Influence of the Cyp1B1 L432V Gene Polymorphism and Exposure to Tobacco Smoke on Cyp1B1 mRNA Expression in Human Leukocytes

Simone Helmig, Bahar Hadzaad,1 Juliane Döhrel, and Joachim Schneider
Institut und Poliklinik für Arbeits- und Sozialmedizin, Justus-Liebig-Universität, Giessen, Germany

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ABSTRACT:
Cytochrome P450 1B1 (CYP1B1), a phase I enzyme, is involved in the activation of a broad spectrum of procarcinogens. An association of the Cyp1B1 L432V polymorphism with diverse types of cancer, as well as an impact on the catalytic activity of the enzyme, has been described. To show the functional impact of the allelic variant Cyp1B1*3, we investigated the quantitative Cyp1B1 mRNA expression in a population of smokers, nonsmokers, and ex-smokers and determined their genotypes. Detection of the L432V polymorphism in exon 3 of the Cyp1B1 gene was performed by rapid capillary polymerase chain reaction (PCR) with melting curve analysis. For qualitative comparison of Cyp1B1 mRNA levels, realtime PCR was performed using SYBR Green fluorescence in a LightCycler system. Calculations of expression were made with the 2−ΔΔCT method. In comparing relative Cyp1B1 mRNA expression, highly significant differences between the two homozygote genotypes *1/*1 and *3/*3 (0.185 ± 0.027, n = 118 versus 0.071 ± 0.013, n = 56; p = 0.000), as well as between the heterozygote genotype *1/*3 and the homozygote genotype *3/*3 (0.178 ± 0.025, n = 171 versus 0.071 ± 0.013, n = 56; p = 0.000), were revealed. Significant differences between the genotypes were also detected within the subgroups of smokers, nonsmokers, and ex-smokers. No significant differences were determined in comparing the relative Cyp1B1 mRNA expression with regard to tobacco smoke exposure. Our results suggest that genotypes carrying the C allele (*1/*1 and *1/*3) at Cyp1B1 L432V polymorphism have a significantly higher Cyp1B1 mRNA expression compared with the genotype without the C allele (*3/*3). Gene expression of Cyp1B1 mRNA cannot be used as a biomarker for exposure of tobacco smoke.

To have a carcinogenic effect most environmental compounds require a metabolic initiation to reactivate electrophilic intermediates by the cytochrome P450 (P450) monooxygenase system (Bartsch et al., 1997). CYP1B1 is a phase I enzyme that belongs to a multigene superfamily of monomeric mixed-function monoxygenases (Sutter et al., 1994). It is involved in the activation of a broad spectrum of procarcinogens, for example, components of tobacco smoke such as polycyclic aromatic hydrocarbons (PAHs). The expression of the Cyp1B1 gene is induced through the aromatic hydrocarbon receptor (AhR), which is activated by PAHs (Furukawa et al., 2004). Because Cyp1B1 expression can easily be determined in peripheral blood lymphocytes, it is often promoted as a biomarker of exposure to environmental AhR agonists (van Duursen et al., 2005b).

CYP1B1 is expressed in many normal human tissues, including peripheral blood cells (Hanaoka et al., 2002). In addition, Cyp1B1 is found to be overexpressed in a variety of human tumors (Murray et al., 1997).


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ABBREVIATIONS: P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; AhR, aromatic hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PCR, polymerase chain reaction; B2M, β2-microglobulin.
incidence of head and neck squamous cancer (Ko et al., 2001), lung cancer susceptibility (Wenzlaff et al., 2005), renal cancer (Sasaki et al., 2004), and colorectal cancer (Fritsche et al., 1999) has been reported. The ability to identify individuals at highest risk of cancer is important for cancer control strategies. The fact that 20% of lung cancer cases occur in nonsmokers is often attributed to a combination of genetic factors (Gorlova et al., 2007). Therefore, we investigate the functional impact of the allelic variant Cyp1B1*3 on quantitative Cyp1B1 mRNA expression in a population of smokers, nonsmokers, and ex-smokers in relation to their Cyp1B1 genotypes.

Materials and Methods

Subjects. The study population consisted of 365 whites. Volunteers were personnel of the Universitätsklinikum Giessen and Marburg GmbH, students of the Justus-Liebig-Universität Giessen, and patients recruited at the Poliklinik of the Institute for Arbeits- and Sozialmedizin, Giessen, Germany.

All of the subjects included in this study were interviewed using a questionnaire to obtain information on lifestyle, including smoking habits, alcohol consumption, indoor air pollution (coal heating and cooking), eating habits, lifetime occupational history, and disease history. The group comprised 271 males (mean age, 56 years; range, 20–91 years) and 93 females (mean age, 40 years; range, 16–82 years). The subgroup smokers (n = 107) was defined as people who smoke daily; nonsmokers (n = 103) were defined as people who had never smoked; and ex-smokers (n = 155) were defined as people who had quit smoking at least 3 months ago. The ethics committees of the University Hospital, Giessen, Germany approved the study.

Real-Time Polymerase Chain Reaction and Polymorphism Detection. Blood samples (approximately 3 ml) were obtained from all the subjects. Genomic DNA was isolated from whole blood using the versaGene DNA Blood samples (approximately 3 ml) were obtained from all the subjects. The ethics committees of the University/H11032 probed. The primers Cyp1B1-forward (5'-GAAATAAGAATTTTGCTCACT-... many) synthesized the PCR primers and the fluorescent-labeled detection Probes (Roche Diagnostics), primers Cyp1B1-forward (0.55 M), sensor (0.275 M), anchor (0.275 M), MgCl2 (3 mM), and 25 ng of genomic DNA in a 10-μl reaction mixture. PCR contamination was checked by the inclusion of a negative control. PCRs were initially denatured at 95°C for 10 min and subjected to 50 cycles of amplification with 5 s of denaturing at 95°C, 20 s of annealing at 56°C, and 35 s of extension at 72°C. The melting curve was achieved by 2 s at 95°C, 30 s at 40°C, and 1 s at 90°C, 0.1°C/s.

**RNA Extraction and Reverse Transcription.** White blood cells were isolated from 10 ml of peripheral blood using Ficoll (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as described by the manufacturer. Total RNA was isolated from white blood cells using a commercial RNA isolation reagent (TRI Reagent; Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Isolated RNA was resuspended in 10 μl of RNase-free water. Each sample was treated twice with 2 μl of RNase-free DNase, 1 unit/μl (QIAGEN, Valencia, CA), for 10 min at 37°C to eliminate remaining DNA. The prepared RNA was reverse-transcribed to synthesize cDNA. Reverse-transcriptase reactions were performed for 1 h at 37°C in a final volume of 10 μl using 1 μg of RNA, 500 ng of oligo(dT)15 (Promega, Mannheim, Germany), 10 mM dNTPs (Fermentas Inc., Glen Burnie, MD), 8 units of RNasin (Promega), and 3 units of AMV Reverse Transcriptase and 1× AMV Reverse Transcriptase Reaction buffer (Promega).

**Real-Time PCR.** For quantitative comparison of Cyp1B1 mRNA levels real-time PCR was performed using SYBR Green fluorescence in a LightCycler System (Roche Diagnostics). After optimization of PCR conditions, amplification efficiency was tested in standard curves using serial cDNA dilutions. The correlation coefficient had to be greater than 0.9 and the slope around ~3.5. Amplification specificity was checked using melting curves. Both negative and positive controls were included in each PCR reaction. All the assays were carried out three times as independent PCR runs for each cDNA sample. Gene expression was always related to expression of β-2-microglobulin (B2M) as housekeeping gene, which is known to be a good choice for normalization of the expression levels of leukocytes (Vandesompele et al., 2002). Calculations of expression levels were performed with the 2-ΔΔCT method according to Pfaffl et al. (2001). The gene expression levels were rounded to three decimal places. The sequence of the specific primers for CYP1B1 and B2M was CYP1B1-forward (5'-AACGGCAACTTCTGCA-GCACTT-3') and CYP1B1-reverse (5'-CTTAGAGGTTCTTCCGCAAATG-3') amplify a 441-base pair fragment of the CYP1B1 gene which contains codon 432 containing the sequences CTG or GTG, coding for leucine or valine, respectively. The fluorescein-labeled probe (ATGACCCACTGAAGTGACCTA-PH) bind to nucleotides 8123 through 8147 of Cyp1B1 L432V polymorphism and Cyp1B1 mRNA expression

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Homozygote Wild-Type</th>
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<tr>
<td>Amino acids</td>
<td>Cyp1B1*1/*1 GCC/Leu</td>
<td>Cyp1B1*1/*3</td>
<td>Cyp1B1*3/*3 G/G</td>
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<tr>
<td>Nucleotides</td>
<td>GCC/Leu</td>
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**Statistical Analysis.** Smokers were considered current smokers at the time of blood withdrawal. Ex-smokers were all the individuals who were previously smokers but had quit smoking at least 3 months before blood withdrawal. Information was collected on the usual number of cigarettes smoked per day, the age at which the subject started smoking, and, if the person was an ex-smoker, the age and time at which the subject stopped smoking. One pack-year was defined as smoking 20 cigarettes daily for 1 year. All of the statistical analyses were performed using the statistical software package SPSS 15.0 (SPSS Inc., Chicago, IL). Allelic and genotype frequencies were obtained by direct counting. Hardy-Weinberg equilibrium was assessed by a χ² test with 1 degree of freedom. Results are expressed as mean ± S.E.M. Means in
different subgroups were analyzed by Student’s t test, one-way analysis of variance, followed by Duncan and Student-Newman-Keuls post hoc analysis, and U test. A value of \( p < 0.05 \) was regarded as significant.

**Results**

**Allele Frequencies.** To determine whether our studied population does not deviate from Hardy-Weinberg equilibrium and whether allele frequencies were within other published frequencies, we investigated allele and genotype frequencies in the total population as well as in all the subgroups. In the total population the allele frequency for quantitative \((Cyp1B1^{*3})\) was 0.415. This allele frequency is similar to other observed frequencies in various healthy white populations and did not significantly deviate from the Hardy-Weinberg equilibrium \((p = 0.1 = 1 - \alpha)\). The allele frequencies of \(Cyp1B1^{*3}\) were not statistically different within the subgroups smokers quantitative \((Cyp1B1^{*3}) = 0.43\), nonsmokers quantitative \((Cyp1B1^{*3}) = 0.40\), and ex-smokers quantitative \((Cyp1B1^{*3}) = 0.42\), and they did not deviate from the Hardy-Weinberg equilibrium \((p = 0.1 = 1 - \alpha)\). Allele frequencies were also not different within the male quantitative \((Cyp1B1^{*3}) = 0.415\) or female quantitative \((Cyp1B1^{*3}) = 0.414\) subgroups, and they did not deviate from the Hardy-Weinberg equilibrium \(p = 0.1 = 1 - \alpha\).

**Cyp1B1 mRNA Expression.** To investigate the functional impact of the Cyp1B1 allelic variants the quantitative Cyp1B1 mRNA expression among the different genotypes for Cyp1B1 L432V polymorphism \({}^{*1/*1}, { }^{*1/*3}, { }^{*3/*3}\) was analyzed. Comparing the mean \pm S.E. of the relative Cyp1B1 mRNA expression revealed significant differences between the two homozygote genotypes \({}^{*1/*1}\) and \({ }^{*3/*3}\) \((0.185 \pm 0.027, n = 118 \text{ versus } 0.071 \pm 0.013, n = 56; p = 0.000)\) and between the heterozygote genotype \({}^{*1/*3}\) and \({ }^{*3/*3}\) \((0.178 \pm 0.025, n = 171 \text{ versus } 0.071 \pm 0.013, n = 56; p = 0.000)\). These results suggest that genotypes carrying the C allele \({}^{*1/*1}\) and \({ }^{*1/*3}\) have a significantly higher Cyp1B1 mRNA expression compared with the genotype without the C allele \({ }^{*3/*3}\) (Fig. 2).

To further investigate the functional impact of the Cyp1B1 allelic variants and to exclude a tobacco smoke influence, the quantitative Cyp1B1 mRNA expression was analyzed within the subgroups smokers, nonsmokers, and ex-smokers. In each subgroup the relative Cyp1B1 mRNA expression was compared between the three different genotypes \({}^{*1/*1}, { }^{*1/*3}, \text{ and } { }^{*3/*3}\). A significant difference of the mean \pm S.E. between the homozygote genotypes \({}^{*1/*1}\) and \({ }^{*3/*3}\) was detected within the subgroup smokers \((0.169 \pm 0.043, n = 33 \text{ versus } 0.056 \pm 0.016, n = 19; p = 0.020)\) and nonsmokers \((0.219 \pm 0.063, n = 34 \text{ versus } 0.064 \pm 0.019, n = 14; p = 0.025)\), respectively. Significant differences of the mean \pm S.E. between the heterozygote genotype \({}^{*1/*3}\) and the homozygote genotype \({ }^{*3/*3}\) were revealed within the subgroups smokers \((0.205 \pm 0.058, n = 50 \text{ versus } 0.056 \pm 0.016, n = 19; p = 0.018)\) and ex-smokers \((0.181 \pm 0.084, n = 72 \text{ versus } 0.084 \pm 0.027, n = 24; p = 0.024)\), respectively (Fig. 3). These findings confirm that the genotypes containing a C allele have a significantly higher Cyp1B1 mRNA expression compared with the genotype without a C allele.

To determine whether Cyp1B1 is activated in leukocytes by components of tobacco smoke, we analyzed the quantitative mRNA ex-
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The expression of Cyp1B1 in relation to smoking habits. Comparing the mean and the S.E. of the relative Cyp1B1 mRNA expression between smokers (0.167 ± 0.033, n = 101), nonsmokers (0.191 ± 0.037, n = 98), and ex-smokers (0.149 ± 0.017, n = 148) did not reveal any significant differences (Fig. 4).

Furthermore, the quantitative Cyp1B1 mRNA expression was analyzed within the different genotypes (*1/*1, *1/*3, *3/*3) within the subgroups smokers and nonsmokers and between the genotypes *1/*3 and *3/*3 within the subgroups nonsmokers and ex-smokers. Therefore, genotypes containing the C allele had a significantly higher Cyp1B1 mRNA expression within the subgroups. Data are shown as mean ± S.E., *p < 0.05.

Cyp1B1 mRNA expression in females compared with males is worth noting (Fig. 6).

Discussion

In this study, we show an influence of the Cyp1B1 L432V polymorphism on its Cyp1B1 mRNA expression in human lymphocytes in relation to interindividual differences by PAH induction through tobacco smoke. Therefore, we investigated the quantitative mRNA expression and the L432V genotypes of Cyp1B1 in 365 white volunteers. The allele frequencies for the studied genotypes did not deviate from the Hardy-Weinberg equilibrium, and allele frequencies were similar to other published frequencies (Bailey et al., 1998; Brüning et al., 1999; Tang et al., 2000; Ko et al., 2001).
We found significant differences in comparing the relative Cyp1B1 mRNA expression between the different Cyp1B1 genotypes, suggesting that genotypes carrying the wild-type C allele (Cyp1B1*1 allele) have a significantly higher Cyp1B1 mRNA expression compared with the genotype without the C allele. These findings were also confirmed when we divided the population into the subpopulations smokers, non-smokers, and ex-smokers.

Hanaoka et al. (2002) found a significant correlation between Cyp1B1 mRNA expression and urinary 1-hydroxyphrene in subjects with the Cyp1B1 432 Leu/Leu wild-type genotype (Cyp1B1*1/*1) (Hanaoka et al., 2002). On the other hand, Hu et al. (2006) found a significantly higher mRNA expression in Taiwanese municipal waste incinerator workers carrying the Cyp1B1*3 allele (n = 9) compared with workers carrying the wild-type allele (n = 34). This finding was confirmed in workers exposed to high levels of PAH/dioxin only. Workers exposed to medium levels or controls showed an insignificantly lower Cyp1B1 mRNA expression when carrying the Cyp1B1*3 allele (n = 15 versus n = 48) (Hu et al., 2006). However, van Duursen et al. (2005a) found no statistically significant effects of Cyp1B1 L432V polymorphism on mRNA expression levels when studying human blood lymphocytes of 10 nonsmoking females (van Duursen et al., 2005b). In addition, no statistically significant differences were found in Cyp1B1 mRNA expression when comparing the Cyp1B1 L432V polymorphism of 14 individuals living in a polychlorinated biphenyl-polluted area in Slovakia (van Duursen et al., 2005a). In contrast, subjects heterozygous or homozygous for the Cyp1B1*3 allele showed a higher TCDD-induced Cyp1B1 mRNA expression in mitogen-treated lymphocytes than subjects homozygous for the Cyp1B1*1 allele (Landi et al., 2005). The low sample size in some studies and the different distribution of the Cyp1B1 polymorphism among Chinese and whites (Tang et al., 2000) should be considered. The fact that mitogens cause lymphocytes to proliferate, which occurs concomitantly with an activation of several cell-signaling pathways and increases in gene transcription, should also be mentioned. Even when housekeeping genes are used for normalization, expression levels of Cyp1B1 can be higher in mitogen-stimulated cells than in nonstimulated cells (van Duursen et al., 2005b). The genetic impact on the expression pattern of Cyp1B1 is only one contributing factor, especially because post-transcriptional mechanisms have been suggested to be involved in regulation of Cyp1B1 (McFadyen et al., 2003). Although the consequences of our findings are not yet clear, further studies need to consider the Cyp1B1 L432V polymorphism when investigating gene and protein expression of Cyp1B1.

We did not find any significant differences in comparing the Cyp1B1 expression of smokers, nonsmokers, and ex-smokers. Furthermore, we did not find any differences in Cyp1B1 expression of smokers, nonsmokers, and ex-smokers when we divided the population into subpopulations by their different Cyp1B1 genotypes. Therefore, we conclude that Cyp1B1 gene expression levels in lymphocytes are not suitable to use as biomarkers of exposure to environmental or tobacco smoke PAHs. Nevertheless, a higher expression of Cyp1B1 was observed in bronchial epithelial cells of smokers than in nonsmokers (Willey et al., 1997; Kim et al., 2004).

Blood lymphocytes do not function as a target organ. Accordingly, Dassi et al. (1998) estimated Cyp1B1 mRNA levels in blood monocytes from 25 smokers and 50 nonsmokers without finding any differences between the two groups. In a group of volunteers, no significant association between self-reported PAH exposure and Cyp1B1 mRNA expression was detected (Tuominen et al., 2003). In addition, Lin et al. (2003) did not find any significant differences in gene expression level between Taiwanese male smokers (n = 12) and nonsmokers (n = 10). For other AhR agonists, similar results were obtained in diverse studies.

When investigating cultured lymphocytes of 121 subjects from Seveso, Italy accidentally exposed to TCDD, no association between Cyp1B1 mRNA expression and TCDD plasma levels was detected (Landi et al., 2003). Furthermore, the concentration of dioxin or dioxin-like compounds necessary to evoke an in vitro effect on Cyp1B1 expression is much higher than the human blood levels found in vivo (van Duursen et al., 2005b). Within a population of Slovaks from a region polluted with polychlorinated biphenyls, there was no significant correlation between polychlorinated biphenyl levels and Cyp1B1 mRNA expression (van Duursen et al., 2005a). On the other hand, Hanaoka et al. (2002) found significantly higher Cyp1B1 mRNA levels in peripheral blood of Chinese coke oven workers exposed to PAHs at high levels (n = 9) compared with workers exposed to lower PAH levels (n = 16) and controls (n = 13). In addition, a significantly higher mRNA expression of Cyp1B1 was found in leukocytes of Taiwanese municipal waste incinerator workers (n = 77) frequently exposed to PAHs and dioxins compared with controls (n = 35) (Hu et al., 2006). A possible explanation for the contrasting findings is that occupational and environmental exposure may differ in magnitude, chemical composition, and frequency. Moreover, the studies differ in sample size, time period between exposure, and measurement of RNA and exposure registration.

In our study, there was a higher mRNA expression of Cyp1B1 in females compared with males. The low sample size of females we investigated in this study should be considered for these results. However, Lin et al. (2003) reported similar results when comparing dimethyl sulfoxide-treated lymphocytes from female nonsmokers with dimethyl sulfoxide-treated lymphocytes from male nonsmokers. In addition, in Taiwanese females the mean Cyp1B1 mRNA expression was significantly higher than in males (Hu et al., 2006). Several epidemiological studies have indicated that female smokers are at higher risk of lung cancer than male smokers (Engeland, 1996; Zang and Wynder, 1996; Prescott et al., 1997, 1998a,b). It is possible that the higher Cyp1B1 expression in females could account for their elevated lung cancer risk. These findings require further investigation.

In conclusion, we showed in this study that the Cyp1B1*3/*3 genotype is associated with a decreased Cyp1B1 mRNA expression compared with the Cyp1B1*1/*1 and Cyp1B1*1/*3 genotypes. We also observed a different Cyp1B1 mRNA expression between females
and males. However, we did not find any statistically significant effect of tobacco smoke on Cyp1B1 mRNA expression in leucocytes.

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