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Human *CYP1A1* Gene: Cosegregation of the Enzyme Inducibility Phenotype and an RFLP

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Summary

The human *CYP1A1* (cytochrome P_1450) gene encodes an enzyme involved in the activation of procarcinogens, such as benzo[a]pyrene, to the ultimate reactive intermediate. Approximately 10% of the human population exhibit high *CYP1A1* inducibility, and Kouri et al. reported that the high-inducibility phenotype might be at greater risk than low-inducibility individuals for cigarette smoke–induced bronchogenic carcinoma. In one 3-generation family of 15 individuals, we show here that the high-*CYP1A1*-inducibility phenotype segregates concordantly with an infrequent polymorphic site located 450 bases downstream from the *CYP1A1* gene. Our findings are consistent with the study of Kawajiri et al., who demonstrated an association between this polymorphism and an increased incidence of squamous-cell lung cancer. Our data suggest that the *CYP1A1* structural gene, or a region near this gene, might be correlated with the inducibility phenotype.

Introduction

Cytochrome P450 enzymes are involved in the oxidative metabolism of endogenous compounds such as steroids, fatty acids, leukotrienes, and prostaglandins and in the metabolism of foreign chemicals such as drugs, carcinogens, and other environmental pollutants (Schuster 1989). The P450 superfamily presently comprises >153 genes in 27 families, 10 of which genes exist in all mammals (Gonzalez and Nebert 1990; Nebert et al. 1991). The human *CYP1* family consists of two functional genes: *CYP1A1,* involved in polycyclic hydrocarbon metabolism, and *CYP1A2,* involved in arylamine metabolism (Nebert and Gonzalez 1987). Both genes are up-regulated (induced) by certain foreign chemicals such as benzo[a]pyrene, 3-methylcholanthrene, β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. As the inducibility of *CYP1A1* increases, so does the metabolism of polycylic hydrocarbon procarcinogens to reactive carcinogenic intermediates; enhanced metabolism often leads to a higher risk of malignancies in the mouse (Nebert 1989). In clinical studies, the *CYP1A1* high-inducibility phenotype has also been correlated with an increased risk of bronchogenic carcinoma, when compared with a control group matched for age, sex, and cigarette-smoking history (Kellermann et al. 1973; Kouri et al. 1982).

The human *CYP1A1* cDNA (Jaiswal et al. 1985*a*) and gene (Jaiswal et al. 1985*b;* Kawajiri et al. 1986) have been cloned, sequenced, and localized to chromosomal 15 near the *MPI* locus (Hildebrand et al. 1985). Several *CYP1A1* RFLP patterns have been described (Jaiswal and Nebert 1986; Bale et al. 1987; Spurr et al. 1987; Haugen et al. 1990). If the *CYP1A1* inducibility phenotype could be easily assessed, this might be helpful in predicting individual risk of cancer and toxicity. Determination of the *CYP1A1* inducibility phenotype by the enzyme assay is very laborious and cumbersome, however, because the procedure requires ≥40 cc of blood, the isolation and culturing of lymphocytes for 3–4 d, and a spectrophotofluorometric assay involving hazardous chemicals (Kouri et al. 1979*a,* 1979*b,* 1982; Jaiswal et al. 1985*b*). In the

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present study of 15 individuals in one 3-generation family, we demonstrate a correlation between the *CYP1A1* enzyme inducibility phenotype and an RFLP pattern at the 3′ end of the *CYP1A1* gene.

Material and Methods

Blood Collection and Lymphocyte Freezing

The sources of all materials have been given elsewhere (Kouri et al. 1979*b,* 1982). Blood samples (≤50 cc) were obtained from each individual by venipuncture on at least two separate occasions. While still at room temperature the blood was mixed with an equal volume of CMF–Hank's balanced salt solution and 2,000 units of heparin, and lymphocytes were isolated via a Ficoll-Hypaque gradient (Kouri et al. 1982) on the same day the blood was drawn. The isolated lymphocytes were frozen to − 90°C at a rate of 1 degree C/min and then were transferred to a liquid-nitrogen freezer.

Lymphocyte Cultures

As needed, the frozen lymphocyte samples were removed from liquid nitrogen and thawed rapidly during a 2-min period in a 37°C water bath. Fresh or previously frozen lymphocytes were then cultured in 95% air:5% CO_2, according to a method described elsewhere (Kouri et al. 1979*b,* 1982), in RPMI 1640 growth medium supplemented with 10% heat-inactivated human AB serum, HEPES buffer, 1% phytohemagglutinin-M, a mixture of penicillin and streptomycin, and 1.5 μM 3-methylcholanthrene as the *CYP1A1* inducer. Cells were collected at 72 and 96 h from the time of culture initiation, when the inducible CYP1A1 enzyme activity is known to be maximal (Kouri et al. 1982). Four flasks were incubated per individual: two for CYP1A1 activity and NADH-cytochrome *c* reductase activity at 72 h and the other two for these enzyme activities at 96 h.

Enzyme Assays

Culture flasks were removed from the incubator after 72 or 96 h, and the cells were collected by centrifugation and were stored at − 70°C until assayed. The assay for lymphocyte benzo[a]pyrene hydroxylase (CYP1A1) activity has been described elsewhere (Nebert 1978). One unit of CYP1A1 activity is defined as that amount of enzyme catalyzing, in 1 min at 37°C, hydroxylated products having the fluorescence equivalent to 1.0 pmol of the recrystallized 3-hydroxybenzo[a]pyrene standard (Nebert 1978). One unit of NADH-cytochrome *c* reductase activity is defined as that amount of enzyme catalyzing, in 1 min at 25°C, the reduction of 1.0 nmol of cytochrome *c* (Kouri et al. 1979*a*). This reductase is known not to be induced by 3-methylcholanthrene (Kouri et al. 1979*a*) and is an accurate measurement of the amount of endoplasmic reticulum, in which the CYP1A1 protein is also embedded. The endoplasmic reticulum appears in these cells at 55–60 h in culture, at which time the lymphocytes have been converted by the mitogen to lymphoblasts (Kouri et al. 1979*b*). The maximal value of CYP1A1 inducibility per unit of reductase activity is known to occur at 72 h in some individuals and at 96 h in others (Kouri et al. 1979*a,* 1979*b,* 1982; Jaiswal et al. 1985*b*).

Isolation of DNA and Probe Preparation

As needed, 5-ml aliquots of blood preserved with citrate (Gustafson et al. 1987) were thawed, and the nuclei were isolated by disruption of the cells in a buffer containing 10 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 250 mM sucrose, and 0.1% Triton X-100 and were centrifuged at 1,000 *g* for 5 min. The nuclei were further purified by sedimentation through 1.0 M sucrose cushions in the same buffer. The nuclei were lysed by a 120-min incubation at 50°C in a buffer containing 10 mM HEPES (pH 8.0), 10 mM EDTA, 0.5% SDS, proteinase K (100 μg/ml), and RNase A (20 μg/ml). Proteins were removed by phenol/chloroform extraction. The aqueous phase was adjusted to 2.0 M ammonium acetate, and 2 vol ethanol was added. The resulting DNA was removed to a new tube with a glass loop and was washed once with 70% ethanol. The DNA was then dissolved in 10 mM Tris (pH 8.0) and 0.1 mM EDTA, and DNA concentrations and purity were estimated by scanning UV spectrophotometry.

Leukocyte DNA (5 μg/lane) was digested to completion with one of a battery of restriction endonucleases, and Southern blot transfer analysis (McBride et al. 1986) was carried out using BioTrace membranes (Gelman). The hybridization probes were prepared by random hexamer–primed synthesis using [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham [1 Ci = 37 GBq]) to generate a specific activity of $1–3 \times 10^9$ cpm/μg DNA (Feinberg and Vogelstein 1983).

Results

Screening for CYP1A1 *Inducibility Phenotype*

A total of 47 unrelated volunteers in the Bethesda,

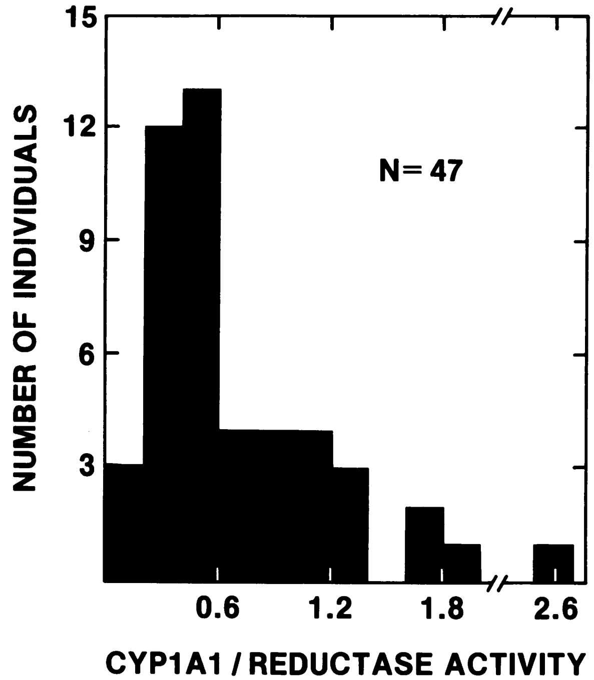

Figure 1 Maximally induced CYP1A1 enzyme activity per unit of NADH-cytochrome *c* reductase activity in mitogen-activated, 3-methylcholanthrene-treated lymphocytes from 47 unrelated individuals. These values represent the average highest activities observed in two or more separate experiments; each individual exhibited <23% variation among the four or more samples tested in each of two or more separate experiments. Environmental factors, such as the number of cigarettes smoked at the time the blood was drawn, do not influence this assay, which specifically determines the inducibility phenotype (Kellermann et al. 1973; Atlas et al. 1976; Kouri et al. 1982).

MD, area were examined for maximally induced CYP1A1 per unit of reductase activity (fig. 1). A similarly skewed-to-the-left distribution has been observed in other studies of normal populations (Kellermann et al. 1973; Kouri et al. 1974, 1982; Atlas et al. 1976). We found four individuals who consistently exhibited CYP1A1/reductase ratios >1.6: two of 19 Caucasians tested, one of 15 Asians (two American Indians, five Chinese, seven Japanese, and one Korean), none of seven African-Americans, and one of six Middle Eastern individuals (two from India, two from Egypt, and two from Lebanon).

Screening for RFLP Patterns

The human *CYP1A1* (Jaiswal et al. 1985*b;* Kawajiri et al. 1986) and *CYP1A2* (Quattrochi et al. 1986; Ikeya et al. 1989) genes each span 6–8 kb, and both map to 15q22-qter (Hildebrand et al. 1985; Bale et al.

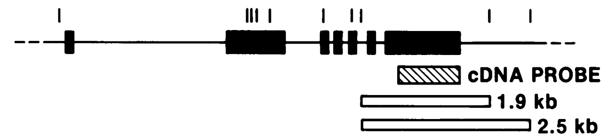

Figure 2 Intron-exon structure of human *CYP1A1* gene (Jaiswal et al. 1985*b;* Kawajiri et al. 1986) and 920-bp *Eco*RI 3′-specific cDNA probe used. Closed rectangles represent the seven exons and the joining line represents introns and flanking regions. One *Msp*I site 101 bases upstream from the transcription initiation site, seven *Msp*I sites inside the gene, and two *Msp*I sites downstream are illustrated at top by vertical lines. The *Msp*I site 450 bases 3′ of the polyadenylation site accounts for the 1.9-kb *Msp* fragment.

1987). Filters of 80 random blood donors' DNAs that had been cleaved with each of 12 restriction endonucleases were probed with *CYP1A1* and *CYP1A2* 5′ flanking probes, one cross-hybridizing 5′ cDNA probe, and 3′-specific cDNA probes from both genes. We found that three restriction endonucleases were helpful in revealing polymorphisms, and all three were detected only with the *CYP1A1* 3′-specific cDNA probe. Allelic frequencies of *Msp*I 2.5- and 1.9-kb fragments were 88% and 12%, respectively, and those of *Eco*RI 14- and 20-kb fragments were 95% and 5%, respectively; combining both polymorphisms, we found haplotype frequencies of 82%, 11%, 6%, and 1% (Bale et al. 1987). We also found a *Pst*I polymorphism in two of 80 individuals (Bale et al. 1987), which has been confirmed recently (Haugen et al 1990).

Population and Family Screening for the CYP1A1 *Inducibility Phenotype and* MspI *Polymorphism*

The *Msp*I polymorphism with the *CYP1A1* 3′-specific cDNA probe detects the 12% allelic frequency of an *Msp*I site 450 bases downstream from the gene (fig. 2). Using eight individuals from the figure 1 data as propositi, we screened 41 individuals in eight families for their *CYP1A1* inducibility phenotype and *Msp*I RFLP pattern. Propositi of seven families (one Chinese, two black, and four Caucasian) had CYP1A1/reductase ratios ≤1.4, and the 26 individuals tested within those families all exhibited both CYP1A1/reductase ratios ≤1.4 and homozygosity for the 2.5-kb *Msp*I fragment. These seven families are thus regarded as uninformative.

One of the four individuals with a CYP1A1/reductase ratio ≥1.6 (fig. 1) was the propositus of an eastern Mediterranean family. When 15 individuals from this 3-generation family were examined for this *Msp*I poly-

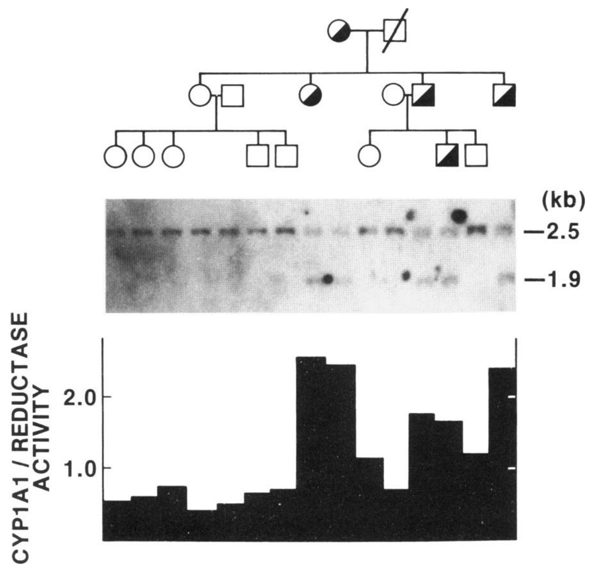

Figure 3 Segregation of 1.9-kb *Msp*I fragment with increased *CYP1A1* inducibility among 15 members of one 3-generation family. The half-blackened circles and half-blackened squares denote heterozygote females and males, respectively. The individual in lane 9 (the grandmother) is included in the fig. 1 histogram, at the far right.

morphism, presence of the 1.9-kb fragment occurred only in those five individuals having a CYP1A1/reductase ratio ≥1.6 (fig. 3). It is interesting that all five individuals were heterozygotes.

Discussion

The present report demonstrates segregation of the high-CYP1A1-inducibility phenotype and the *Msp*I 1.9-kb fragment detected by the *CYP1A1* 3′-specific cDNA probe. We found seven informative meioses in one family and no individuals homozygous for the 1.9-kb allele. Kawajiri et al. (1990) recently reported genotype frequencies of .49 for the *Msp*I 2.5-kb/2.5-kb homozygote, .40 for the heterozygote, and .11 for the 1.9-kb/1.9-kb homozygote among 104 Japanese. This distribution represents a 31% allelic frequency of the *Msp*I fragment in the Japanese population studied, whereas we found a 12% allelic frequency among 80 blood bank samples. These findings suggest that the allelic frequency of the 1.9-kb fragment might be two to three times higher in Japan than in the population that we studied. Although these authors did not measure *CYP1A1* expression in cultured lymphocytes, they did discover among lung cancer patients that the frequency of the 1.9-kb/1.9-kb homozygote was about threefold higher than that among noncancer patients. This observation would be compatible with the hypothesis that greater *CYP1A1* inducibility might be associated with increased risk of cigarette smoke–induced lung cancer (Kellermann et al. 1973; Kouri et al. 1982). This observation is also consistent with the present study, in which we have shown that the 1.9-kb *Msp*I allele segregates with high *CYP1A1* inducibility in one 3-generation family.

Most studies characterizing expression of the mouse, rat, and human *CYP1A1* gene have located regulatory response elements between 1,100 bases upstream of the transcription initiation site and exon 1, as well as in the first intron (Hines et al. 1988; Nebert and Jones 1989). The *CYP1A1* induction process is believed to require interaction of the inducer-receptor complex with the *a*romatic *h*ydrocarbon (Ah)–responsive element (Denison et al. 1988; Fujisawa-Sehara et al. 1988; Nebert and Jones 1989). Mice having allelic differences in the Ah receptor gene—a regulatory gene controlling *CYP1A1* inducibility—exhibit high- and low-affinity Ah receptor proteins and different risks of polycyclic hydrocarbon–caused toxicity and malignancies (Nebert 1989). It is expected that allelic differences in the human Ah receptor gene might also provide information about the high- and low-*CYP1A1*-inducibility phenotype (Nebert 1988). The present study suggests, however, that either the *CYP1A1* gene itself or some element tightly linked to it might be the cause of the high-inducibility phenotype in this family.

The *Msp*I 1.9-kb fragment is the result of an *Msp*I site 450 bases downstream from the polyadenylation sites in the 3′ end of exon 7 of the *CYP1A1* structural gene (fig. 2). How might the presence of this *Msp*I site up-regulate the inducibility of the *CYP1A1* gene? Regulatory genes encoding factors that affect *CYP1A1* transcription (e.g., the Ah receptor) do not appear to be linked to the *CYP1A1* structural gene. One possible explanation is that transcription might run through the *Msp*I site 450 bases downstream from the polyadenylation sites; if this is the case, there are several feasible mechanisms that could affect *CYP1A1* inducibility (e.g., either rate of polyadenylation or stabilization of the transcript). Further studies are underway to explore these mechanisms.

Another possible explanation would be linkage disequilibrium (Hill and Robertson 1968; Chakravarti et al. 1984; Leitersdorf et al. 1989), where there might exist an important regulatory region that includes the *Msp*I site located 450 bases downstream from exon 7.

This putative regulatory region would be physically so close to the *CYP1A1* gene that the chance for recombination would be extremely infrequent. The presence of a mutated *Msp*I site might even be directly responsible for the impaired *CYP1A1* inducibility, because of the increased (or decreased) ability of regulatory protein(s) to bind to this region. Further population and family studies will be necessary to test this hypothesis.

The enzyme assay for determination of the inducibility phenotype is a laborious procedure requiring 40 cc of blood and 4 d of mitogen-activated lymphocyte cultures (Kouri et al. 1982; Jaiswal et al. 1985*b*). A noninvasive RFLP screening test, such as a study of PCR-amplified DNA from buccal mucosal cells (Lench et al. 1988), in combination with linkage analysis in each family, might be helpful in determining the *CYP1A1* inducibility phenotype. Such tests might be useful in the future, for predicting and possibly avoiding individual risk of environmentally caused malignancy or toxicity caused by cigarette smoke and other combustion products.

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