Original Paper

Individual Differences in Gene Expression in Primary Cultured Renal Cortex Cells Derived from Japanese Subjects

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We used microarrays to examine individual-based differences in gene expression in primary cultures of renal tubular cells derived from Japanese subjects. The subjects had solitary tumors in the kidney or urinary tract, which were diagnosed pathologically as renal cell carcinoma or transitional cell carcinoma. Renal tissue samples collected from a non-tumorous portion of the tissue were regarded as normal tissues, as there were no abnormal microscopic findings and no evidence of renal dysfunction from the clinical laboratory data. The genome-wide gene expression profiles of nine human renal cell cultures were analyzed using the Affymetrix GeneChip HG-U133A and HG-U133B arrays. Approximately 8,500 transcripts exhibited significant differential expression \((p < 0.05)\) among the subjects, and the coefficients of variation for 1,338 transcripts were greater than 50%. Some of these transcripts encode drug-metabolizing enzymes (e.g., UGT1A8 and UGT1A9) or sodium/phosphate co-transporters (e.g., PDZK1). These data provide the basis for toxicogenomic studies using primary cultured renal cortical cells from Japanese subjects.

1. Introduction

We use toxicogenomics to clarify toxicity mechanisms and to identify biomarkers that predict kidney toxicity. Toxicogenomics involves the application of microarrays to analyzing gene expression patterns after exposure to toxic compounds, which can contribute to establishing the functional profile of the genome and the discovery of useful markers of toxicity \(^1\),\(^2\). In addition, the information derived from toxicogenomic studies may improve predictions of the toxicity of new compounds and provide clues to the mechanisms of toxicity. The current trend in toxicogenomic studies is to analyze gene expression profiles after exposing animals, such as rodents, to toxic chemicals or medicines in order to examine toxicity-related gene expression. However, adverse events in humans are often not predicted from animal studies conducted during the drug development process. Therefore, research techniques that employ human tissues are necessary. However, due to the scarcity and size of suitable human tissue samples, it is difficult to repeat exposure experiments using the same batch of tissue. Thus, it is necessary to perform such studies with a small number of human samples. While it is known that inherited and environmental individual differences occur in humans, it is unclear whether the data obtained using small numbers of human samples are applicable to general toxicity-related gene expression research. Information is also lacking on the extent of individual differences in gene expression. To understand gene expression patterns using human tissues that originate from few individuals, it is important to examine the individual differences in gene expression of cultured human tissues. The use of human tissues collected during surgical procedures is restricted in Japan. It is also prohibited to use human organs removed for organ transplantation from a brain dead donor but not transplanted into a recipient, even if the donor had expressed a willingness to donate the organ to research and the organ was kept in very good condition post-mortem. Japanese tissues are not available from any commercial source. Moreover, the National BioResource Center of Japan (http://www.nbrp.jp) does not supply Japanese human tissues that have been harvested by surgery. Therefore, to use primary Japanese human tissues, researchers must prepare themselves. In addition, it is becoming more and more difficult to obtain primary tissues from celiotomy incision, as the current trend in the treatment of solitary renal or urinary tract tumors is laparoscopy, which is not suitable for the harvesting of tissues for biochemical research. In this study, we used the cultured cells because warm...
ischemic time was often more than 30 minutes and thereby the quality of RNA extracted from the kidney was defective without cell viability recovery by culturing for a few weeks. The kidney has many functions, such as the excretion of soluble waste substances, homeostatic maintenance, endocrine secretion, and metabolism. It is also known that some of these functions have genetic polymorphisms, which result in individual differences. Therefore, for our toxicogenomic study, it is important to collect gene expression data on all subjects, to understand these individual differences. In this study, we analyzed the gene expression patterns of primary renal tubular cells, and searched for differential gene expression among individuals. This is the first study to examine individual-based differences in the gene expression of primary cultured cells from Japanese subjects.

2. Materials and Methods

2.1 Subjects and Sampling of Renal Tubular Cells

Japanese patients of at least 18 years of age, who were admitted to the Jichi Medical School Hospital for renal resection of a confirmed solitary tumor in one kidney, the renal pelvis or the urethra, were eligible for this study. Patients with renal dysfunction (i.e., serum creatinine levels > 2.0 mg/ml or abnormalities in serum levels of sodium, potassium, or chloride) were excluded. Our institutional review boards, including the Bioethics Committee of Jichi Medical University and the Bioethics Committee for Human Gene Analysis, approved the study protocol. All patients or their legally authorized representatives gave written informed consent before enrolment in the study, which was carried out in accordance with the principles of the Declaration of Helsinki (as revised, 1996). The preparation and cryopreservation of the primary cultured cells were conducted using previously described methods, and the function and formation of these cells were confirmed previously. In brief, a few grams of tissue were chopped and washed with Euro-Collins solution (Kobayshi Pharmaceutical Co., Ltd., Osaka, Japan). After 60 min of continuous agitation in an intracellular-like solution with 1,500 U/ml dispase (Godo Shusei Co., Ltd., Tokyo, Japan), the cells were incubated in 0.05% trypsin and 0.53 mM sodium ethylenediamine tetraacetate (EDTA) (Invitrogen Corp., Carlsbad, CA, USA) at room temperature. The cells showed a uniform morphology of epithelial cells, which suggested that the purified cells were of uniform origin and characteristics. Expression analysis revealed that five kidney-specific genes–KL, SLC17A3, AQP2, SLC22A2, and KCNJ–were present in these cells. The cells exhibited gamma-GTP enzymatic activity and Glut2 antigen expression, which suggests renal tubular origin. Thus, the majority of the purified and cultured cells originated from the proximal renal tubule and retained at least some of the characteristics of the original tissue. Details of the individuals and sample preparation are described in the supplemental data.

2.2 Gene Expression Analysis

Primary renal cortical cell cultures were derived from 11 individuals (n = 3 cultures for each individual) and grown to confluence before harvesting. Total RNA samples were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. The purified RNA samples were quantified on a U-2000 spectrometer (Hitachi Instruments Service Co., Ltd., Tokyo, Japan). Double-strand cDNA was synthesized from 20 µg of purified RNA and used to prepare biotin-labeled complementary RNA for hybridization on the Test3, HG-U133A, and HG-U133B microarray chips of the GeneChip system (Affymetrix, Santa Clara, CA, USA), which contain 45,000 oligonucleotide probe sets that correspond to approximately 39,000 transcripts. Hybridization and washing of the arrays and detection of the signals were performed using the GeneChip system according to the manufacturer’s instructions (Affymetrix). We measured the 3’-end to 5’-end ratios of housekeeping genes, such as β-actin and GAPDH. This ratio gives an indication of the integrity of the starting RNA, efficiency of first-strand cDNA synthesis, and in vitro transcription of cRNA. The signal obtained for each probe set reflects the probe sequences and their hybridization properties (GeneChip Expression Analysis Technical Manual, Affymetrix). When the 3’-end to 5’-end ratio was > 3.0, the data were excluded from analysis. Details of the cDNA, cRNA preparation, labeling, hybridization and scanning procedures are described in the supplemental data.

2.3 Normalization

Normalization was performed using the GeneSpring®version 7.1 software (Silicon Ge-
Table 1 Patient characteristics.

| UPN | Age | Gender | Prior Treatment | Prescribed Medication | Histological Diagnosis | Histological Findings other than tumor |
|-----|-----|--------|-----------------|-----------------------|------------------------|----------------------------------------|
| #01 | 78  | M      | No              | cefcapene             | TCC                    | normal                                 |
| #02 | 66  | M      | No              | No                    | RCC                    | normal                                 |
| #03 | 57  | M      | No              | tamsulosin, brotizolam| TCC                    | normal                                 |
| #04 | 35  | M      | No              | No                    | RCC                    | normal                                 |
| #05 | 66  | F      | No              | No                    | RCC                    | normal                                 |
| #06 | 47  | M      | No              | No                    | RCC                    | normal                                 |
| #07 | 77  | M      | No              | No                    | RCC                    | normal                                 |
| #08 | 67  | M      | No              | No                    | TCC                    | normal                                 |
| #11 | 85  | F      | No              | brotizolam            | RCC                    | normal                                 |

M, male; F, female; TCC, transitional cell carcinoma; RCC, renal cell carcinoma; UPN, unique patient number

Table 2 Clinical laboratory findings for subjects included in the analysis.

| UPN | BUN (mg/dl) | Cr (mmole/l) | Na (mmole/l) | K (mmole/l) | Cl (mmole/l) |
|-----|-------------|--------------|--------------|-------------|--------------|
| #01 | 16          | 0.86         | 142          | 3.8         | 104          |
| #02 | 13          | 0.73         | 139          | 4.4         | 103          |
| #03 | 13          | 0.84         | 144          | 4.1         | 106          |
| #04 | 14          | 0.80         | 139          | 4.1         | 101          |
| #05 | 15          | 0.91         | 139          | 3.9         | 102          |
| #06 | 13          | 0.83         | 140          | 4.1         | 105          |
| #07 | 18          | 0.78         | 140          | 4.1         | 105          |
| #08 | 14          | 0.79         | 140          | 4.0         | 103          |
| #11 | 13          | 0.55         | 138          | 3.8         | 102          |

Normal range 8-20 0.63-1.03 136-148 3.6-5.0 96-108

UPN, unique patient number; BUN, blood urea nitrogen; Cr, serum creatinine; Na, sodium; K, potassium; Cl, chloride

2.4 Statistical Analysis

The transcripts were accepted only if 60% or more of the samples indicated ‘present’ or ‘marginal’ flags, and the data were further refined using Cross Gene Error Modeling. Transcripts with control signals that were lower than the calculated base/proportion value were removed from the analysis. The fold-changes and C.V. values among the individuals were calculated as follows:

\[
C.V. (%) = \frac{\text{standard deviation}}{\text{mean of gene expression}} \times 100
\]

Based on the null hypothesis that there was no difference on the average of the gene expression between individuals, \( p \) value was calculated using a non-parametric test and the Benjamini and Hochberg false discovery rate procedure for multiple testing.

2.5 Comparison of the Gene Expression with Cancer Genome Anatomy Project (CGAP) Resource

Among gene expression data from 27 samples in total, the genes with ‘positive’ or ‘marginal’ flag in at least 60% of samples were listed in ‘high expressing gene list’. Rests of them was listed in ‘negative expressing gene list’. On the other hand, the gene expression data originated from normal kidney was obtained from CGAP resource. Based on the null hypothesis that there was no relation between our experimental data and CGAP expression data, Fisher exact test was conducted.

3. Results

3.1 Subjects

We analyzed the comprehensive gene expression profiles of primary cultures of renal cortical cells derived from nine Japanese subjects who were admitted for kidney resection. Table 1 and Table 2 summarize the patients’ characteristics and the clinical laboratory find-
Table 3  The number of genes exhibiting differential expression in the primary cultured renal cortex cells of one or more individuals. *Fold-change in expression level compared with the mean expression level for all subjects. **Number of individuals that exhibit differential expression for the same gene.

| Number of individuals** | Up or down regulation | Fold change* |
|------------------------|-----------------------|--------------|
| 1                      | Up                    | 2,885 × 2 × 3 × 4 × 5 |
|                        | Down                  | 566 × 36, 990 |
| 2                      | Up                    | 649 × 12 |
|                        | Down                  | 620 × 42 |
| 3                      | Up                    | 138 × 6 |
|                        | Down                  | 209 × 10 |
| 4                      | Up                    | 7 × 1 |
|                        | Down                  | 24 × 3 |
| 5                      | Up                    | 0 × 0 |
|                        | Down                  | 1 × 0 |

Up, up-regulation; Down, down-regulation.

Table 4  Classification of genes by p-value indicating significantly different expression levels among the subjects.

| p-value | Number of genes | Number of false-positive genes |
|---------|-----------------|-------------------------------|
| p < 0.05 | 8,596           | 748                           |
| p < 0.01 | 4,319           | 149                           |
| p < 0.001 | 1,212           | 15                            |

Fig. 1  Classification of genes according to the coefficient of variation (C.V.) among the subjects. Histogram showing the number of transcripts with the indicated C.V. levels

Table 5  The number of transcripts with C.V. values that exceeded the indicated thresholds.

| C.V. value (%) | Number of genes |
|----------------|-----------------|
| > 50           | 1,338           |
| > 70           | 212             |
| > 80           | 96              |
| > 90           | 44              |
| > 100          | 27              |

1,212 transcripts (Table 4). The estimated numbers of false-positive transcripts, which by chance give values less than the indicated p-value, were: 748 for p < 0.05; 149 for p < 0.01; and 15 for p < 0.001 (Table 4).

We examined the coefficient of variation (C.V.) for the gene expression levels among the subjects (Fig. 1). The C.V. was > 50% for 1,338 transcripts, > 70% for 212 transcripts, and > 90% for 44 transcripts (Table 5). Included among the transcripts with C.V. values > 90% were PDZK1, UGT1A8, and UGT1A9, which are associated with drug metabolism.

3.3 Comparison of the Gene Expression with CGAP Resource

According to Section 2 Fisher exact test was conducted. p-value was below 0.0001 and the null hypothesis was rejected. Therefore, it was considered that there was a significant correlation between our experimental data and CGAP expression data.
4. Discussion

In this study, we used microarray analysis to investigate individual differences in the gene expression of primary cultured renal cortical cells from Japanese subjects. The current social structure and environment in Japan are not favorable to the use of human tissues collected during surgical procedures in biochemical research. Therefore, Japanese tissues are not available from commercial sources or from the National BioResource Center. Primary Japanese human tissues can be used only for limited projects in limited facilities. It has become more and more difficult to obtain kidney samples from surgery, due to recent changes in operational procedures. Therefore, studies such as the present one, using Japanese primary tissues, are rare. Primary cultures were prepared from the non-tumor portions of extirpated kidneys. The non-tumor portions showed no pathological abnormalities. All of the subjects had normal Na, K, and BUN values and none of the subjects exceeded the upper limit for creatinine (Cr) content. There was no evidence to suggest any underlying disease, such as hypertension or diabetes mellitus, which might lead to renal dysfunction. No patient was prescribed renal-toxic medicines before the operation. Thus, the renal tissues that we harvested were regarded as normal. However, it is possible that these tissues had an unknown genetic factor(s) and they may have been exposed to environmental factors related to carcinogenesis, as the samples were from the non-tumor tissues of cancer patients. As primary cultured renal cortical cells consist mainly of renal tubular cells, the gene expression profiles in this study were expected to reflect those of renal tubular cells. We analyzed the gene expression patterns of nine primary cultures of renal tubular cells. Of the 39,000 transcripts analyzed by the microarray system, 8,569 (approximately 22%) exhibited significantly different expression levels among the subjects. In pharmacokinetics, high inter-subject variability is considered present when the C.V. of a pharmacokinetic parameter (such as the maximum plasma concentration, the area under the plasma concentration-time curve, or clearance) is > 50% \(^7,8\). When we classified the genes based on their C.V. values, we found that 44 transcripts, including the PDZK1, UGT1A8, and UGT1A9 transcripts, had C.V. values > 90%. PDZK1 encodes a protein that is regulated by dietary phosphate, increases phosphate uptake through a sodium/phosphate cotransporter, and plays an important role in cellular phosphate regulation \(^9\). The UGT1A8 and UGT1A9 transcripts belong to the UGT1A glycosyltransferase gene family and catalyze the conjugation of glucuronic acid to various endobiotics and xenobiotics \(^10\). Thus, some individual differences are seen for genes that encode drug-metabolizing enzymes or transporters. These genes may be associated with individual differences in drug metabolism, distribution, and excretion.

We analyzed the gene expression profiles of primary cultures of cells from Japanese individuals, to determine individual differences using various statistical methods. Caution is necessary in the identification of these genes, which showed individual-based differences or were listed as outliers form the data obtained from primary renal cell cultures. Thus, these data are useful in interpreting and understanding the toxicogenomics of primary cultured renal cortical cells from Japanese subjects.

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