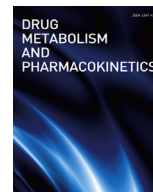




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## Regular Article

## Sex- and age-dependent gene expression in human liver: An implication for drug-metabolizing enzymes

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## ABSTRACT

Sex and age differences in hepatic expression of drug-metabolizing enzyme genes could cause variations in drug metabolism, but has not been fully elucidated, especially in Asian population. In this study, the global expression of human hepatic genes was analyzed by microarrays in 40 Japanese subjects (27 males and 13 females). Thirty-five sex-biased genes were identified ( $P < 0.005$ ). Whereas, 60 age-biased genes in two age groups,  $<60$  years and  $\geq 70$  years ( $P < 0.001$ ), were identified in males. By Gene Ontology analysis, the sex-biased genes were related to protein catabolism and modification, while the age-biased genes were related to transcription regulation and cell death. Quantitative polymerase chain reaction confirmed the female-biased expression of drug-metabolizing enzyme genes *BChE*, *CYP4X1*, and *SULT1E1* ( $\geq 1.5$ -fold,  $P < 0.05$ ). Further analysis of drug-metabolizing enzyme genes indicated that expression of *CYP2A6* and *CYP3A4* in females in the  $\geq 70$  age group was less than in the  $<60$  age group ( $\geq 1.5$ -fold,  $P < 0.05$ ), and this trend was also observed for *PXR* expression in males ( $\geq 1.5$ -fold,  $P < 0.05$ ). The results presented provide important insights into hepatic physiology and function, especially drug metabolism, with respect to sex and age.

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## 1. Introduction

The liver is the major drug-metabolizing organ, in which sex- and age-dependent differences have been observed in human drug metabolism [1,2]. Cytochrome P450 (CYP) is a gene family comprising 57 functional genes encoding enzymes that metabolize drugs, foreign chemicals, and endogenous compounds, and enzymes involved in cholesterol metabolism, bile acid biosynthesis,

synthesis and metabolism of steroids and vitamin D3, and retinoic acid hydroxylation [3]. Within the CYP family, CYP3A4 is considered to be one of the most important drug-metabolizing enzymes, as CYP3A4 (together with CYP3A5) accounts for more than half of the total CYP content in human liver and metabolizes more than half of all prescription drugs [4]. CYP3A-mediated erythromycin *N*-demethylation is higher in women than in men [5]. Moreover, females have significantly higher CYP3A4 content and CYP3A4-mediated ifosfamide *N*-dechloroethylation [6]. Gene expression, protein expression, and activity of human CYP3A4 are higher in female liver than in male liver [7]. These results suggest that sex dimorphism in CYP3A4-mediated activities is at least partly attributable to the difference in CYP3A4 expression between males and females.

Aging is essential to understanding the human physiology and the pathology of diseases in humans. Recent studies of the

*Abbreviations:* BChE, butyrylcholinesterase; CYP, cytochrome P450; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PXR, pregnane X receptor; RT-PCR, reverse transcriptase PCR; SULT, sulfotransferase.

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molecular mechanism of aging have revealed that oxidative damage caused by free radicals in the mitochondria [8] and telomere shortening [9] play roles in aging. Moreover, genetic studies have shown that mutations in insulin-like growth factor receptor, tyrosine kinase receptor, DNA helicase, or p53 alter animal lifespan [10]. Microarray analysis successfully identified age-biased genes expressed in muscle [11] and brain [12] in mouse, and retina [13] and muscle [14] in human. Drug metabolism is also affected by aging, as clearance generally decreases with age, and CYP2E and CYP3A content is lower in the elderly [2]. In contrast to phase I CYP enzymes, drug metabolism mediated by phase II enzymes does not differ in the elderly [2].

Identification of sex-biased genes could help to better understand physiological differences between males and females, and the molecular basis of such differences. Global expression analysis in mouse revealed that thousands of genes are expressed differentially between males and females, in a variety of tissues, including brain, liver, fat, and muscle [15]. Another study also identified sex-biased genes in kidney, liver, and hypothalamus tissues, many of which play roles in drug and steroid metabolism, including CYPs [16]. Similarly, in human, sex-biased genes have been reported in tissues such as heart [17,18], kidney [19], skeletal muscle [20,21], and brain [22]. Although sex-biased genes have been also found in human liver [23–25], those studies analyzed the samples from Caucasians or Hispanics. Considering the ethnic variability of drug metabolism, it is important to investigate sex-biased genes in Asians.

In this study, sex- and age-biased genes in human liver were identified by microarray analysis using 27 male and 13 female livers of Japanese subjects. These genes were classified functionally using Gene Ontology. To assess the impact of the results on drug metabolism, expression of these and other drug-metabolizing enzyme genes (mainly CYP genes) was analyzed by quantitative polymerase chain reaction (qPCR). These data collectively describe the sex and age dependent expression of genes involved in human liver physiology, especially drug metabolism.

## 2. Materials and methods

### 2.1. Materials

Oligonucleotides were synthesized by Invitrogen (Tokyo, Japan), and TaqMan probes were synthesized by Applied Biosystems (Foster City, CA) or Biosearch Technology Japan (Tokyo, Japan). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO) or Wako (Osaka, Japan) unless otherwise specified.

### 2.2. Tissue samples and RNA extraction

Human liver samples were collected from 40 patients undergoing hepatic surgery (Supplemental Table 1). Every possible effort was made to collect only normal tissue, which was frozen in liquid nitrogen as quickly as possible. This study was reviewed and approved by the institutional ethical review committee and informed consent was received from all patients. Total RNA was extracted from liver samples using TRIzol (Invitrogen, Carlsbad, CA), treated with DNaseI (Takara, Tokyo, Japan), and purified using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols.

### 2.3. Microarray analysis

The control pool of RNA samples contained an equal amount of total RNA extracted from each human liver sample. Ten micrograms of pooled total RNA was reverse transcribed and labeled with Cy3.

Each RNA sample was also reverse transcribed and labeled with Cy5. A description of the cDNA microarrays used and hybridization and transcript quantification procedures have been described previously [26,27]. Sex-biased genes were identified using Random Permutation Test [28]. To avoid the bias potentially created by the sample size difference between males and females, male samples were randomly divided into two groups, Group 1 (14 males,  $64.9 \pm 9.5$  years) and Group 2 (13 males,  $64.9 \pm 12.1$  years). The female group was compared with each male group, and differentially expressed genes were identified in accordance with two criteria: First, a difference in the median expression between two groups greater than 1.4-fold; and, second, a statistically significant difference between two groups with random permutation tests ( $P < 0.005$ ). Genes meeting both criteria were designated as sex-biased genes.

A combination of random permutation test and pattern analysis was carried out to identify age-biased genes. The menopausal status of females was not known, therefore only samples from male patients were analyzed. The males were divided into three age groups, <60 years ( $n = 7$ ), 60–69 years ( $n = 9$ ), and  $\geq 70$  years ( $n = 11$ ). First, we selected genes with more than two fold difference in median gene expression between the male <60 and  $\geq 70$  age groups. Second, a random permutation test was performed, and we chose genes with  $P < 0.001$ . For these genes, pattern analysis was carried out for three age groups, <60 years, 60–69 years, and  $\geq 70$  years, to select the genes with expression levels that decrease or increase gradually with age as age-biased genes, and, those showing an intermittent pattern of expression in the 60–69 age group.

### 2.4. Gene Ontology analysis

The 175 sex-biased genes ( $\geq 1.4$ -fold,  $P < 0.05$ ), and 720 age-biased genes in the male <60 and the  $\geq 70$  age groups ( $\geq 2.0$ -fold,  $P < 0.05$ ) were subjected to GO classification. Overrepresented functional categories were determined using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2.0 (<http://david.abcc.ncifcrf.gov/>) according to the protocol. Significantly overrepresented categories for Biological process, Molecular function, and Cellular process were selected ( $P < 0.05$ ).

### 2.5. Quantitative PCR

Expression of drug-metabolizing enzyme genes was measured by qPCR with all the samples used for microarray analysis. Real-time reverse transcription (RT)-PCR was carried out as described previously [29] using TaqMan Universal PCR Master Mix (Applied Biosystems) or SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers and probes (Supplemental Table 2). Expression levels were normalized to the 18S ribosomal RNA level based on three independent amplifications. Statistical significance of differences in gene expression levels was determined using a two-tailed unpaired Student's *t* test. Values were presented as mean  $\pm$  S.D. of all liver samples, and were considered significant if  $P$  was  $< 0.05$ .

## 3. Results

### 3.1. Identification of sex- and age-biased genes

This study included 27 males ( $64.9 \pm 10.6$  years) and 13 females ( $56.3 \pm 15.1$  years), Japanese patients who underwent hepatic surgery for colorectal, hepatocellular, or other types of carcinoma, and for other reasons such as hepatic calculi (Supplemental Table 1). Because the samples from healthy subjects were not

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