Decreased Cyp2E1 mRNA expression in human leucocytes in patients with fibrotic and inflammatory lung diseases

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Abstract. Cytochrome P450 2E1 (CYP2E1), a phase I enzyme, is involved in the activation of a broad spectrum of procarcinogens. The importance of this enzyme for pharmacology is derived from its unique substrate spectrum. Large interindividual differences in the toxicity of its substrates are known. We investigated the quantitative Cyp2E1 mRNA expression in a cohort of patients with fibrotic and inflammatory lung diseases (n=137) and healthy controls (n=221) considering tobacco-smoke, exposure to asbestos, silica, organic dust or chemical irritating particles in relation to the C1053T (RsaI) polymorphism of Cyp2E1. For quantitative comparison of Cyp2E1 mRNA levels real-time PCR was performed using a LightCycler® System. Calculations of expression were made with the 2 -ΔΔCT method. Detection of the C1053T (RsaI) polymorphism of the Cyp2E1 gene was performed by rapid capillary PCR with melting curve analysis. Fibrotic (0.016±0.002; n=52; p=0.000) and inflammatory (0.013±0.002; n=16, p=0.000) lung disease patients showed a significantly reduced Cyp2E1 mRNA expression compared to healthy controls (0.026±0.002; n=206). Exposure to asbestos (0.019±0.003; n=40; p=0.016), organic dust (0.016±0.003; n=39; p=0.000) and irritative chemicals (0.016±0.002; n=22; p=0.000) revealed a highly significant reduced Cyp2E1 expression. Cyp2E1 mRNA expression of occupational disease (OD) patients suffering from lung or pleural asbestosis OD-No. 4103 (0.018±0.003; n=31; p=0.024), allergic obstructive airway diseases OD-No. 4301 (0.013±0.002; n=23; p=0.000), exogenous allergic alveolitis OD-No. 4201 (0.009±0.002; n=6; p=0.000) and obstructive airway diseases caused by irritative chemicals OD-No. 4302 (0.013±0.001; n=11; p=0.000) was highly significantly reduced. In conclusion, we demonstrated a significantly decreased mRNA expression in patients with fibrotic and inflammatory lung diseases compared to healthy controls.

Introduction

Increased anthropogenic pollution of the environment leads to a steady growth in cancer incidence world-wide. Long-term intake of carcinogenic compounds e.g. contained in tobacco smoke or occupational dust contributes to development of malignancies (1). The cytochrome P450 mono-oxygenase system (CYP) plays a major role in the metabolic initiation of xenobiotics into carcinogenic metabolites (2). Cytochrome P450 2E1 (CYP2E1) plays an important role in biotransformation of procarcinogens. The importance of this enzyme in occupational and environmental medicine is derived from its unique substrate spectrum, that includes a number of high production chemicals, such as hydrocarbons, solvent and industrial monomers (3). Cyp2E1 also participates in metabolism of many compounds, including N-nitrosamines, benzene, acetaldehyde and phenol, which are amongst other ingredients of tobacco smoke (4). Its ability to reduce molecular oxygen to highly reactive compounds, even in the absence of substrates, leads to intensified damage of macromolecules and formation of DNA-adducts (1). The highest expression levels of Cyp2E1 are found in the liver (5) but it is also expressed in nasal and oropharyngeal epithelium (6), bronchial epithelium (7) and peripheral blood lymphocytes (8).

Molecular epidemiology can have an important impact when analyzing the complexity of gene-environment interactions at the molecular level. While gene mutations of high penetration often result in a high risk for cancer (9), polymorphisms of genes with low penetration (such as Cyp2E1), are thought to predispose the individual risk if there is exposure to a certain chemical (10).

Since the Cyp2E1 gene is polymorphic and significant individual variability was found for its gene expression level (11), it can be assumed that polymorphisms, at least partially, explain the inherited individual susceptibility to development of malignancies (1).

A single nucleotide polymorphism (C/T) was identified in 5'-regulatory region of Cyp2E1 at position -1053, which is accompanied with the loss of a RsaI restriction site (accession no. gene J02843). Gene analyses indicate that the rare c2 allele is accompanied with a higher expression (12,13).
bronchial hyperreactivity (UBH) and asthma; LD 3, 16 people including chronic obstructive lung disease (COLD), unspecific disease; LD 2, 67 people with obstructive airway diseases fibrosis including asbestosis, silicosis and idiopathic pulmonary

Occupational diseases consisted LD 1, 54 people with lung composed 137 people (mean age 62 years; range, 18-85 years).

excluded Table I).

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221 unrelated healthy subjects (mean age 27 years; range,

and disease history. The control group composed of

smoking habits, alcohol consumption, indoor air pollution (coal heating and cooking), eating habits and lifetime occupational

To demonstrate the functional impact of occupational or environmental factors we investigated the quantitative Cyp2E1 mRNA expression in a cohort of people with fibrotic and inflammatory lung disease (n=137) as well as in a cohort of healthy controls (n=221) tobacco use, exposure to asbestos, silica, organic dust or irritating chemical particles in relation to the C1053T (RsaI) polymorphism of Cyp2E1.

Materials and methods

Subjects. The study population consisted of a total of

358 Caucasians (mean age 37 years; range, 15-85 years). Volunteers were personnel of the Universitätsklinikum Giessen und Marburg GmbH, students of the Justus-Liebig-Universität Giessen as well as patients recruited at the Policlinic of the Institute for Arbeits- and Sozialmedizin Giessen, Germany.

All 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 and lifetime occupational and disease history. The control group composed of 221 unrelated healthy subjects (mean age 27 years; range, 15-77 years) without known diseases. Any subject with diseases related to potential lung disorders (chronic obstructive lung disease, etc.) or any benign or malignant tumours were excluded Table I).

The fibrotic and inflammatory lung diseases (LD) group composed 137 people (mean age 62 years; range, 18-85 years). Occupational diseases consisted LD 1, 54 people with lung fibrosis including asbestosis, silicosis and idiopathic pulmonary disease; LD 2, 67 people with obstructive airway diseases including chronic obstructive lung disease (COLD), unspecific bronchial hyperreactivity (UBH) and asthma; LD 3, 16 people with other inflammatory lung diseases including sarcoidosis, tuberculosis, (broncho-) pneumonia, (tracheo-) bronchitis, floride exogenic allergic alveolitis (farmer lung disease) and pleural empyema. To allow further discrimination between different kinds of exposure, several subgroups with exposure to asbestos, silica, organic dust and irritating chemicals were created. In addition patients with recognized occupational diseases were compared by their occupational disease (OD) numbers (Table II).

Smokers (n=112) were defined as people who smoke daily, non-smokers (n=178) were defined as people who had never smoked and ex-smokers (n=68) were defined as people who had quit smoking at least 3 months previously.

The ethics committee of the university hospital, Giessen, Germany, approved the study. All subjects gave written informed consent for the study.

Real-time PCR and polymorphism detection. Blood samples (ca. 3 ml) were obtained from all subjects. Genomic DNA was isolated from whole blood using the Versagen™ DNA Purification Kit (Genta Systems, Minneapolis, USA) according to the manufactures instruction. Detection of the C1053T (RsaI) polymorphism of the Cyp2E1 gene was performed by rapid capillary PCR, with melting curve analysis, using fluorescence-labelled hybridisation probes in a LightCycler® System (14). The wild-type allele (C) containing the RsaI site is referred to as c1 and the rare allele (T) without the RsaI site is referred to as c2 (15). At the home page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (www.cypalleles.ki.se/) the polymorphism was named Cyp2E1*5B, while the wild-type allele was designated as Cyp2E1*1A (16).

Both negative and positive controls were included in each PCR reaction. TIB Molbiol, Berlin, Germany, synthesized the PCR primers as well as the fluorescent-labelled detection probes. The primers Cyp2E1-sense (5'-AGAATGGCATAA CTCAAATTCC-3') and Cyp2E1-antisense (5'-CAGACCC TCTTCACATTTAT-3') amplify a 364-bp fragment of the Cyp2E1 5' flanking region. The fluorescence-labeled probe (TTCTATATTAACTGACTGTC-FL) and the LC-Red640-labeled probe (LC-Red640-CAACCTATGAAT TTCATTGTTAATATAAAAGTACAAAATT-FL) bind to nucleotides 1751-1778 and 1780-1814 within the human Cyp2E1 gene (GenBank entry J02843), respectively. The expected melting curve in the case of the wild-type C allele (c1) was higher than in the case of the rare T allele (c2). The homozygous samples for the C allele (Cyp2E1 c1/c1) contain a single peak and a

Table I. Demographic parameters of healthy controls (HC) and lung disease patients (LD).

<table>
<thead>
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<th></th>
<th>Overall</th>
<th>Healthy controls</th>
<th>Occupational disease</th>
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<tbody>
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<td>221</td>
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<tr>
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<tr>
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<td>210</td>
<td>125</td>
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<tr>
<td>wt/mt</td>
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<td>11</td>
<td>11</td>
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<tr>
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<td>88</td>
<td>24</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>178</td>
<td>122</td>
<td>56</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>68</td>
<td>11</td>
<td>57</td>
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</table>

Table II. Exposure at the workplace and compensated occupational disease according to the numbers as defined in the list of occupational diseases (BKV).

<table>
<thead>
<tr>
<th>Exposure to</th>
<th>N</th>
<th>BKV No.</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestos</td>
<td>42</td>
<td>4103</td>
<td>32</td>
</tr>
<tr>
<td>Silica</td>
<td>13</td>
<td>4101</td>
<td>8</td>
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<tr>
<td>Organic dust</td>
<td>41</td>
<td>4301</td>
<td>24</td>
</tr>
<tr>
<td>Irritative chemical</td>
<td>22</td>
<td>4302</td>
<td>11</td>
</tr>
</tbody>
</table>

To demonstrate the functional impact of occupational or environmental factors we investigated the quantitative Cyp2E1 mRNA expression in a cohort of people with fibrotic and inflammatory lung disease (n=137) as well as in a cohort of healthy controls (n=221) tobacco use, exposure to asbestos, silica, organic dust or irritating chemical particles in relation to the C1053T (RsaI) polymorphism of Cyp2E1.

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melting temperature of 55˚C, and homozygous samples for the T allele (Cyp2E1 c2/c2) show a single peak at 47.5˚C. Heterozygous (Cyp2E1 c1/c2) samples give rise to biphasic melting curves (Fig. 1). In the following homozygous genotype Cyp2E1 c1/c1 is denoted 'c1/c1', heterozygous genotype Cyp2E1 c1/c2 is denoted 'c1/c2' and homozygous genotype Cyp2E1 c2/c2 is denoted 'c2/c2'.

Reaction mixture (modified) (14) comprised 1x FastStart DNA Master Hybridization Probes (Roche Diagnostics, Mannheim, Germany), primers Cyp2E1-sense (1.5 μM) and Cyp2E1-antisense (1.5 μM), sensor (0.25 μM), anchor (0.25 μM), MgCl2 (4 mM), 25 ng of genomic DNA in a 10 μl reaction mixture. PCR contamination was checked by the inclusion of a negative control. PCR was initially denatured at 95˚C for 10 min and subjected to 50 cycles of amplification with 2 sec of denaturing at 95˚C, 10 sec of annealing at 55˚C, and 20 sec of extension at 72˚C. The melting curve was achieved by 2 sec at 95˚C, 30 sec at 36˚C and 1 sec at 90˚C (0.1˚C/sec).

RNA extraction and reverse transcription. White blood cells (WBC) were isolated from 10 ml peripheral blood using Ficoll® (GE Healthcare) as described by the manufacturer. Total RNA was isolated from WBCs using a commercial RNA isolation reagent (TRI Reagent®, Sigma) 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 RNase-free Dnase 1 unit/μl (Qiagen) for 10 min at 37˚C to eliminate remaining DNA. Prepared RNA was reverse-transcribed as described (17).

Real-time polymerase chain reaction (PCR). For quantitative comparison of Cyp2E1 mRNA levels real-time PCR was performed using SYBR-green fluorescence in a LightCycler® System (Roche Diagnostic GmbH). After optimization of PCR conditions, amplification efficiency was tested in standard curves using serial cDNA dilutions. The correlation coefficient had to be >0.9 and the slope about -3.5. Amplification specificity was checked using melting curves. Both negative and positive controls were included in each PCR reaction. All assays were carried out three times as independent PCR runs for each cDNA sample. Gene expression was always related to the expression of B-2-microglobulin (B2M) as the housekeeping gene, which is known as a suitable choice for normalization of leukocyte expression levels (18). Calculations of expression were performed with the 2-ΔΔCT method according to Pfaffl (19). Gene expression levels were rounded to three decimal places. Sequences of the specific primers for CYP2E1 and B2M were CYP2E1-SP (5’CCT ACA TGG ATG CTG TGG TG-3’), CYP2E1-ASP (5’TGG GGA TGA GGT ATC CTC TG-3’), B2M-for (5’-ACTGAATTCACCCCCACTGA-3’), B2M-rev (5’-CCTCCATGATGATGCTTACA-3’). PCR reactions were carried out in a final volume of 20 μl using 1x Absolut™ QPCR SYBR Green Capillary Mixes (Abgene), 300 nM of B2M primers or 600 nM of Cyp2E1 primers and 2 μl cDNA. The PCR conditions for Cyp2E1 were as follows: initial denaturation 15 min at 95˚C, touch-down PCR 2 cycles of 95˚C for 10 sec, 65˚C for 10 sec, 72˚C for 25 sec; 2 cycles of 95˚C for 10 sec, 63˚C for 10 sec, 72˚C for 25 sec; 2 cycles of 95˚C for 10 sec, 61˚C for 10 sec, 72˚C for 25 sec; 45 cycles of 95˚C for 10 sec, 59˚C for 10 sec, 72˚C for 25 sec. PCR conditions for B2M were as follows, initial denaturation for 15 min at 95˚C, PCR 55 cycles of 95˚C for 10 sec, 65˚C for 10 sec, 72˚C for 25 sec; 2 cycles of 95˚C for 10 sec, 63˚C for 10 sec, 72˚C for 25 sec; 2 cycles of 95˚C for 10 sec, 61˚C for 10 sec, 72˚C for 25 sec; 45 cycles of 95˚C for 10 sec, 59˚C for 10 sec, 72˚C for 25 sec. PCR conditions for B2M were as follows, initial denaturation for 15 min at 95˚C, PCR 55 cycles of 95˚C for 10 sec, 63˚C for 10 sec, 72˚C for 10 sec.

All measurements were made without information about the origin of the samples and were performed in triplicate. After amplification reaction products were controlled and separated on 2% agarose gels, stained with SYBR Safe, and photographed under UV illumination.

Statistical analysis. All statistical analyses were performed using the statistical software package, SSPS 15.0 (SPSS Inc., Chicago, IL, USA). Allelic and genotype frequencies were obtained by direct counting. Hardy-Weinberg equilibrium was assessed by the χ² test with 1 degree of freedom. Results are expressed as mean ±standard error of the mean (SE).
Means in different subgroups were analysed by Student’s t-test and one-way ANOVA. A value of \( p<0.05 \) was regarded as significant.

**Results**

**Allele frequencies.** To determine whether our studied population deviated from the Hardy-Weinberg equilibrium and if allele frequencies were within other published frequencies, we investigated allele and genotype frequencies in the total population as well as in the control and patient groups.

In the total population the allele frequency for \( q(Cyp2E1 \, c2) \) was 0.034. This allele frequency is similar to other observed frequencies in various healthy Caucasian populations and did not significantly deviate from the Hardy-Weinberg equilibrium.

The allele frequencies within the healthy control group was \( q(Cyp2E1 \, c2)=0.025 \) and within the patient group was \( q(Cyp2E1 \, c2)=0.052 \). Neither group deviated from the Hardy-Weinberg equilibrium.

**Cyp2E1 allelic variants and Cyp2E1 mRNA expression.** To investigate the functional impact of the Cyp2E1 allelic variants, the quantitative Cyp2E1 mRNA expression among different genotypes for Cyp2E1 C-1053T polymorphism (\( c1/c1, \, c1/c2, \, c2/c2 \)) was analysed. Since the allele frequency of the \( c2 \) allele is low within the Caucasian population, we combined all genotypes, carrying a \( c2 \) allele, in one group. Comparing the means ± SE of the relative Cyp2E1 mRNA expression did not reveal any significant differences between the homozygote genotypes (\( c1/c1 \)) and the \( c2 \) allele carrying genotypes (\( c1/c2, \, c2/c2 \)). The \( c2 \) allele carrying genotypes tends to result in higher mRNA expression for the total population (0.023±0.001; \( n=317 \) vs. 0.062±0.024; \( n=22 \)), as well as for the healthy control (0.026±0.002; \( n=198 \) vs. 0.172±0.103; \( n=11 \)) and the patient group (0.018±0.002; \( n=120 \) vs. 0.052±0.019; \( n=12 \)). This suggests that genotypes carrying the \( c2 \) allele have a higher Cyp2E1 mRNA expression compared to the genotype without the \( c2 \) allele (Fig. 1).

**Possible functional impacts on Cyp2E1 mRNA expression.** We further investigated the functional impact of the smoking status, gender, body mass index (BMI) and age on the quantitative Cyp2E1 mRNA expression. Analysis in the total population as well as in the subgroups did not reveal any relevant differences in Cyp2E1 mRNA expression between smokers, non-smokers and ex-smokers or between males and females (data not shown). Relative Cyp2E1 mRNA expression in context with the BMI produced a coefficient of determination \( R^2=0.032 \) overall, \( R^2=0.00001 \) healthy subgroup and \( R^2=0.02 \) patient group. Relative Cyp2E1 mRNA expression in context of age produced a coefficient of determination \( R^2=0.011 \) overall, \( R^2=0.003 \) healthy subgroup and \( R^2=0.012 \) patient group. Therefore we conclude that the smoking habits, gender, BMI and age do not influence Cyp2E1 mRNA expression.

**Ethiologly and Cyp2E1 mRNA expression.** The lung diseases (LD) group was further divided in fibrotic lung diseases (LD 1) including asbestosis, silicosis and idiopathic pulmonary disease, in obstructive lung diseases (LD 2) including chronic obstructive lung disease (COLD), unspecific bronchial hyperreactivity (UBH) and asthma as well as in inflammatory lung diseases (LD 3), including sarcoidosis, tuberculosis, (broncho-) pneumonia, (tracheo-) bronchitis, floride exogenic allergic alveolitis (farmer lung disease) and pleural empyma. The two subgroups, LD 1 (0.016±0.002; \( n=52; \, p=0.000 \)) and LD 3 (0.013±0.002; \( n=16; \, p=0.000 \)), showed significantly reduced Cyp2E1 mRNA expression compared to the healthy control group (0.026±0.002; \( n=206. \)). This was also seen in the subgroup LD 2 (0.020±0.002; \( n=64; \, p=0.053 \)) however, a significance was not reached (Fig. 2).

**Exposure and Cyp2E1 mRNA expression.** For further investigations subgroups with exposure to asbestos, silica, organic dust and irritative chemicals were formed. Cyp2E1 mRNA expression of these subgroups was compared to healthy controls without any known exposure at the workplace.
The asbestos exposure group (0.019±0.003; n=40; p=0.016) but not the silica exposure group (0.016±0.005; n=13; p=0.095) showed a significantly reduced Cyp2E1 mRNA expression compared to the healthy controls (0.026±0.002; n=197). Additionally, exposure to organic dust (0.016±0.003; n=39; p=0.000) and irritative chemicals (0.016±0.002; n=22; p=0.000) revealed a highly significant reduced Cyp2E1 mRNA expression (Fig. 3).

**Discussion**

In our study we demonstrated a higher mRNA expression for genotypes carrying at least one of the rare c2 allele. Since the allele frequency (c2) was low at 0.034 we determined only 22 c2 allele carriers. Therefore, significance was not reached. Still our result is affirmed by gene analyses, which indicate that the rare c2 allele is accompanied with a higher mRNA expression (12,13). Increased mRNA expression does not necessarily reflect enzyme activity level, but still evidence was obtained on increased catalytic activity of the enzyme coded by the c2 allele (13,20). Cyp2E1 is involved in the metabolism of many procarcinogens and possesses the ability to reduce molecular oxygen to highly reactive compounds, leading to intensified lipid and protein peroxidation, DNA damage and carcinogenesis (21). Therefore a higher cancer risk would be expected to associate with the c2 allele.

Instead an increased risk for the development of esophageal cancer in the Chinese population, associated with the c1/c1
genotype has been reported (22,23). An association between the c1/c1 genotype and an increased lung cancer risk was reported as well (24,25). Among Asians the c2 allele of Cyp2E1 is suggested to be a decreased risk factor for developing lung cancer (26). Striking inter-ethnic differences, complicating analysis, appear with respect to the rare c2 alleles frequency.

The rare allele frequencies reported are 24-30% for Asians (15,27,28), 2-3% for Caucasians (20,27), 0.3-7% for Afro-Americans (27,29,30), 15% for Mexican-Americans (29) and 18% for Taiwanese (31).

We demonstrated in our study a significant reduced Cyp2E1 mRNA expression of patients with fibrotic (LD 1) and inflammatory lung diseases (LD 3) compared to a healthy control group. In patients with an exposure to asbestos, organic dust and irritative chemicals Cyp2E1 mRNA expression was significantly reduced. These results were confirmed in patients with compensated occupational disease (OD). Smoking status, gender, body mass index (BMI) or age did not influence the quantitative Cyp2E1 mRNA expression in our study.

Transcription of the Cyp2E1 gene is primarily due to the binding of the hepatocyte-specific transcription factor HNF-1 upstream of the start site for RNA synthesis (32). The enzyme Cyp2E1 is regulated by transcriptional activation, mRNA stabilization, increased mRNA translatability and decreased protein degradation (33).

Inflammatory cytokines such as interleukin-6, -1B and tumor necrosis factor α (TNF-α) are reported to inhibit Cyp2E1 expression in rat hepatoma cells (34). Additionally a remarkable decrease of Cyp2E1 mRNA according to the fibrosis stage progression of chronic hepatitis C patients with liver fibrosis was also reported. This decrease was associated with the decreased mRNA levels on its protein. Additionally, it needs to be determined if results gained from leucocytes account as a marker for processes of organs standing the basis for pharmacological and toxicological development. Further studies will be needed to detect inflammatory cytokine levels and to determine the effect of the decreased mRNA levels on its protein. Additionally, it needs to be determined if results gained from leucocytes account as a marker for processes of organs such as the lung.

In conclusion, we demonstrated significantly decreased mRNA expression in patients with fibrotic and inflammatory lung diseases compared to healthy controls.

References


