Relative Quantification of *Cytochrome P450 1B1* Gene Expression in Peripheral Leukocytes Using LightCycler

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Abstract. *Background: The cytochrome P450 oxidase system is a multigene family of inducible enzymes that play a central role in the metabolic activation of various xenobiotics, including polycyclic hydrocarbons (PAH). To investigate the considerable variability of cytochrome P450 1B1 (Cyp1B1) expression due to the exogenous influence of tobacco smoke or the endogenous influence of genetic polymorphism, a sensitive quantitative determination of gene expression is necessary. Materials and Methods: A method is introduced for the analysis of Cyp 1B1 gene expression using real-time quantitative PCR and the comparative ΔΔCT (threshold cycle) method. Blood samples from a smoker and non smoker were collected and total RNA was analysed in comparison to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results: The expression of Cyp 1B1 was 16.43 times higher in the smoker than in the non-smoker. Conclusion: This approach provides a manageable method for examining large quantities of samples and could possibly be used to evaluate gene environmental interactions on the basics of gene expression analysis.*

The cytochrome P450 oxidase system is a multigene family of inducible enzymes that play a central role in the metabolic activation of various xenobiotics, including polycyclic hydrocarbons (PAHs). Human lung cancer for example may result from exposure to carcinogens such as PAHs present mainly in cigarette smoke. Therefore genetic polymorphism within the cytochrome P450 enzyme, leading to changes in gene expression or enzyme activity, could be associated with a different interindividual susceptibility to lung cancer (1-2).

Host factors influence susceptibility to the effects of tobacco smoking. One type of host factor are the pleiotropic genes of the cytochrome P450 system, which control the oxidative metabolism of environmental carcinogens. Most environmental carcinogens require metabolic activation by phase I enzymes, cytochrome P450s, to their reactive electrophilic intermediates. Phase I intermediate compounds covalently bind to nucleic acids and proteins and form carcinogen-DNA adducts, which may initiate carcinogenesis. The metabolism of many compounds involves the action of a number of enzymes, each with different environmental and genetic determinants of activity.

Several forms of P450 have been identified as playing a role in lung carcinogenesis and are associated with individual differences in susceptibility to carcinogens. P450s are expressed in human tissue and metabolize PAH. Therefore P450s are responsible for the metabolic activation of benzo[α]pyrene and other carcinogens in cigarette smoke. In particular, the phase I enzyme cytochrome P450 oxidase 1B1 (Cyp1B1) is known to catalyse a number of PAHs to carcinogenic epoxides in several organs (3). To investigate the considerable variability of Cyp1B1 expression due to the exogenous influence of tobacco smoke or the endogenous influence of genetic polymorphism, a sensitive quantitative determination of gene expression is necessary.

Therefore real-time quantitative PCR and the comparative ΔΔCT method were introduced for the analysis of Cyp1B1 gene expression (4). Human peripheral blood was chosen for this study since this material might be exposed to PAHs within the lungs and is easily available. Hence the Cyp1B1 level in peripheral blood might be a useful biomarker of exposure to biologically effective doses of PAHs.

Materials and Methods

A 10 ml peripheral blood sample from a smoker, A, and a never-smoker, B, were collected and the total RNA was isolated by the Trizol® method. This procedure is an improvement of the single-step method reported by Chomczynski and Sacchi (5) for total RNA isolation and is based on a guanidine-thiocyanate-phenol-chloroform extraction. After DNAse treatment, the total RNA was precipitated. At this step, it is very important to confirm that the isolated total

*Some of the results are included in the thesis of B. Hadzaad.

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Key Words: CYP1B1, mRNA, gene expression.

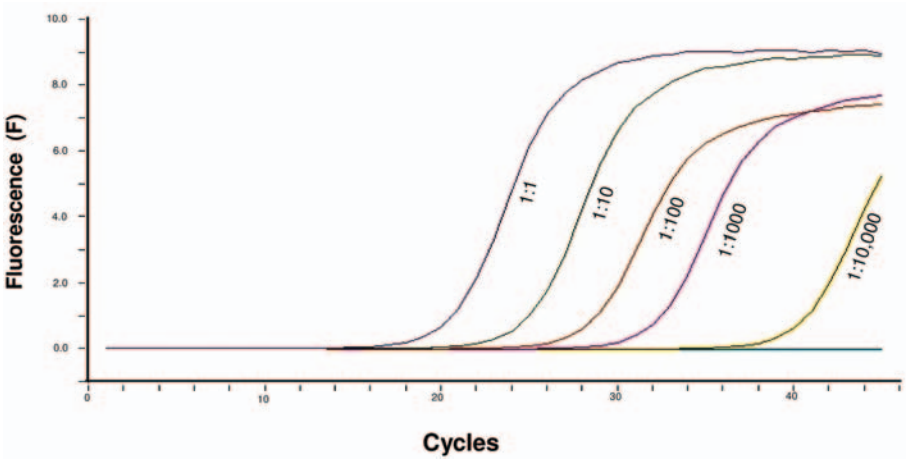

Figure 1. *Serial dilutions of cDNA amplified with Cyp1B1 primer are used to create a standard curve (here for example for Cyp1B1) with the LightCycler instrument.*

RNA is free of any DNA, since DNA can adulterate the results of the subsequent quantitative PCR. Therefore, the RNA was amplified by regular PCR, for example with β-actin primers, to confirm that the isolated RNA was free of DNA. Any other established primers could be used as well. Pure RNA cannot serve as a template in regular PCR amplification. Therefore the RNA template should not reveal any product, unless the sample still contains DNA residues. For further analysis, the RNA was reverse transcribed and the quality of the cDNA was confirmed again by β-actin PCR and gel electrophoresis. The established β-actin primers served as quality control in our laboratory, since they are designed to span an intron and therefore reveal products of different length for genomic DNA and RNA. If only one band appeared in the gel electrophoresis, it was concluded that the cDNA synthesis had been successful and the sample, free of genomic DNA, could be used for quantitative PCR.

To reveal gene expression and to compare the expression patterns between the smoker and the non-smoker, quantification was performed using real-time PCR with SYBR Green I detection. Optimal conditions for the target *Cyp1B1* gene and the reference gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) have been established (6).

The master mix, including all the PCR ingredients but the cDNA, was prepared in a separate room to prevent contamination. All the PCR contents needed such as PCR mix, primers, magnesium chloride stored at –20˚C were thawed, vortexed and briefly centrifuged (1,000 rpm, 4 s) prior to use. It was very important to aliquot the PCR ingredients into small amounts to avoid repeated freeze-thawing cycles. The final amplification occurred in 1 x Absolute QPCR SYBR Green Capillary Mix® Fa. Abgene which contains an optimised reaction buffer and DNA Polymerase and is ideal for use with the Roche Light Cycler using glass capillaries, 600 nmol of the *Cyp1B1* primer (MWG, Ebersberg, Germany) or 300 nmol of the *GAPDH* primer (Invitrogen, Karlsruhe, Germany) plus 2 μl cDNA in a total volume of 20 μl.

The sequence of the specific primers for *CYP1B1* and *GAPDH* were *CYP1B1* forward (5'-AACCGCAACTTCAGCAACTT-3'), *CYP1B1* reverse (5'-GAGGATAAAGGCGTCCATCA-3'), and *GAPDH* forward (5'- TGCACCACCAACTGCTTAGC-3'), *GAPDH* reverse (5'-GGCATGGACTGTGGTCATGAG-3').

To avoid aerosol contamination of the negative control while preparing the sample it is recommended to seal the negative control immediately before proceeding with the sample preparation. For *Cyp1B1* amplification a touch down PCR program was used. Therefore the temperature (starting at 67˚C) was reduced in three steps of two cycles, each time for 2˚C before the amplification was carried out at 61˚C (45 cycles). The amplification of the housekeeping gene *GAPDH* occurred at 61˚C (45 cycles).

Since SYBR Green I detects double-strand DNA in general, melting curve analysis was used to check for primer dimers or unspecific PCR products. Serial dilutions of the cDNA (1:1; 1:10; 1:100; 1:1000; 1:10,000) with nuclease-free water were used to generate a standard curve (Figure 1) for *Cyp1B1* as well as *GAPDH* which covered 3-5 orders of magnitude in the range of the samples in order to calculate the specific efficiency (E) using LightCycler Software 3.5. After completion of PCR, the LightCycler software calculates the copy number of target molecules by plotting logarithm of fluorescence *versus* cycle number and setting a baseline x-axis. The baseline identifies the cycle in which the log-linear signal can be distinguished from the background for each sample. The x-axis crossing point of each standard is measured and plotted against the logarithm of concentration to produce a standard curve. The concentrations of target sequence in the samples are extrapolated from the standard curve. The LightCycler Software 3.5. displays only the slope of a standard curve, which can be used to calculate the efficiency using the equation $E=10^{-1}/\text{slope}$.

Results

The efficiency (E) of 1.85 was calculated using the slope -3.7 of the standard curve. The Comparative or ΔΔCT method was used to compare the smoker and non-smoker samples. Firstly the CT (threshold cycle) values for each sample were determined at the respective CP (crossing point) indicating the cycle at which PCR amplification begins its exponential

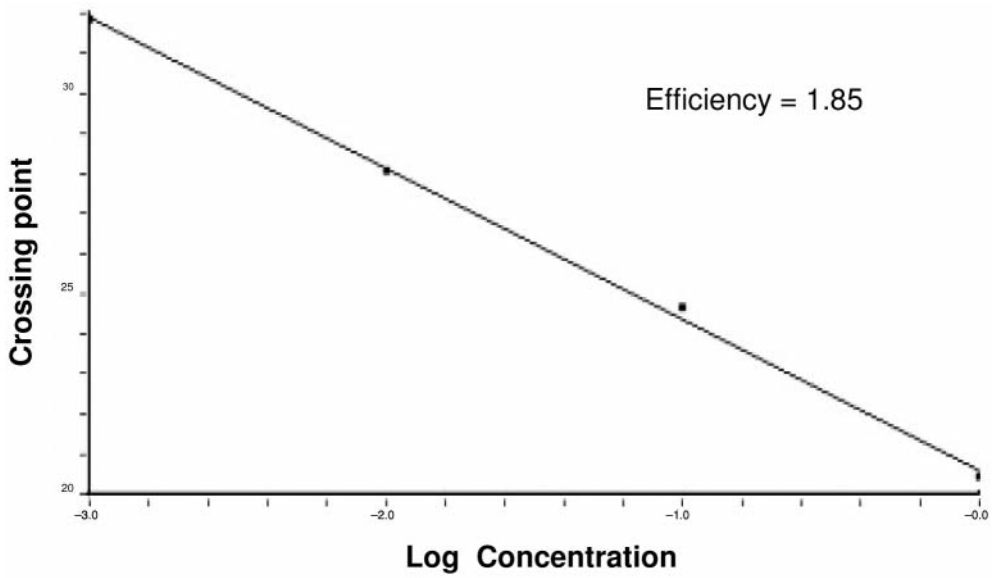

Figure 2. *Standard curve for amplification with Cyp1B1 primer. This figure shows the standard curve based on serial dilutions of cDNA amplified with Cyp1B1 primer. The Efficiency of 1.85 was calculated using the slope –3.7 of this standard curve.*

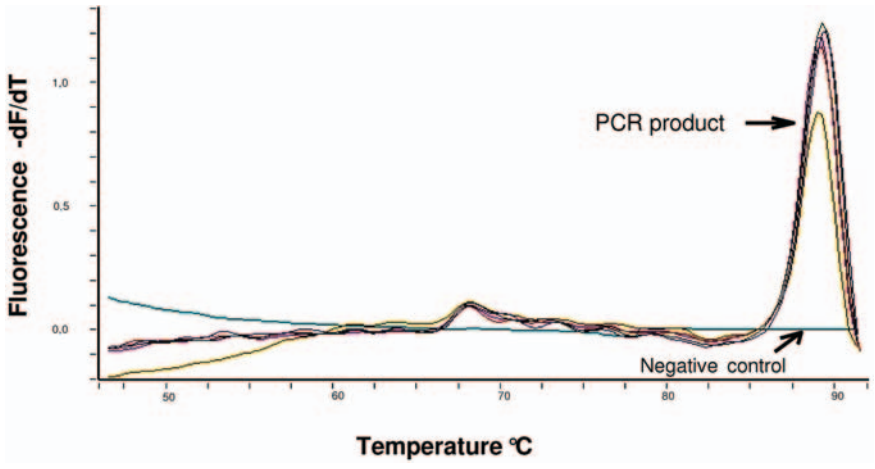

Figure 3. *Melting curve analyses for Cyp1B1 gene expression. Fluorescence data were converted to derivative melting curves by plotting the negative derivate of the fluorescence with respect to temperatur vs. temperatur [-(dF/dT) vs. T] and show one PCR product peak at 89°C visible for the target PCR. The negative control shows no visible PCR product.*

phase. The revealed slope for the standard curve (*Cyp1B1* as well as *GAPDH*) was –3.7 and the efficiency was

$E_{Cyp1B1}=10^{-1/-3.7}=1.85$
$E_{GAPDH}=10^{-1/-3.7}=1.85$

(Figures 1 and 2). Therefore the efficiency (E) for *Cyp1B1* as well as for *GAPDH* was E=1.85.

The melting curve analyses of the *Cyp1B1* PCR (see Figure 3) as well as the *GAPDH* PCR revealed only one specific product.

Subsequently the ΔCT for each sample was determined using the equation $\Delta CT=CT_{target\ gene} - CT_{housekeeping\ gene}$. Thus $\Delta CT\ A=CT\ A_{Cyp1B1} - CT\ A_{GAPDH}=0.5$ and $\Delta CT\ B=CT\ B_{Cyp1B1} - CT\ B_{GAPDH}=5.05$
Once the ΔCT for each sample was known, the ΔΔCT was calculated as follows: $\Delta\Delta CT=\Delta CT_{sample} - CT_{control}$
$\Delta\Delta CT=\Delta CT_{sample\ A} - CT_{sample\ B}=–4.55$

The relative quantification describes the change in expression of the target gene (*Cyp1B1*) in sample A (smoker) relative to that in sample B (non-smoker). The CT value for *Cyp1B1* and *GAPDH* in the smoker (A) and non-

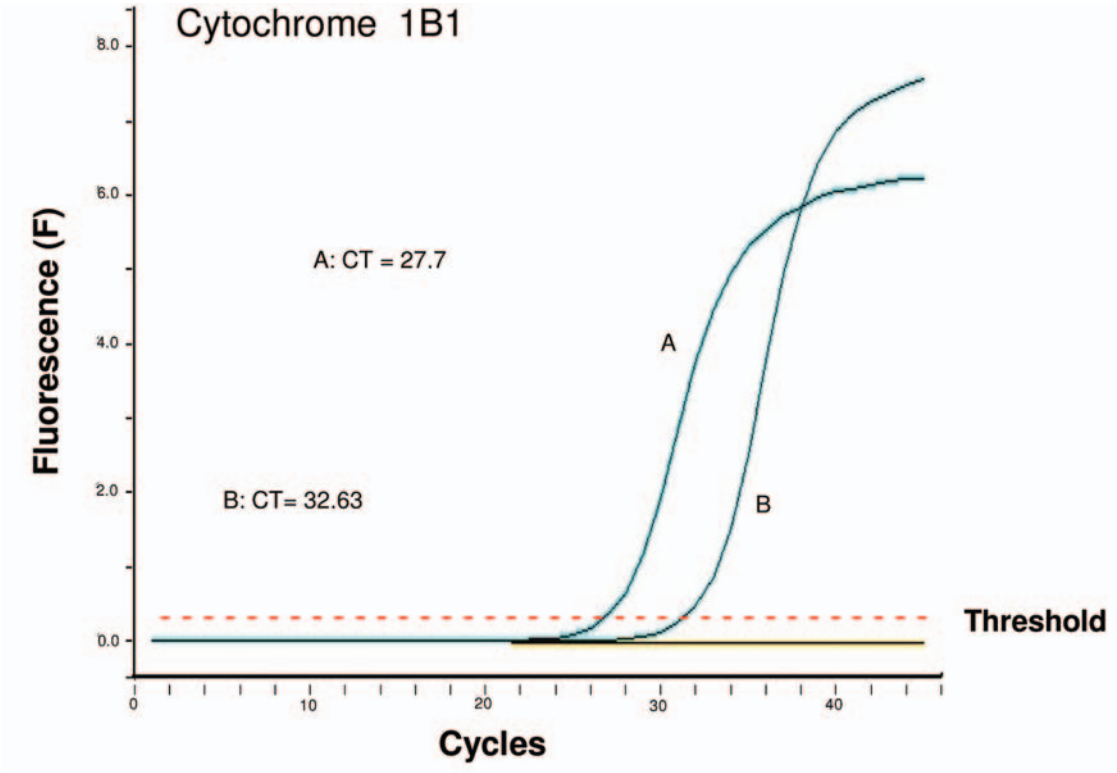

Figure 4. *Quantification of Cyp1B1 gene expression in sample A (smoker) and sample B (non-smoker).*

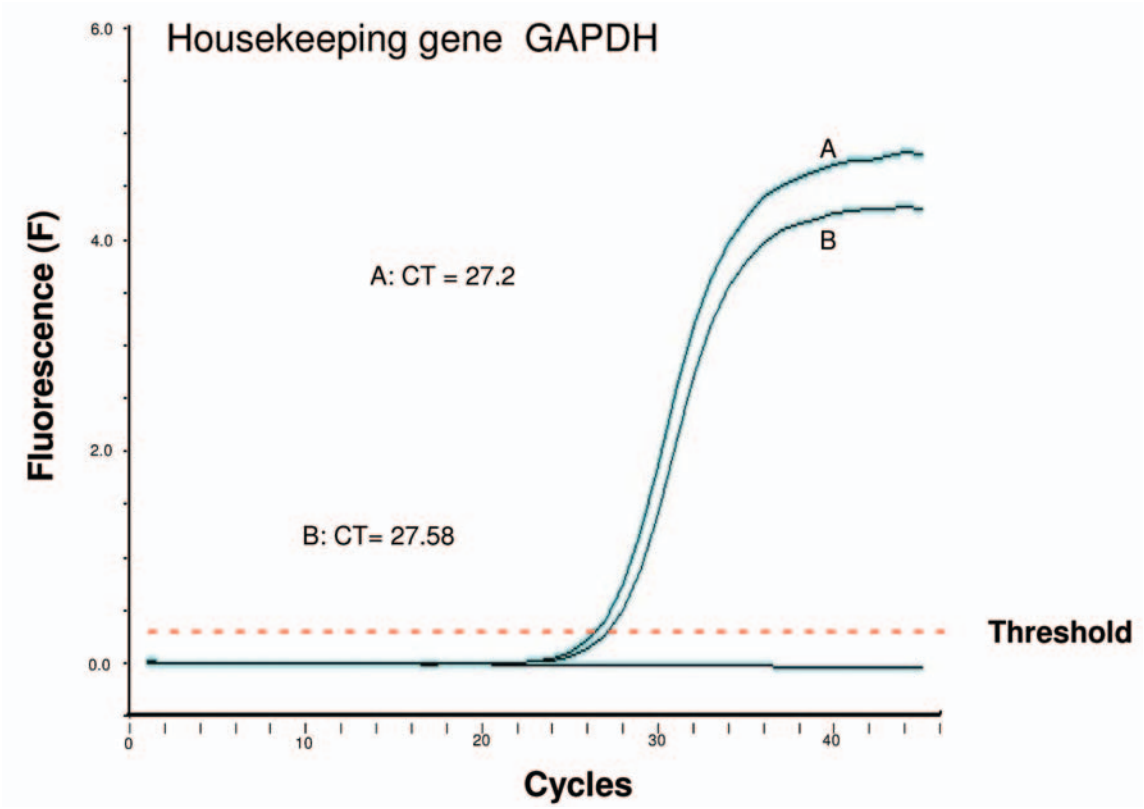

Figure 5. *Quantification of GAPDH housekeeping gene expression in sample A (smoker) and sample B (non-smoker).*

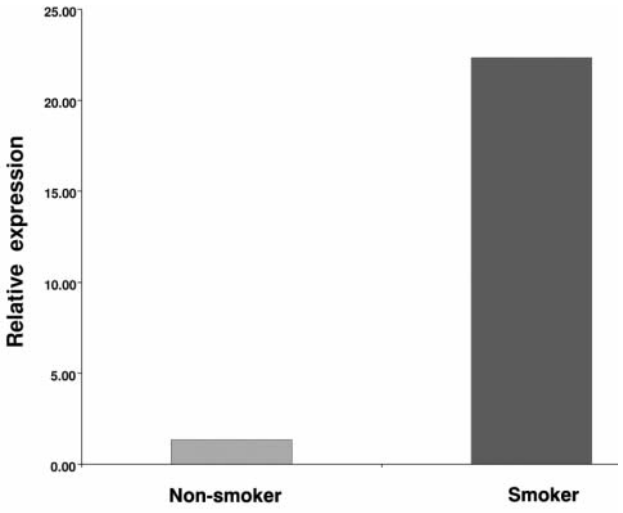

Figure 6. Relative expression of Cyp 1B1 in a smoker and non-smoker.

smoker (B) samples are shown in Figures 4 and 5. The relative expression of the target gene was estimated using the equation, relative expression=$E^{-\Delta\Delta CT}$. Thus the relative expression=$1.85^{-(-4.55)}$=16.43.

Therefore the expression of Cyp1B1 was 16.43 times higher in sample A (smoker) than in sample B (non-smoker) (Figure 6).

The relative expression of the ΔCT for each sample is displayed, according to $E^{-\Delta CT}$ *i.e.* sample A $1.85^{-0.5}$ and sample B $1.85^{-5.05}$.

Discussion and Conclusion

The relative quantification of *Cyp1B1* in the sample from a smoker compared to the sample from a non-smoker showed a more than 16-fold increase as a result of a gene expression induction. For statistical significance, a larger population needed to be screened. Nevertheless this approach showed a manageable method for examining large quantities of samples.

Using this method it would be possible to evaluate the influence of gene polymorphisms as well as exogenous events on gene expression and relevant polymorphisms could be identified. The functional impact of *Cyp1B1* mRNA expression in human lymphocytes considering interindividual differences by PAH (polycyclic aromatic hydrocarbons) induction through tobacco smoke was examined. While lung cancer risk is related to tobacco smoking (1), it may be possible to estimate the individual risk of illness when analyzing gene–environment interactions at the molecular level.

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