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QUARTERLY

Cytochrome P4502C9 genotype in Southeast Anatolia and possible relation with some serum tumour markers and cytokines

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Substrates for CYP2C9 include fluoxetine, phenytoin, warfarin, losartam and numerous nonsteroidal anti-inflammatory drugs. Polymorphisms in the coding region of the *CYP2C9* gene produce variants at amino-acid residues 144 Arg/Cys and 359 Ile/Leu of the CYP2C9 protein. Individuals homozygous for Leu359 have markedly diminished metabolic capacities for most CYP2C9 substrates, the frequency of this allele is, however, rather low. Consistently with the modulation of enzyme activity by genetic and other factors, wide interindividual variability occurs in the elimination and/or dosage requirements of prototypic CYP2C9 substrates. The polymorphic enzyme CYP2C9 takes part in the metabolism of alkylating agents and polycyclic aromatic hydrocarbons like benzo(*a*)pyrene, a carcinogen present in tobacco smoke. Although the impact of impaired enzyme activity in metabolism of carcinogens and procarcinogens has not been fully defined, an association of *CYP2C9* variant alleles to DNA adduct levels in lung tissues as well as to lung cancer risk have been reported.

In this study 64 healthy subjects (44M/22F) were analysed for *CYP2C9* genotype with PCR-RFLP and for serum carcinoembryonic antigen (CEA), α-fetoprotein (AFP), CA 19-9, CA 15-3, ferritin, IL-6, IL-8 concentrations by chemiluminescence or electrochemiluminescence methods.

***CYP2C9*1* was found to be the most prevalent allele and *CYP2C9*1/CYP2C9*1* was the most frequent genotype represented in 64% of the population in southeastern Anatolia (Gaziantep). Although slight differences in serum tumour marker and cytokine concentrations were observed for *CYP2C9* genotypes the differences were statistically insignificant ($P > 0.05$).**

This could be due to the complexity of the role of *CYP2C9* in benzo(*a*)pyrene metabolism as well as from other contributing factors like interindividual variability of diverse enzymes participating in the same metabolic pathway, unequal expression of

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Abbreviations: AFP, α-fetoprotein; CEA; carcinoembryonic antigen; IL, interleukin.

the variant alleles and differences in exposure to carcinogens. However, determination of CYP2C9 phenotypes in a larger group of subjects might clarify these slight differences.

Interindividual variability in the activity of enzymes involved in biotransformation of xenobiotics may affect metabolic response to environmental carcinogens and indirectly predicts cancer risk. Genetic polymorphism of cytochrome P-450 (CYP)1A1, CYP2D6, CYP2E1, *N*-acetyltransferase (NAT)1, NAT2, glutathione-*S*-transferase (GST)M1, GSTT1 and GSTP1 are associated with susceptibility to malignant diseases such as lung, bladder, hepatocellular and colorectal cancers [1].

Cytochrome P-4502C9 (CYP2C9) metabolises a wide variety of clinically important drugs including phenytoin, warfarin, tolbutamide and a large number of nonsteroidal anti-inflammatory drugs [2]. It also appears to play a role in the metabolism of polycyclic aromatic hydrocarbons to mutagenic diol epoxides, like benzo(*a*)pyrene a carcinogen present in tobacco smoke. To date six different human *CYP2C9* cDNA sequences have been reported but only three appear to be alleles: *CYP2C9*1* the wild type, *CYP2C9*2* with residue 144 Arg/Cys and *CYP2C9*3* with residue 359 Ile/Leu substitution [2–4]. Variant alleles result in altered metabolic clearance of therapeutic agents as well as of natural substrates [5–7]. Recently an association between levels of carcinogen–DNA adducts and *CYP2C9*3* variant allele was reported in normal bronchial tissues of patients with malign and benign pulmonary diseases [3]. A slightly increased or decreased risk of lung cancer has also been suggested to be associated with the *CYP2C9*2* and *CYP2C9*3* variant alleles, respectively, in a population in Los Angeles County (California) [6, 8].

Tumour markers have gained wide clinical acceptance for monitoring progression of malignant disease and its response to therapy. Among them, carcinoembryonic antigen (CEA), α-fetoprotein (AFP), CA19-9, CA 125, CA 15-3 and ferritin are of the highest value in diagnosis and management of colorectal, hepatocellular, pancreas, ovarian, breast and hepatocellular carcinoma, respectively [9–13]. Elevation of CEA, CA 125 and CA 15-3 are applied for management of lung carcinoma, as well [9, 10, 14, 15]. A variety of factors like smoking, menstrual cycle and age have been suggested to contribute to the interindividual or intraindividual variability of tumour markers [16–19]. Recently we have demonstrated an association of *NAT2* genotypes with higher colorectal malignancy incidence (rapidly acetylating *4/5B* and *4/6A* genotypes) and higher serum CEA levels suggesting that xenobiotic metabolising enzyme genotypes influence tumour marker levels [20]. However, the possible effect of genotypes of other xenobiotic metabolising enzymes has not been investigated yet.

Although cytokines do not fulfil the classical criteria for tumour markers, IL-6 inhibits the growth of a variety of human cell lines derived from malignant tumours of breast and ovary. Conversely, it is a potent growth factor for plasmocytoma and myeloma cells suggesting its possible role in regulation of tumour cell growth and differentiation. IL-8, an integral component of host defence as a chemoattractant molecule, has not been fully investigated for its function as a pathogenic mediator in cancer [21].

The aim of the present study is to determine, as a part of an ongoing study, the frequencies of *CYP2C9* alleles and genotypes in south-eastern Anatolia (Gaziantep) and investigate whether an individual's *CYP2C9* genotype has an impact on concentrations of some serum tumour markers and cytokines.

MATERIAL AND METHODS

Subjects. In this study 64 unrelated healthy adult subjects (44M/22F) with a mean age of 26.4 years (range: 17–62) from south-eastern

Anatolia (Gaziantep, Turkey) were studied after their informed consent according to the Helsinki declaration as revised in 1996. All subjects were outpatients without any malign or benign disease known to affect tumour marker or cytokine concentrations like hepatitis, endometriosis, inflammatory bowel disease, acute or chronic infection, autoimmune diseases, psoriasis, pulmonary and pleural diseases etc. [10, 13, 21] as confirmed by clinical and laboratory assessment. None of them regularly consumed alcohol but 26 were tobacco smokers. None of the female subjects was either postmenopausal or menstruating at the time of sample collection [22].

Serum tumour marker and cytokine analysis. Blood samples were collected using a standard venipuncture technique between 9:30–11:00 a.m. Serum was separated by centrifugation of blood at 4°C, 1500 × ***g*** for 10 min and stored at –20°C until analysis which was performed on the same run in order to avoid inter-run analytical variation. Serum CEA, AFP, CA 125 and ferritin levels were determined by electrochemiluminescence immunoassay method enhanced with magnetic capture on an Elecsys 2010 Immunoassay Analyser (Boehringer Mannheim-Roche®, Germany). Serum CA 19-9, CA 15-3, IL-6 and IL-8 levels were determined by chemiluminescence immunometric assays on an Immulite® Immunoassay Analyser (Immulite GI-MA®, BR-MA®, IL-6®, IL-8® assays, DPC, LA, CA, U.S.A.). Control sera Precicontrol® (Boehringer Mannheim-Roche®, Germany) for CEA, ferritin, BRCO® for CA15-3, LGIC® for CA19-9 and LILCM® for IL-6, IL-8 assays (DPC, LA, CA, U.S.A.) were included in each analytical run. Intraassay and interassay precision performance of the assays was determined on ten replicates in a single run and in ten different runs, respectively, from quality control data of the laboratory. The CVs obtained were within 3.0–9.5% range.

Genotyping and identification of* CYP2C9 *mutations. Venous blood samples, 5–10 ml, drawn in EDTA as anticoagulant was obtained from each subject. DNA was extracted from leukocytes manually by 3-step phenol/chloroform extraction. DNA samples were dissolved in 10 mM Tris/1 mM EDTA, pH 8.0, and stored at –4°C until PCR analysis. *CYP2C9* exon 3 mutation 432 C/T which is responsible for amino-acid change 144 Arg/Cys was detected by RCR-RFLP as described earlier using primers CLI (5′-CACTGCTGAAAGAGCTAACAGAG) and CR1 (5′-GTGATATGGAGTAGGGTCACCCAC) to amplify a 372-bp amplicon in a 50 μl PCR mix comprising 10 mM Tris/HCl, pH 8.3, 1.25 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 0.2 μM each of the primers, 2.5 U Taq polymerase (AmpliTaq^{tm}, Perkin Elmer), and 1 μl of genomic DNA. PCR was performed with an initial denaturation for 2 min at 97°C followed by 37 cycles of 30 s at 94°C, 10 s at 60°C, 1 min at 72°C and a terminal extension for 7 min at 72°C. For 144 Arg/Cys detection, 20 μl of PCR product was digested overnight with restriction endonuclease *Sau*961 (New England Biolabs, Schwalbach, Germany), analysed by 3% agarose gel electrophoresis and documented with a video system. Wild type alleles (Arg) were cut into fragments of 179, 119 and 74 bp, whereas mutant alleles (Cys) showed fragments of 253 and 119 bp by loss of one restriction site. Exon 7 mutation 1077 A/T which codes for the amino-acid change 359 Ile/Leu was detected by a PCR-RFLP assay using primers C5 (5′-AGGAAGAGATTGAACGTGTGA) and C6 (5′-GGCAGGCTGGTGGGGAGAAGGCCAA). PCR conditions were the same as described above. A 130-bp amplicon was digested with StyI (New England Biolabs); wild type alleles (Ile) remained uncut, but mutant alleles (Leu) were cleaved into two fragments of 104 and 26 bp. As a control in each assay series a DNA sample known to be positive for StyI restriction site was included to confirm the activity of the respective batch of StyI 23.

Data analysis. Data are usually presented as mean ± standard error of the mean (S.E.M.). Comparison of tumour marker and

cytokine levels of *CYP2C9* genotypes was performed by Kruskal Wallis analysis. Two tailed P values < 0.05 were considerate significant. Statistical analyses were performed with software SSPS^{TM} for Windows (SPSS Inc., Chicago, U.S.A.) statistical program.

RESULTS

Frequencies of *CYP2C9* alleles

The frequencies of *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3* alleles in the population examined from southeast of Anatolia (Gaziantep, Turkey) were 0.79, 0.10 and 0.11, respectively. Subjects homozygous for **1* allele (**1/*1*) constituted 64%, heterozygous for **2* allele (**1/*2*) 14%, heterozygous for **3* allele (**1/*3*) 16%, homozygous for **2* allele (**2/*2*) 3%, homozygous for **3* allele (**3/*3*) 3% of the population studied (Table 1).

Serum tumour marker and cytokine concentrations in *CYP2C9* genotypes

Subjects with **1/*2* and **1/*3* genotypes had the highest (1.6±0.3 and 1.6 ± 0.4 ng/ml) while **2/*2* had the lowest (1.1 ± 0.8 ng/ml) serum CEA levels, but the differences between groups were not significant (P > 0.05). The highest and the lowest serum AFP levels were observed in subjects homozygous for **2* and **3* alleles (2.8 ± 0.6 and 1.2 ± 0.3, respectively) the difference being statistically insignificant (P > 0.05). For CA 19-9, serum levels were relatively close except for the **2/*2* genotype with values lower (12.3 ± 5.5, P > 0.05) than in the other groups. Serum CA 125, CA 15–3, and ferritin values did not show any obvious difference between groups (P > 0.05). Although statistically insignificant, the lowest IL-6 levels (8.5 ± 1.0 pg/ml) and the highest IL-8 levels (57.7 ± 37.8 pg/ml) were observed in **2/*2* and **1/*2* genotypes, respectively (P > 0.05) (Table 1).

Subjects were grouped for gender and smoking habits. No significant difference (P>0.05) in serum tumour marker and cytokine concentrations for *CYP2C9* genotypes was observed between male/female and smoking/nonsmoking subgroups (not shown).

DISCUSSION

Genetic polymorphism of CYP2C9 has been identified in humans, with marked interracial variations in the frequency of variant alleles. Earlier studies have indicated that differences in CYP2C9 enzyme activity may exist not only between the major races but also among different Caucasian populations. Consequently, further studies on different Caucasian ethnic groups seem to be urgently needed to provide important genotypic data for facilitating a rational approach to evaluation of their impact. The frequencies of *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3* alleles in the population examined from southeast of Anatolia (Gaziantep, Tur-

Table 1. Serum tumour marker and cytokine concentrations of *CYP2C9* genotypes (mean ±S.E.M)

CYP2C9 genotype	**1/*1*	**1/*2*	**1/*3*	**2/*2*	**3/*3*
N (%)	41 (%64)	9 (%14)	10 (%16)	2 (%3)	2 (%3)
CEA ng/ml	1.4 ± 0.1	1.6 ± 0.3	1.6 ± 0.4	1.1 ± 0.8	1.4 ± 0.4
AFP U/ml	2.4 ± 0.4	2.0 ± 0.3	2.5 ± 0.7	2.8 ± 0.6	1.2 ± 0.3
CA 19-9 U/ml	17.4 ± 2.6	20.8 ± 7.0	19.1 ± 8.2	12.3 ± 5.5	21.7 ± 14.2
CA 125 U/ml	15.7 ± 1.2	18.0 ± 2.7	12.5 ± 1.2	11.3 ± 2.1	20.2 ± 6.5
CA 15-3 U/ml	40.5 ± 5.1	43.9 ± 8.6	56.7 ± 12	53.2 ± 26.5	41.6 ± 3.6
Ferritin U/ml	23.5 ± 2.1	36.4 ± 11.8	29.1 ± 5.9	31.7 ± 9.9	23.1 ± 11.8
IL-6 pg/ml	12.6 ± 1.2	16.5 ± 3.3	14.4 ± 4.0	8.5 ± 1.0	11.7 ± 1.7
IL-8 pg/ml	23.1 ± 5.4	57.7 ± 37.8	20.0 ± 7.7	24.5 ± 19.1	19.5 ± 6.0

key) were not significantly different from those of other Caucasian populations [2, 23, 24].

CYP2C family is distributed widely and organ specifically, and is variably expressed in neoplastic and normal tissues [25–27]. Along with CYP1A1 and CYP1A2, CYP2C9 appears to play a role in the oxidative metabolism of benzo(*a*)pyrene, a polycyclic aromatic hydrocarbon carcinogen present in tobacco smoke, thus, it does influence benzo(*a*)pyrene related lung and larynx cancer risk [3, 6, 8, 25]. In spite of low content of CYP2C9 in human lung tissues, a relatively high capacity of benzo(*a*)pyrene activation by cDNA expressed CYP2C9 has been reported. This could be partly explained by higher affinity of CYP2C9 for benzo(*a*)pyrene compared to CYP1A and CYP2E that are most abundant pulmonary CYPs. Experimental studies indicate that proximate carcinogens formed through the metabolism of benzo(*a*)pyrene are transported to target tissues by circulation and that CYP2C9 is likely to influence the metabolism of tobacco smoke polycyclic aromatic hydrocarbon in lungs as well as in liver, where CYP2C9 is most abundantly expressed. *CYP2C9* variant alleles are also associated with altered metabolism of alkylating agents that are well-established mutagens [7, 28].

There is a considerable interest in elucidating the normal function of tumour markers and in determining whether due to altered amount or structure this function is subverted in malignancy. Anti-adhesion properties of the CA 15-3 antigen reducing both cell–cell and cell–extracellular matrix interactions evoke the possibility that this antigen could promote metastasis. The antigenic determinant for CA19-9, sialyl Le^a, is suggested to be involved in the hematogenous metastasis by the E-selectin mediated binding of tumour cells to the endothelium. We have recently found an association between *NAT2* genotypes and serum CEA levels suggesting a relationship between xenobiotic metabolising enzyme genotypes and tumour marker levels [20, 29, 30].

In our experiments neither a significant difference in the content of tumour markers or cytokines in any of the *CYP2C9* genotypes, or in the presence of each variant allele was observed. Previous studies have shown that even relatively conservative changes in the amino-acid composition of CYP2C9 may affect both its activity and substrate specificity, or may have no effect on the metabolism of certain substrates. Although *CYP2C9*2* and *CYP2C9*3* alleles have been shown to be associated with impaired metabolic clearance of warfarin, tolbutamide and phenytoin, their metabolic response to natural substrates has not been fully defined [2, 4, 5, 7, 23, 31]. CYP2C9 catalyses both detoxification and activation steps in the metabolism of benzopyrene to ultimate carcinogens thus, the final result of altered enzyme activity remains obscure [3, 6, 8]. Furthermore, differences in expression of variant alleles may affect the metabolism of a particular substrate. Bhasker *et al.* [4] have reported a 5- to 10-fold greater expression of the Cys over Arg 144 allele in some of the heterozygous subjects. In this study the metabolising phenotype of the heterozygous subjects was not considered as the number of homozygous subjects in stratified data was very small. Moreover, many enzymes act as catalysts of a particular substrate altering biological activity of other enzymes by an enhancing, suppressing or synergical effect [2, 32].

Cytokines form a part of mechanisms controlling cellular growth and replication that are damaged in cancer. Some protooncogenes and oncogens code for normal or abnormal components of cytokine receptor or signal transduction pathways. IL-6 has growth inhibitory activity directly on cancer cells of breast and ovary and on myeloid cells; it causes tumour regression and enhances anti-tumour immune effects. An interaction of cytokine and/or chemokine release and *CYP2C9* gene expression in several conditions, e.g. in acute-

phase response and sudden infant death syndrome, has been reported [21, 33–36].

In practice, preanalytical and analytical error that may influence cytokine assays should be considered. Short half-life of cytokines, very low normal circulating levels, high intraindividual variation, presence of circadian rhythm, *in vitro* stimulation of cytokine production by microorganisms, degradation in collection containers, etc. are the main problems experienced in laboratory work that limit our ability to define the exact role of cytokines in pathophysiological conditions [21].

CONCLUSION

According to presented data, *CYP2C9*1* was the most prevalent allele and *CYP2C9*1/CYP2C9*1* was the most frequent genotype representing 64% of the population in southeastern Anatolia. No significant difference between *CYP2C9* genotypes in concentration of serum tumour markers and cytokines investigated was found. However, determination of CYP2C9 phenotypes in a larger study group may be necessary to clarify the observed slight differences.

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