Next generation sequencing of sex-specific genes in the livers of obese ZSF1 rats

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ABSTRACT

Type 2 diabetes induces pathophysiological changes in the liver. The aim of this study was to identify differently expressed genes in the livers of male and female ZSF1 rats (ZDFxSHHF-hybrid, generation F1), a model for type 2 diabetes.

Gene expression was investigated using next-generation sequencing (NGS). Selected candidate genes were verified by real-time PCR in the livers of obese and lean rats.

103 sex-different genes, associated to pathways "response to chemical stimulus", "lipid metabolism", and "response to organic substance", were identified. Male-specific genes were involved in hepatic metabolism, detoxification, and secretion, e.g. cytochrome P450 2c11 (Cyp2c11), Cyp4a2, glutathione S-transferases mu 2 (Gstm2), and Slc22a8 (organic anion transporter 3, Oat3). Most female-specific genes were associated to lipid metabolism (e.g. glycerol-3-phosphate acyltransferase 1, Gpam) or glycolysis (e.g. glucokinase, Gck).

Our data suggest the necessity to pay attention to sex- and diabetes-dependent changes in pre-clinical testing of hepatic metabolized and secreted drugs.

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1. Introduction

Type 2 diabetes is associated with histopathological and pathophysiological changes in the human liver [1]. A high prevalence to develop nonalcoholic fatty liver disease, liver cirrhosis, and hepatocellular carcinoma was identified in patients with type 2 diabetes [1–3]. In rat liver, oxidative stress, pathological changes of hepatic architecture, inflammation and focal necrosis were shown to be induced in early stages of diabetes [4].

The liver is the major organ responsible for drug metabolism and drug clearance. Women have a greater risk of suffering adverse drug reactions (ADRs) than men [5]. In 427 human liver samples, 80 sex-dependently expressed genes were identified and genes encoding hepatic drug transporter, organic anion transporter 2 (OAT2; gene name: SLC22A7) and organic cation transporter 1 (OCT1; SLC22A1), were found to be female-specifically expressed [6]. In patients with polymorphisms in SLC22A1 lower effects of metformin, an antidiabetic drug and OCT1 substrate, were observed [7]. Other antidiabetic drugs, such as sitagliptine, repaglinide, and rosiglitazone, inhibited OCT1-mediated transport of metformin in vitro [8,9]. In our previous study,

only a weak inhibition of OAT2-mediated cGMP uptake by sitagliptin was observed, and OAT2 function was not affected by miglitol [10]. In contrast, sitagliptin transport was shown to be OAT3-mediated [11]. Sex-differences were found for the expression of genes involved in several hepatic drug detoxification and metabolism pathways, in human liver as well as in several rat models for diabetes [6,12,13]. Furthermore, it was postulated that the increased bile acid pool in diabetic rats stimulated the transcription of anion transporters in the sinusoidal membrane of hepatocytes [14].

The aim of this study was to identify transcriptional differences between male and female rat livers, with the focus on hepatic drug metabolizing enzymes and drug transporters from the Slc22a gene family. For this purpose, liver samples of male and female obese ZSF1 rats (ZDFxSHHF-hybrid, generation F1) were investigated using next-generation sequencing (NGS). ZSF1 rats are first generation hybrid between rat strains with different mutations in the leptin receptor gene, Zucker diabetic fatty (ZDF) rats and spontaneously hypertensive heart failure (SHHF) rats [15]. The initial designation of this rat model was ZDFxSHHF-hydrid rats [16]. ZSF1 rats are an established animal model for type 2 diabetes, hyperlipidemia, nephropathy, and metabolic syndrome, exhibiting symptoms comparable to humans [15,17]. Male obese (diabetic) ZSF1 rats were reported to have significantly higher body weight, liver weight, plasma cholesterol, and plasma triglycerides at 8 and 41 weeks of age compared to their lean (non-diabetic) controls

[15,17–19]. At 8 weeks of age, lean and obese male ZSF1 rats have similar blood glucose level [18]. Elevated blood glucose levels were observed between 12 and 41 weeks of age in obese, but not in lean male ZSF1 rats [17,18]. In general, liver weight, blood glucose, plasma cholesterol, and plasma triglycerides were lower in obese female compared to obese male ZSF1 rats, but higher compared to lean males at 41 weeks of age [17]. Sex-dependent changes of hepatic gene expression were not investigated in lean and obese ZSF1 rats so far.

2. Material and methods

2.1. Animals and liver preservation

Obese male and female ZSF1 (ZSF1-Lepr^{fa}Lepr^{cp}/Crl) rats, and an equivalent number of lean controls for each sex were kept in the animal facility of Charles River Laboratories in New York State under conventional housing conditions (22 °C, 55% humidity, and 12 h day/night cycle) with free access to water and conventional rat chow till 16 weeks of age. The livers of ZSF1 rats were removed post mortem in accordance to US federal law, conserved in RNAlater® (Life Technologies, Germany), and shipped to our laboratory. For the experimental design ≥6 rat individuals for each group to be compared were tested, which is known for achieving robust RNA-Seq results [20]. Technical batch effects were minimized by preparing samples, sequencing libraries and sequencing run at the same time, respectively.

2.2. RNA isolation, cDNA library preparation, and next-generation sequencing (NGS)

For NGS experiments total RNA was isolated from the livers of 6 male and 6 female obese ZSF1 rats using TRIzol® reagent (Life Technologies) according to the manufacturer's recommendations and was digested with RNase-Free DNase I in order to remove DNA contamination (Sigma Aldrich, Germany). Quantity and quality of extracted RNA were analyzed using microfluidic electrophoresis (Bioanalyzer; Agilent Technologies USA) and NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, USA), following the manufacturer's protocol. 800 ng of each total RNA samples with a RNA integrity number (RIN) >8 was used as starting material. TrueSeq RNA Sample Preparation Kit (Illumina, Netherlands) was used to prepare samples for NGS analysis. Accurate quantitation of cDNA libraries was performed by using the QuantiFluorTM dsDNA System (Promega, Germany). The size range of cDNA libraries was determined applying the DNA 1000 chip on the Bioanalyzer 2100 from Agilent (280 bp). cDNA libraries were amplified and sequenced by using the cBot and HiSeq 2000 from Illumina (SR, 1 × 50 bp, 10–45 million reads per sample). Sequence images were transformed with Illumina software BaseCaller, which were demultiplexed with CASAVA (version 1.8.2). Quality check was done via FastQC (Babraham Bioinformatics, United Kingdom).

2.3. Gene expression analysis

The sequenced reads were mapped to the rat UCSC reference genome (rn4) [21] using bowtie2 (2.0.2) [22]. Conversion of SAM to BAM and corresponding sorting was done via SAMtools [23]. Counting the reads to each gene was done via HTSeq (0.5.3p3) (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). Normalization of read counts to the library size, estimation of dispersions and testing for differentially expressed (DE) genes based on a statistical test assuming negative binomial data distribution were computed in the R/Bioconductor environment (version 2.15.2) loading DESeq (1.10.1) [24] and biomaRt (2.14.0) [25] packages. DESeq normalized data were shown to produce the smallest coefficient of variation in terms of diverse library sizes and compositions compared to other common analysis methods [26]. Significant sex-specific genes were determined as log2 fold change (log2FC) >1 or <− 1, base mean <1000, and false discovery rate (FDR)-corrected p value <0.05 with multiple testing correction according to Benjamini and Hochberg [27]. The data were generated conforming to MIAME standards and have been submitted to the Gene Expression Omnibus (GEO) database (GSE57598).

2.4. cDNA synthesis and TaqMan® real-time PCR

For real-time PCR experiments RNA was used from the livers of 6 lean and obese males, and 8 lean and obese female ZSF1 rats. Reverse transcription of RNA was performed using Superscript™ II Reverse Transcriptase (Life Technologies) and Oligo dT-Primers (Eurofin MWG Operon, Germany). TaqMan® Master Mix and TaqMan® Gene Expression Assays (Life Technologies) were used for the analysis of genes of interest: glutathione S-transferase mu 2 (Gstm2), Rn00598597_m1; 11β-hydroxysteroid dehydrogenase type 1 (Hsd11b1), Rn00567167_m1; cytochrome P450 4a2 (Cyp4a2), Rn01417066_m1; Cyp2c11, Rn01502203_m1; leukemia inhibitory factor receptor (Lifr), Rn00579104_m1; 3-oxo-5-alpha-steroid 4-dehydrogenase 1 (Srd5a1), Rn00567064_m1; glucokinase (Gck), Rn00561265_m1; glycerol-3-phosphate acyltransferase 1 (Gpam), Rn00568620_m1; Oct1, Rn00562250_m1; Oat2, Rn00585513_m1; Oat3, Rn00580082_m1. Hypoxanthine phosphoribosyltransferase 1 (Hprt1, Rn01527840_m1) and β-actin (Rn00667869_m1) were analyzed as housekeeping genes for sample normalization. Real-time PCR conditions were: 2 min at 50 °C followed by 10 min at 95 °C and 40 amplification cycles (95 °C for 15 s and 60 °C for 60 s) using Mx3000P™ real-time PCR cycler (Agilent Technologies). The amplification efficiencies of the TaqMan® Gene Expression Assays were 100 ± 10%, in accordance with manufacturer's information. The data were analyzed as ΔCt = housekeeping gene (Hprt1)-gene of interest, according to the $2^{-\Delta\Delta Ct}$ method [28].

2.5. Statistical analysis of real-time PCR data

Real-time PCR data are presented as mean ± SEM. For multiple comparisons, males vs. females, and lean vs. obese ZSF1 rats, the statistical analysis was performed with two-way analysis of variance (ANOVA) and followed by Bonferroni test (GraphPad Prism 4, version 4.03; GraphPad Software, USA). Differences were considered as significant at the level of p < 0.05.

3. Results

Sex-dependent expression of all investigated genes in the livers of obese ZSF1 rats was summarized in the volcano plot, showing statistically significant female- and male-specific genes marked in red (Fig. 1A). The principal component analysis (PCA) plot shows individual variations of liver samples revealing no differences between male and female livers (Fig. 1B). Interestingly, the gene expression of one male liver (L4) was found to be different in comparison to other males (L1, L2, L3, L5, and L6) (Fig. 1B).

Within male and female liver samples from obese ZSF1 rats, 469 out of 15,027 genes sequenced were sex-dependently expressed. The exclusion of genes with low expression (base mean < 1000) resulted in 103 sex-differently expressed hepatic genes in obese male and female ZSF1 rats (Table 1).

The largest portion of sex-dependent genes was localized on chromosome 1, followed by genes localized on chromosome 2 and 5 (Fig. 2). Most female-specific genes were localized on chromosome 1 (42.5% of 40 female-specific genes) and the highest number of male-specific genes were localized on chromosome 5 (19% of 63 male-specific genes) (Fig. 2).

In addition, candidate genes were analyzed using Gene Ontology (GO) [29]. The highest number of sex-dependent genes was associated to the GO terms "response to chemical stimulus", "lipid metabolic