with digoxigenin (DIG)-uridine triphosphate by SP6 RNA polymerase and T7 RNA polymerase using the DIG RNA Labeling Kit (Roche, Penzberg, Germany). For ISH, formalin-fixed and paraffin-embedded tissue was cut at 6 μm. ISH was carried out using the Ventana HX system Discovery (Ventana Japan), an automated slide staining system. Sections were hybridized for 6 h at 66°C, and the concentration of cRNA probes was 250 ng/mL.

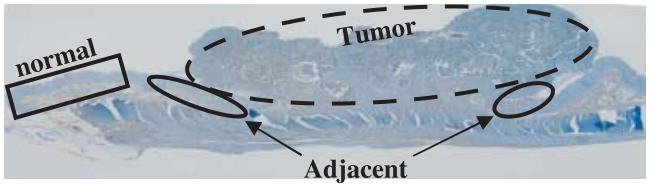

Fig. 1. Slide glass appearance of immunohistochemistry for CYP2A6. CYP2A6 immunoreactivity was examined in the carcinoma region (dotted oval), adjacent mucosa (oval) and normal mucosa (rectangle).

Statistics. All quantitative data are mean ± SD values. Differences among areas and cases were assessed using post-hoc analysis, and P-values < 0.05 were considered to be statistically significant.

Results

Immunohistochemistry for CYP2A6. In all cases, normal mucosa showed almost no CYP2A6 expression (Fig. 2b). In adenomas and adenocarcinomas (Fig. 2d,f,j,l), tumor cells demonstrated cytoplasmic granular immunoreactivity for CYP2A6. CYP2A6 immunoreactivity was shown to be stronger inside of compared with outside of the gland. In adenocarcinoma cases, immediately adjacent mucosa also showed CYP2A6 expression (Fig. 2d, arrowhead).

CYP2A6 indices. A summary of CYP2A6 indices is shown in Fig. 3. In adenomas and carcinomas in adenoma, CYP2A6 indices were higher than in adjacent mucosa and normal mucosa. However, adenocarcinomas and adjacent mucosa showed similar CYP2A6 indices, higher than in normal mucosa and also higher than in other tumors. Sex, age, location, differentiation, tumor stage and lymph node metastasis all showed no significant relationship with CYP2A6 indices.

In situ hybridization for CYP2A6 in human adenocarcinoma cases. Figure 2g,h illustrates the results of ISH. Human adenocarcinoma cases showed CYP2A6 mRNA expression in the cytoplasm of carcinoma cells.

Table 2. Sequences of CYP2A6 primers used in the probe preparation for *in situ* hybridization

	Primer sequences (5′–3′)
First polymerase chain reaction	
Forward (SP6 + CYP 2A6)	ATTTAGGTGACACTATAGAATGGGTCTTCAAAGGCTATGG
Reverse (T7 + CYP 2A6)	AATACGACTCACTATAGGGCCATGCGGATGAGAAAGGAGT
Second polymerase chain reaction	
Forward (SP6)	TTGTGCGGCCATTTAGGTGACACTATAGAA
Reverse (T7)	GAGCGCGCGTAATACGACTCACTATAGGGC

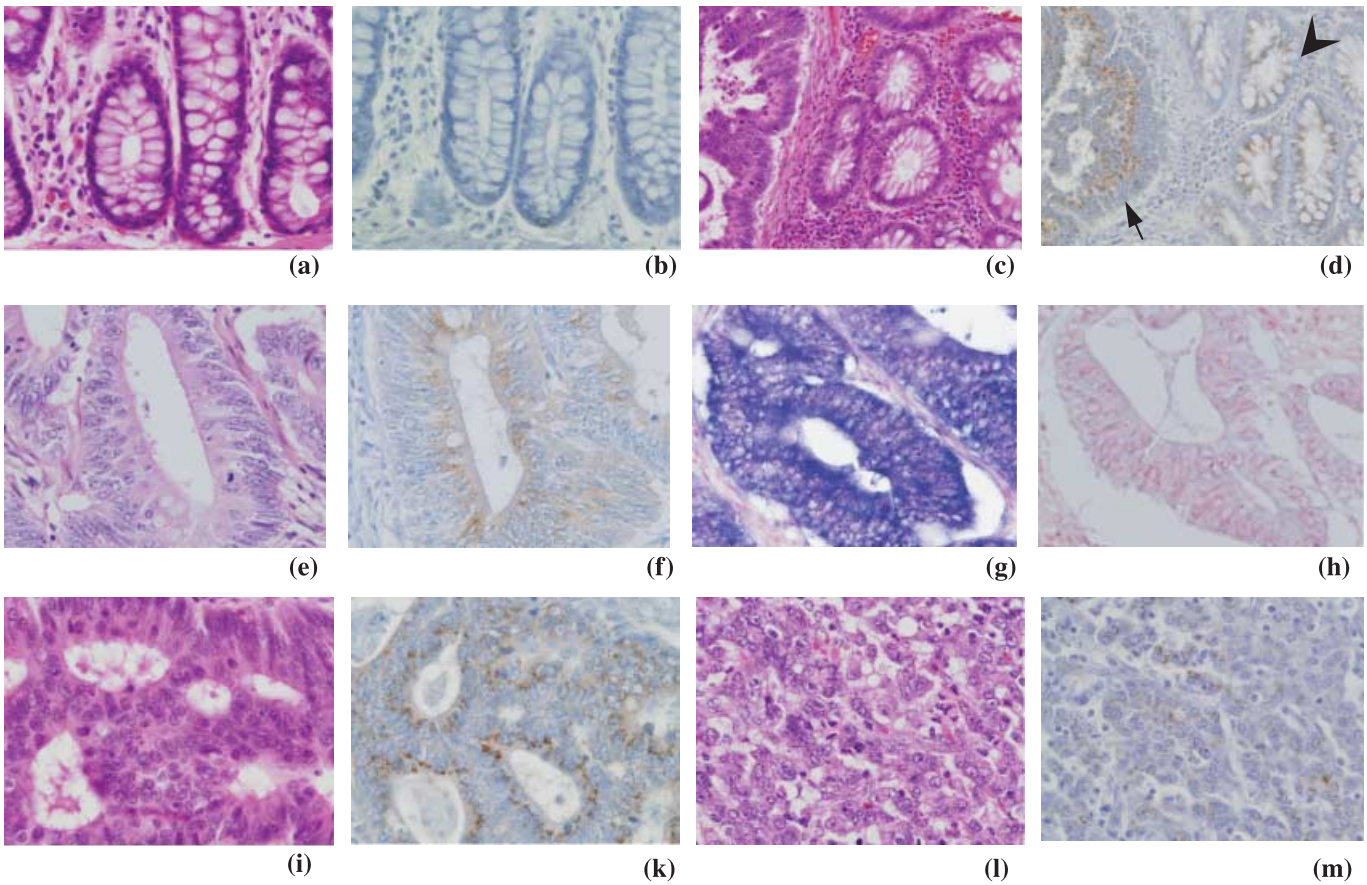

Fig. 2. Representative cases of CYP2A6 staining. (a,b) Normal colonic glandular epithelium. (c,d) Adenocarcinoma and adjacent mucosa. (e–h) Well-differentiated adenocarcinoma. (i,j) Moderately differentiated adenocarcinoma. (k,l) Poorly differentiated adenocarcinoma. (a,c,e,i,k) Hematoxylin–eosin staining. (b,d,f,j,l) Immunohistochemistry for CYP2A6. Carcinoma cells (arrow) and adjacent mucosa (arrowhead) show cytoplasmic granular CYP2A6 expression. (g) *In situ* hybridization for the CYP2A6 antisense probe shows cytoplasmic diffuse staining (blue). (h) *In situ* hybridization for the CYP2A6 sense probe shows no staining.

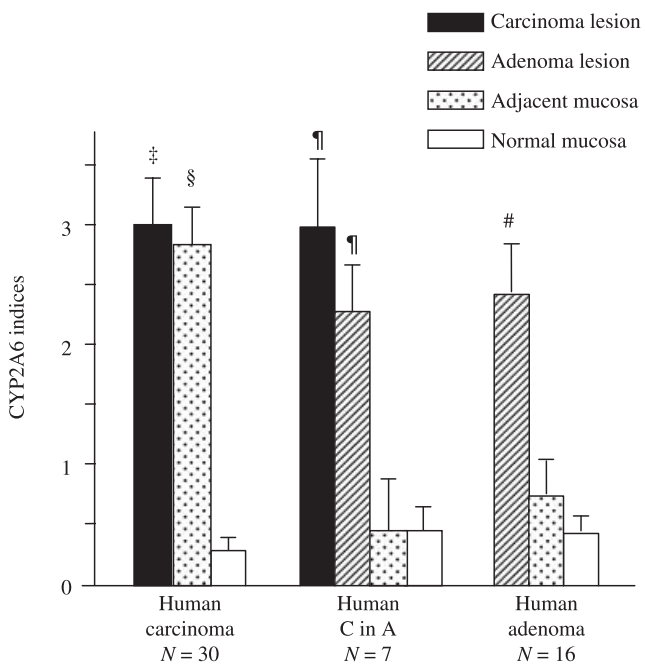

Fig. 3. Summary of CYP2A6 indices. ‡$P < 0.05$ versus normal mucosa of carcinomas. §$P < 0.005$ versus normal mucosa of carcinomas and $P < 0.05$ versus adjacent lesion of carcinomas in adenoma and adenomas. ¶$P < 0.05$ versus adjacent lesion and normal mucosa of carcinomas in adenoma. #$P < 0.05$ versus adjacent lesion and normal mucosa of adenomas.

Discussion

To our knowledge, this is the first report of CYP2A6 expression in human colorectal tumors and normal epithelium identified by immunohistochemistry. Our results showed that human colorectal adenocarcinomas, carcinomas in adenoma, and adenomas had stronger CYP2A6 immunoreactivity than control tissue. Colorectal adenocarcinoma and immediately adjacent mucosa showed stronger immunoreactivity for CYP2A6 than control tissue. This was not evident with carcinoma in adenoma, or in adenoma cases.

Previous studies have shown a relationship between colorectal cancer risk and CYP2A6 activity, measured by the urinary caffeine metabolite ratio.[27,28] Therefore, CYP2A6 in colon tissue might have a relationship with tumorigenesis or sensitivity to carcinogens. In the present study, carcinoma lesions and adenoma lesions had similar levels of CYP2A6 overexpression, and CYP2A6 immunoreactivity showed no relationship to tumor stage. These results suggest that CYP2A6 might have an important role in the early phase of colorectal tumorgenesis rather than in colorectal tumor progression.

The finding that non-neoplastic epithelium adjacent to colon carcinomas, but not adenomas, showed CYP2A6 overexpression requires comment. It could be a diffusion artifact, given the pronounced elevation in the malignant tumors. Alternatively: (1) non-neoplastic cells adjacent to human carcinoma cells may have properties resembling human carcinoma cells; or (2) CYP2A6 overexpression in adjacent mucosa may play an important role in the microenvironment surrounding the colon carcinoma, especially in tumor progression.

doi: 10.1111/j.1349-7006.2007.00572.x

Increased CYP2A6 immunoreactivity means increased expression of enzyme protein. A recent study indicated that genetic polymorphisms of CYP2A6 affect enzymatic activity,[20] so strong CYP2A6 immunoreactivity in the present colorectal tumor possibly reflects CYP2A6 genetic alteration, possibly impacting on protein turnover.

Previously, we reported that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung adenomas are suppressed strongly by pretreatment with a potent human CYP2A6 inhibitor.[30,31] In the present study colon carcinomas showed CYP2A6 expression, so this might be a molecular target for treatment, especially in advanced carcinoma cases.

In conclusion, we here revealed CYP2A6 immunoreactivity of human colorectal adenocarcinoma, carcinoma in adenoma and adenoma. The data support possible roles for CYP2A6 probably in colorectal tumorigenesis and progression, and show that it might be a good candidate therapeutic and chemopreventive target.

Acknowledgments

We thank Mr T. Shirai and Ms J. Iwai, Takinomiya General Hospital, for preparation of tissue sections.

References

1 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759–67.
2 Miller EC, Miller JA. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. *Cancer* 1981; **15**: 2327–45.
3 Sinha R, Kulldorff M, Chow WH, Denobile J, Rothman N. Dietary intake of heterocyclic amines, meat-derived mutagenic activity, and risk of colorectal adenomas. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 559–62.
4 Sachse C, Smith G, Wilkie MJ *et al*. A pharmacogenetic study to investigate the role of dietary carcinogens in the etiology of colorectal cancer. *Carcinogenesis* 2002; **23**: 1839–49.
5 Norat T, Riboli E. Meat consumption and colorectal cancer: a review of epidemiologic evidence. *Nutr Rev* 2001; **59**: 37–47.
6 Knekt P, Jarvinen R, Dich J, Hakulinen T. Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: a follow-up study. *Int J Cancer* 1999; **80**: 852–6.
7 Ding X, Kaminsky LS. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol* 2003; **43**: 149–73.
8 Windmill KF, McKinnon RA, Zhu X, Gaedigk A, Grant DM, McManus ME. The role of xenobiotic metabolizing enzymes in arylamine toxicity and carcinogenesis: functional and localization studies. *Mutat Res* 1997; **376**: 153–60.
9 Oyama T, Kagawa N, Kunugita N *et al*. Expression of cytochrome P450 in tumor tissues and its association with cancer development. *Front Biosci* 2004; **9**: 1967–76.
10 Murray GI, Taylor MC, McFadyen MC *et al*. Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res* 1997; **57**: 3026–31.
11 Murayama N, Sai K, Nakajima Y *et al*. Expression of CYP2A6 in tumor cells augments cellular sensitivity to tegafur. *Jpn J Cancer Res* 2001; **92**: 524–8.
12 Yokose T, Doy M, Taniguchi T *et al*. Immunohistochemical study of cytochrome P450 2C and 3A in human non-neoplastic and neoplastic tissues. *Virchows Arch* 1999; **434**: 401–11.
13 Bergheim I, Bode C, Parlesak A. Distribution of cytochrome P450 2C, 2E1, 3A4, and 3A5 in human colon mucosa. *BMC Clin Pharmacol* 2005; **5**: 4.
14 Bergheim I, Bode C, Parlesak A. Decreased expression of cytochrome P450 protein in non-malignant colonic tissue of patients with colonic adenoma. *BMC Gastroenterol* 2005; **5**: 34.
15 Kiss I, Sandor J, Pajkos G, Bogner B, Hegedus G, Ember I. Colorectal cancer risk in relation to genetic polymorphism of cytochrome P450 1A1, 2E1, and glutathione-S-transferase M1 enzymes. *Anticancer Res* 2000; **20**: 519–22.
16 Bigler J, Whitton J, Lampe JW, Fosdick L, Bostick RM, Potter JD. CYP2C9 and UGT1A6 genotypes modulate the protective effect of aspirin on colon adenoma risk. *Cancer Res* 2001; **61**: 3566–9.
17 Slattery ML, Samowtiz W, Ma K *et al*. CYP1A1, cigarette smoking, and colon and rectal cancer. *Am J Epidemiol* 2004; **160**: 842–52.
18 Hagiwara T, Kono S, Yin G *et al*. Genetic polymorphism in cytochrome P450 7A1 and risk of colorectal cancer: the Fukuoka Colorectal Cancer Study. *Cancer Res* 2005; **65**: 2979–82.
19 Murtaugh MA, Sweeney C, Ma KN, Caan BJ, Slattery ML. The CYP1A1 genotype may alter the association of meat consumption patterns and preparation with the risk of colorectal cancer in men and women. *J Nutr* 2005; **135**: 179–86.
20 Fukami T, Nakajima M, Higashi E *et al*. Characterization of novel Cyp2a6 polymorphic alleles (Cyp2a6*18 and Cyp2a6*19) that affect enzymatic activity. *Drug Metab Dispos* 2005; **33**: 1202–10.
21 Kushida H, Fujita K, Suzuki A *et al*. Metabolic activation of N-alkylnitrosamines in genetically engineered *Salmonella typhimurium* expressing CYP2E1 or CYP2A6 together with human NADPH-cytochrome P450 reductase. *Carcinogenesis* 2000; **21**: 1227–32.
22 Hughes R, Cross AJ, Pollock JR, Bingham S. Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation. *Carcinogenesis* 2001; **22**: 199–202.
23 Chao A, Thun MJ, Jacobs EJ, Henley SJ, Rodriguez C, Calle EE. Cigarette smoking and colorectal cancer mortality in the cancer prevention study II. *J Natl Cancer Inst* 2000; **92**: 1888–96.
24 Ariyoshi N, Miyamoto M, Umetsu Y *et al*. Genetic polymorphism of CYP2A6 gene and tobacco-induced lung cancer risk in male smokers. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 890–4.
25 Miyamoto M, Umetsu Y, Dosaka-Akita H *et al*. CYP2A6 gene deletion reduces susceptibility to lung cancer. *Biochem Biophys Res Commun* 1999; **261**: 658–60.
26 Saoo K, Takeuchi H, Matsuda Y *et al*. Expression of CYP2A in the tissue of human lung cancer. *Cancer Sci* 2004; **95** (Suppl): 346–7.
27 Nowell S, Coles B, Sinha R *et al*. Analysis of total meat intake and exposure to individual heterocyclic amines in a case-control study of colorectal cancer: contribution of metabolic variation to risk. *Mutat Res* 2002; **506–507**: 175–85.
28 Sweeney C, Coles BF, Nowell S, Lang NP, Kadlubar FF. Novel markers of susceptibility to carcinogens in diet: associations with colorectal cancer. *Toxicology* 2002; **181–182**: 83–7.
29 Hamilton SR, Aaltonen LA. World Health Organization classification of tumors. *Pathology and Genetics of Tumors of the Digestive System*. France: IARCPress, 2000: 103–43.
30 Takeuchi H, Saoo K, Yokohira M *et al*. Pretreatment with 8-methoxypsoralen, a potent human CYP2A6 inhibitor, strongly inhibits lung tumorigenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in female A/J mice. *Cancer Res* 2003; **63**: 7581–3.
31 Takeuchi H, Saoo K, Matsuda Y *et al*. Dose-dependent inhibitory effects of dietary 8-methoxypsoralen on NNK-induced lung tumorigenesis in female A/J mice. *Cancer Lett* 2006; **234**: 232–8.