**TITLE**

Functional genomics approaches to studies of the Cytochrome P450 superfamily

**SHORT TITLE**

Functional genomics and the Cytochrome P450 superfamily

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**ABSTRACT**

Functional genomics approaches are widely implemented in current research and have found application in many areas of biology. This review will present research fields, novel findings and new tools developed in the cytochrome P450 field using the functional genomics techniques. The most widely used method is microarray technology, which has already greatly contributed to the understanding of the cytochromes P450 function and expression. Several focused CYP microarrays have been developed for genotyping, toxicogenomics and studies of CYP function of many different organisms. Our contribution to the CYP field by development of Steroltalk microarrays to study the cross-talk of cholesterol homeostasis and drug metabolism is also presented.

## INTRODUCTION TO FUNCTIONAL GENOMICS

The era of "omics" has opened new possibilities to the study of organisms. With many sequenced genomes and the onset of genomics, a need for functional annotation and interaction studies has emerged. Functional genomics is a field focused on function-related aspects of the genome. These include mutation and polymorphism analyses as well as a number of "omics" such as transcriptomics (gene expression), proteomics (protein expression), phosphoproteomics, glycomics, and metabolomics. All these high-throughput technologies generate large quantities of data, which need complex analyses. Systems biology computational tools enable integration of such quantitative data from different technology platforms in mathematical models and networks.

Functional genomics techniques are all whole genome, proteome and metabolome oriented. Many of these approaches have been successfully used in research of the cytochrome P450 superfamily. In this review we will limit ourselves to genome and proteome analyses in either studies focused on cytochromes P450 superfamily or on different biological processes in which cytochromes P450 are involved. Whole genome methods include DNA-microarrays, SAGE (serial analysis of gene expression), SADE (SAGE adaptation for downsized extracts), suppression subtractive hybridization and construction of subtractive cDNA library, representational difference analysis and others (Hunt, 2000). Proteome analyses methods are tissue, protein microarrays and two-dimensional gel electrophoresis coupled with mass spectrometry.

## GENOTYPING OF CYTOCHROMES P450 USING DNA-MICROARRAY TECHNOLOGY

Genotyping of cytochromes P450 is important in pharmacogenetic studies of how genetic variation influences drug response by correlating gene expression or single-nucleotide polymorphisms with drug's efficacy and toxicity. Pharmacogenomics is a whole genome approach to genotyping using microarray technology. In 2000 a first paper on genotyping of *CYP2D6* alleles in Korean population using commercial microarrays has been published (Yoon, 2000). Later, two custom microarrays for detection of *CYP2C9* and *CYP3A4* SNPs (single nucleotide polymorphism) were developed (Wen, S., 2004; Wen, S. Y., 2003). An important breakthrough in the field happened with FDA approval of the first diagnostic microarray AmpliChip CYP450 developed by Roche Diagnostics in cooperation with Affymetrix Company (http://www.amplichip.us/). This microarray enables identification of individual's genetic variation in two drug metabolism genes, *CYP2D6* and *CYP2C19*, from the blood. The two CYPs are responsible for metabolism of 25% of prescribed drugs and the enzyme activity is largely affected by inherited variations. This diagnostic microarray is a first step toward personalized medicine, where knowledge of a patient's genotype ensures an optimal drug therapy with maximal efficacy and minimal adverse effects.

Recently a microarray-based platform (thin-film biosensors chip) for genotyping of human *CYP7A1* gene was described (Nakamoto, 2006). CYP7A1 is the rate limiting enzyme of the bile acid biosynthesis and its polymorphisms have been associated with a number of metabolic disorders, including atherosclerosis, gallstone disease, hypercholesterolemia and others. The purpose of this study was to analyze linkage

disequilibrium patterns and haplotype blocks in *CYP7A1* gene. These results would lead to a better design of genetic association studies, which would correlate genetic variations in *CYP7A1* gene to diseases.

## EXPRESSIONAL PROFILING OF CYTOCHROMES P450 USING MICROARRAY TECHNOLOGY

The majority of microarray analyses in the field of the cytochrome P450 superfamily involve gene expression studies. Cytochrome P450 superfamily is a versatile protein family, which is involved in many different physiological and pathophysiological processes. Therefore research fields which use microarrays for studies of cytochrome P450 gene expression span from toxicogenomic studies to gene function discovery (Figure 1).

Toxicogenomics is a research field that uses gene expression profiles to understand and associate a particular mechanism of toxicity with a studied compound. Findings from this field are applicable in all fields of drug research and development, where a potential toxicity of a new drug or any other chemical is being tested. Several custom human and murine microarrays have been developed over the years, many of them by different companies (Bartosiewicz, 2000; de Longueville, 2002; Gerhold, 2001; Hong, 2003; Kier, 2004; Pennie, 2000; Yauk, 2006).

Over two hundred papers have already been published on studies of transcriptional regulation of cytochromes P450 using microarray technology. Main research focuses of these studies are:

- How different xenobiotics and nuclear receptors affect gene expression of drug metabolizing cytochromes P450 (Hartley, 2004; Ishida, 2002; Kiyosawa, 2003; Rae, 2001; Rosenfeld, 2003; Ueda, 2002; Wu, 2004).
- How is cytochromes P450 expression affected by physiological processes such as pregnancy (Ejiri, 2005; He, 2006), and pathophysiological processes like cancer (Achary, 2000; Delsite, 2002; Vondracek, 2002; Yeh, 2006).
- How are cytochromes P450 regulated by different physiological factors such as hormones (Ahluwalia, 2004; Henriquez-Hernandez, 2007), diet (Berger, 2006; Deng, 2004; Kreeft, 2005; Ricketts, 2007), cell type (Braeuning, 2006), and genetics (Becker, 2004; Dyck, 2003; Kreeft, 2005; Phan, 2002).

These studies used either commercial whole-genome or academic microarrays. Also two custom cDNA microarrays were developed using cDNA library from suppression subtraction hybridizations in studies of obesity development and leptin signaling (Liang, 2001; Van Schothorst, 2005).

Application of microarray technology in the cytochrome P450 field has also extended to other organisms beside mammals. One of the research areas is ecotoxicogenomics, the goal of which is to develop tools for identification of possible toxic environmental pollutants using fish (*Platichthys flesus*) (Sheader, 2006; Williams, 2006) or roundworm (*Caenorhabditis elegans*) (Custodia, 2001) as model organisms. Cytochromes P450 are also involved in insect resistance to certain insecticides. Therefore, an important application of microarray technology is in the field of insecticide resistance in mosquito *Anopheles gambie* (David, 2005; Vontas, 2005) and fruit fly *Drosophila melanogaster* (Jensen, 2006; Le Goff, 2003; Pedra, 2004; Willoughby, 2006). Such studies showed a developmental (Strode, 2006), age

(McElwee, 2004) and sex (Le Goff, 2006) dependent expression of detoxification genes in invertebrates.

Discovery of new cytochromes P450 and their functional annotation is another application of the microarray technology. For example, a new insect CYP306A1 was discovered and its function in the steroid hormone biosynthesis in silkworm *Bombyx mori* was shown (Niwa, 2004). CYP707A was implicated in the abscisic acid catabolism in *Arabidopsis* (Kushiro, 2004), and CYP82E2 was implicated in the production of more carcinogenic tobacco in *Nicotiana tabacum* (Siminszky, 2005). Two custom *Arabidopsis thaliana* microarrays have been developed and used in focused studies of cytochrome P450 gene expression (Narusaka, 2004; Xu, 2001).

The study of host-pathogen interactions also applies microarray technology. In plants, CYPs are involved in host defense systems (Glawischnig, 2004; Kim, 2006; Narusaka, 2004; Voelckel, 2004), and in mammals expression of CYPs is modulated by pathogen infection (Boutin, 2004; Fadl, 2007; Fukushima, 2003; Hajjou, 2005). For detoxification studies a custom CYP microarray from white rot fungus *Phanerochaete chrysosporium* was also developed (Doddapaneni, 2005). This fungus is very interesting because it has a large repertoire of cytochromes P450 (at least 150 different), many with unknown functions, and has ability to detoxify a number of chemical pollutants.

## ANALYSES OF CYTOCHROME P450 GENE EXPRESSION USING OTHER FUNCTIONAL GENOMICS METHODS

Beside DNA-microarrays analyses, cytochromes P450 gene expression has been studied using other functional genomics methods. Several studies have successfully used suppression subtractive hybridization to evaluate gene expression and also to

produce cDNA microarrays. Many are in the field of ecotoxicogenomics and examined how different environmental pollutants effect fish gene expression (Reynders, 2006; Straub, 2004; Volz, 2005; Williams, 2006). Two studies were performed in the field of plant cytochrome P450, investigating host-pathogen interactions (Kong, 2005; van Munster, 2007) and adaptations to salt stress (Gu, 2004). Serial analysis of gene expression (SAGE) was used in studies of cancer research (Aldaz, 2002; Aung, 2006), physiological processes (Blomberg, 2005; Friedland, 2006), insecticide resistance (Guerrero, 2007), and pollutant toxicity (Ekman, 2003). Few studies used representational difference analysis to study gene expression of mammalian *CYP2S1*, *CYP3A*, *CYP4F1* and *CYP4B1* (Harris, 1998; Melia, 1998; Rivera, 2002).

## PROTEOMIC ANALYSES OF CYTOCHROMES P450

Proteomic analyses are oriented toward measurement of differential expression of cytochromes P450 isozymes. There are two approaches: the first one uses specific antibodies to detect certain cytochromes P450 using tissue or protein microarrays; the second approach uses mass spectrometry for protein detection.

Tissue microarrays have been used to determine cytochrome P450 protein levels in cancer tissues. These studies showed that certain cytochromes P450 are independent markers of prognosis in osteosarcoma (Dhaini, 2003), colorectal (Kumarakulasingham, 2005) and ovarian cancer (Downie, 2005). However, this approach has limited potential because it needs development of isozyme specific antibodies, which is not always possible (Edwards, 2003; Galeva, 2003).

Usage of mass spectrometry for detection and identification of proteins provides a more reliable and sensitive approach (Galeva, 2003). Prior to mass spectrometry

detection, protein mixture have to be fractionated using either one or two-dimensional gel electrophoresis or high pressure liquid chromatography (HPLC). An excellent paper summarizing methods in proteomic analysis of cytochromes P450 has been published recently (Wang, 2006). Research studies using these approaches measured the presence of different isozymes in the liver (Alterman, 2005); sex-related differences (Nisar, 2004); or isozyme expression after treatment with TCDD (2,3,7,8-tertrachlorodibenzo-p-dioxin) (Sarioglu, 2006), TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene) (Lane, 2007) and phenobarbital (Galeva, 2003; Jenkins, 2006; Kanaeva, 2005). Proteomic analyses of cytochrome P450 have been applied also in cancer research, where levels of cytochromes P450 were being determined in colorectal metastasis (Lane, 2004; Petushkova, 2006) and other diseases (Low, 2004; Zhang, 2007). Proteome analysis of plant cytochromes P450 has been preformed in maize roots (*Zea mays* L.) (Requejo, 2005).

## THE STEROLTALK MICROARAYS: A NEW TOOL IN EXPRESSION STUDIES OF CYTOCHROME P450 SUPERFAMILY

In the field of toxicogenomics many focused DNA-microarrays measuring gene expression of cytochromes P450 have been developed. It has been rationalized before why custom focused microarrays are more appropriate for studies of gene expression oriented toward a specific hypothesis (Yauk, 2006). However, these microarrays contain mainly cytochromes P450 involved in the exogenous metabolism. There is an increasing amount of data showing that certain xenobiotics affect gene expression of not only the drug metabolism, but also of other metabolic pathways in the murine liver (Fletcher, 2005; Kiyosawa, 2004; Ueda, 2002). Therefore, within the frame of the European Union project Steroltalk, our group has developed mouse and human

cDNA microarrays, which represent a bridge between exogenous and endogenous cytochrome P450 metabolism. The Steroltalk microarray is dedicated to studies focused on cholesterol homeostasis and drug metabolism. The human and mouse Steroltalk microarray includes probes from all the families of the cytochrome P450 superfamily. Present are also members from nuclear receptor superfamily, which are important regulators of cytochrome P450 gene expression; different transporters of xenobiotics, cholesterol and bile acids; all genes of cholesterol biosynthesis, transcription factors SREBP (sterol response element binding protein) involved in cholesterol feedback loop, and genes involved in plasma cholesterol transport. Genes involved in circadian rhythm, glucose and fatty acid homeostasis and signaling pathways regulating these two processes are also included. The Steroltalk microarray enables focused studies on how cholesterol, xenobiotics or any other physiological or pathophysiological factor affect the liver metabolism and homeostasis (Figure 2). A prototype microarray Sterolgene v0 has already been used in studies of how fasting and high cholesterol diet, phenobarbital and TNF-α (tumor necrosis factor alpha) treatment affect cholesterol homeostasis and drug metabolism in mouse (Režen et al. unpublished; Fon Tacer et al, unpublished). Using the Sterolgene v0 microarray we have shown that fasting down-regulates cholesterol biosynthesis and drug metabolism, but up-regulates bile acid synthesis. The inflammatory cytokine TNF-α reverses these adaptations and up-regulates cholesterol biosynthesis, down-regulates bile acid synthesis and drug metabolism.

## CONCLUSIONS

Functional genomic methods have been successfully used in the cytochrome P450 field. The most successful areas are pharmacogenomics, toxicogenomics,

ecotoxicogenomics and cancer research. Novel findings using microarrays are especially in studies of CYPs expression under different endogenous and exogenous factors, and functional annotations of new cytochromes P450. Several novel tools were developed for genotyping (CYP SNP chips), toxicogenomic studies (CYP toxo chips), and studies of cross-talksand interactions (Steroltalk microarray). Many microarrays were also developed to enable CYP studies of different organisms (fish, plant, insects, roundworm, etc) in toxicogenomics and ecotoxicogenomics. However, other 'omics' fields, such as are phosphoproteomics, metabolomics, etc. are still lagging behind, waiting to be explored in the near future.

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## FIGURES LEGENDS

**Figure 1:** Different research fields using functional genomics methods in studies of cytochrome P450 superfamily.

**Figure 2:** Steroltalk microarray in studies of liver metabolic homeostasis.

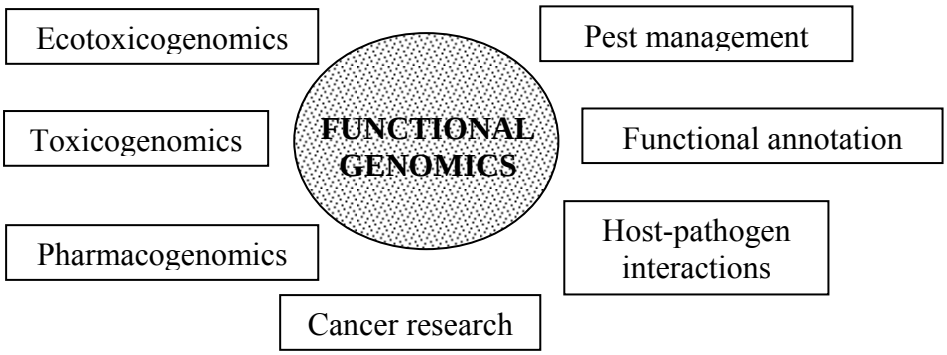


**Figure 1**

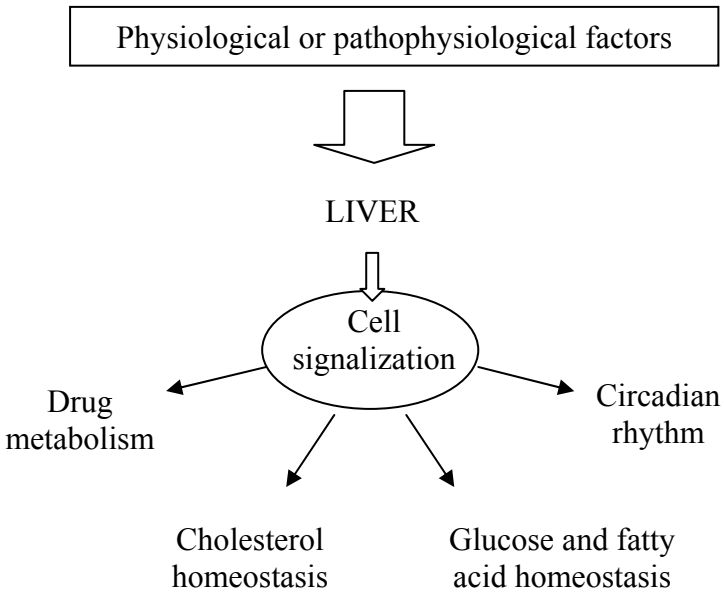


**Figure 2**