Regulation of Pharmacogene Expression by microRNA in The Cancer Genome Atlas (TCGA) Research Network

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Abstract

Individual differences in drug responses are associated with genetic and epigenetic variability of pharmacogene expression. We aimed to identify the relevant miRNAs which regulate pharmacogenes associated with drug responses. The miRNA and mRNA expression profiles derived from data for normal and solid tumor tissues in The Cancer Genome Atlas (TCGA) Research Network. Predicted miRNAs targeted to pharmacogenes were identified using publicly available databases. A total of 95 pharmacogenes were selected from cholangiocarcinoma and colon adenocarcinoma, as well as kidney renal clear cell, liver hepatocellular, and lung squamous cell carcinomas. Through the integration analyses of miRNA and mRNA, 35 miRNAs were found to negatively correlate with mRNA expression levels of 16 pharmacogenes in normal bile duct, colon, and lung tissues (p<0.05). Additionally, 36 miRNAs were related to differential expression of 32 pharmacogene mRNAs in those normal and tumor tissues (p<0.05). These results indicate that changes in expression levels of miRNAs targeted to pharmacogenes in normal and tumor tissues may play a role in determining individual variations in drug response.

Key Words: Epigenomics, microRNAs, Pharmacogenetics, Neoplasms, The Cancer Genome Atlas

INTRODUCTION

Pharmacogenomics focuses on how individual genetic variations influence drug responses, and is helping to develop safer and more effective treatments for patients (Relling and Evans, 2015). Many pharmacogenomic studies have concerned single nucleotide polymorphisms (SNPs) that affect drug responses, and several SNPs have been reported (Georgitsi et al., 2011). However, the diversity of drug responses is not explained by genetic mutation alone. As well as the genetic polymorphisms, drug response may be different due to factors that regulate gene expression.

MicroRNAs (miRNAs) are small, ~21 nucleotide single-strand noncoding RNAs that can regulate gene expression by binding to partially complementary sites in 3’ untranslated regions (3’ UTRs) of messenger RNAs (mRNAs). This miRNA-mRNA interaction governs a variety of mechanisms that control gene expression, including mRNA degradation and translational repression (Wienholds et al., 2005; Pasquinelli, 2012). The mRNAs affected by the miRNAs consequently influence susceptibility to cancer, as well as amentia, autoimmune diseases, and diabetes (Sayed and Abdellatif, 2011). Therefore, miRNAs are becoming recognized as important mediators that affect drug responses, without affecting the genomic sequence. An increasing number of studies on pharmacoeipigenetics and pharmacoeipigenomics support a role for miRNA in regulating expression of genes encoding proteins involved in drug absorption, distribution, metabolism, and excretion (ADME) (Shomron, 2010; Rukov and Shomron, 2011), as well as pharmacodynamics (Yu et al., 2016). One miRNA can regulate various ADME genes via direct and/or indirect targeting, or one ADME gene may be modulated by multiple miRNAs (Yu and Pan, 2012). However, our current understanding of miRNA action was mainly obtained from in vitro cell culture systems and ex vivo systems (Rukov and Shomron, 2011). Moreover, prediction and identification of miRNAs target genes is a time-consuming, labor-intensive, and error-prone process (Huang et al., 2016).
This epigenetic regulation of miRNAs in drug transporters or enzymes has a greater impact on drug responses. The influence of the epigenetic changes in cancer diseases can be expected to be even greater. Thus, we hypothesized that the drug response may be affected by expression changes of pharmacogenes in patients with cancer, in special, in organs involved in drug metabolism. The Cancer Genome Atlas (TCGA) Research Network has profiled and analyzed large numbers of human tumors to discover molecular aberrations at the DNA, RNA, and protein level, and also examined epigenetic changes, including those related to miRNA (Weinstein et al., 2013). Because the TCGA also contains a significant collection of normal tissue samples, it would be an appropriate resource for pharmacogenomic miRNA studies. Tumor-induced miRNA changes are also important in drug responses and toxicity, especially responses to chemotherapy (Zheng et al., 2017).

Therefore, the aim of this study was to explore miRNA expression differences in normal tissues derived from patients with five different cancer types and identify significant miRNAs regulating pharmacogene expression, using an integrated analysis of miRNA and mRNA. In addition, we purposed to assess miRNA expression differences in normal tissues of cancer patient samples.

**MATERIALS AND METHODS**

**miRNA data collection using TCGA datasets**

The miRNA data of normal and tumor tissues was downloaded from the TCGA Research Network portal (cancergenome.nih.gov) which dataset was available as of May 2016. All data for cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), kidney renal clear cell (KIRC), and lung squamous cell carcinoma (LUSC) samples were collected in the United States, whereas liver hepatocellular carcinoma (LIHC) samples originated from patients in the United States, France, Japan, and China, considering various ethnic backgrounds. The miRNA sequencing (miRNAseq) data was gathered using an Illumina® HiSeq 2000 platform at the Michael Smith Genome Sciences Centre (GSC) of the BC Cancer Agency (Vancouver, BC, Canada). From the Illumina® HiSeq RNASeqV2 level 3 dataset, the “normalized_count” (quantile normalized relative standard error of the mean) value of each miRNA was collected. The miRNAseq data was integrated in to a matrix with log2 transformed for the downstream analysis.

**Pharmacogenes selection and mRNA data collection**

Important pharmacogenomic-related genes were searched on the Pharmacogenomics Knowledge Base (Klein et al., 2001). Additional pharmacogenetic genes, derived from the U.S. Food and Drug Administration (FDA) Table of Pharmacogenomic Biomarkers in Drug Labels (http://www.fda.gov/
drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.html), were included. The final phamacogenes for analysis were selected by eliminating duplicates. The public sequencing data of mRNA, associated with selected pharmacogenes, was also collected from the TCGA Research Network portal. RNA sequencing (RNASeq) data were produced by the University of North Carolina (Chapel Hill, NC, USA) using an Illumina® HiSeq 2000 platform. An mRNAseq matrix with log-2 transformation was made for downstream analysis.

Comparison of miRNA expression in normal and tumor tissues

All normal and tumor tissues samples were clustered using a hierarchical method. The clustering dendrogram was drawn using the Ward linkage method. To plot miRNA expression data in a heat map, we selected miRNAs that had >0.1 deviations in expression levels across samples. In addition, a distance matrix for miRNA expression variables in normal tissue samples was constructed using the Euclidean distance and was visualized by multidimensional scaling (MDS). This step was implemented using cmdscale in the R statistics software package.

Correlation analysis of miRNAs and gene expression

We selected only paired data in solid primary tumors and normal tissues to compare the difference in expression of miRNA. We analyzed the correlation between the expression levels of miRNA and mRNA in normal tissues of cancer samples and found a significant negative correlation. In addition, the Pearson’s correlation analysis was performed to identify in tumor specific downregulated miRNA by analyzing the significant association between miRNA and mRNA expression, and correlation coefficients were calculated with adjustment for cancer types.

miRNA target prediction

We next matched the significant correlations with target information using TargetScan (Agarwal et al., 2015), miRANDA (Betel et al., 2008), miRDB (Wong and Wang, 2015), Diana Tools (Paraskevopoulou et al., 2013), miRMap (Vejnjar and Zdobnov, 2012), and miRNAMap (Hsu et al., 2008) as appropriate. Given that no program was consistently superior to the others, and that we aimed to minimize the probability of introducing false positives and/or negatives, we selected genes that were identified by at least three databases as potential targets (Dai and Zhou, 2010). Data extraction and analyses were performed using Python version 3.4 (http://www.python.org/).

Evaluation using GEO dataset

For evaluate with our founding, we collected expression datasets of miRNA and mRNA for tumor and non-tumor tissues derived from colonic adenocarcinoma (GSE29623) (Chen et al., 2012) and intrahepatic cholangiocarcinoma and hepatocellular carcinoma patients (GSE57555) (Murakami et al., 2015).

Statistical analysis

Differences between the number of miRNAs and mRNA expression in each cancer patient were analyzed by Student’s t-test. Pairwise comparisons of miRNA expression levels in normal tissues were analyzed with a paired t-test. Regression analysis tested whether changes in miRNA expression correlated with mRNA expression after adjusted by tissue types. All statistical tests were performed in R Statistics version 3.3.2 (http://www.r-project.org/). Statistical significance was defined as a p-value of less than 0.05. Multiple testing correction was performed by controlling the false discovery rate (Benjamini and Hochberg, 1995) at α=0.05.

Table 2. Comparisons of miRNA and mRNA expression levels between tumors and normal solid tissues derived from cancer patients*

| Cancer                  | Number of patients | Number of miRNAs | Number of mRNAs |
|-------------------------|--------------------|------------------|-----------------|
|                         | Increased in tumors| Decreased in tumors | Increased in tumors | Decreased in tumors |
| Cholangiocarcinoma      | 9                  | 120              | 82              | 21              | 48               |
| Colon adenocarcinoma    | 8                  | 255              | 120             | 8               | 26               |
| Kidney renal clear cell | 67                 | 182              | 270             | 36              | 45               |
| Liver hepatocellular carcinoma  | 48                | 212              | 168             | 19              | 53               |
| Lung squamous cell carcinoma¹ | 43                | 157              | 441             | 26              | 42               |

*Significantly differently expressed miRNAs or mRNAs between tumor tissues and normal solid tissues were determined by paired t-test, respectively (p<0.05). ¹The 36 samples had mRNA expression data.
RESULTS

Pharmacogenes selection

Through searching database, 63 genes were selected and 31 genes were added from FDA table. After adding cytochrome P450 oxidoreductase (POR), a total of 95 genes, including 30 drug-metabolizing enzymes and 12 transporter genes, are listed in Table 1.

Comparison of miRNA expression in normal and tumor tissues

A total of 1,448 samples were downloaded from the TCGA portal (36 CHOL, 458 COAD, 244 KIRC, 373 LIHC, and 337 LUSC samples). After excluding unpaired data, 1,870 miRNAs remained in 9 CHOL, 8 COAD, 67 KIRC, 48 LIHC, and 43 LUSC primary tumor and paired normal tissue samples. Through Ward linkage analysis, the samples were clustered into one of four major groups that each represented a human tissue (Fig. 1). The number of miRNAs expressed at lower levels in primary solid tumors was higher than that seen for normal solid tissues (Table 2). Meanwhile, for KIRC and LUSC the number of miRNAs expressed at higher levels in primary solid tumors was lower than that seen for normal solid tissues. The number of miRNAs having lower expression levels in primary tumor tissues was lower than that for normal tissues in patients with CHOL, COAD, and LIHC.

Based on assessment of miRNA relationships among the 95 pharmacogenes in different tissues, the overall pattern of the MDS plot separated the colon, kidney, liver, and lung into four discrete identities, while bile duct tissues were included into one of four major groups that each represented a human tissue (Fig. 1). The number of miRNAs analyzed at lower levels in primary solid tumors was higher than that seen for normal solid tissues (Table 2). Meanwhile, for KIRC and LUSC the number of miRNAs expressed at higher levels in primary solid tumors was lower than that seen for normal solid tissues. The number of miRNAs having lower expression levels in primary tumor tissues was lower than that for normal tissues in patients with CHOL, COAD, and LIHC.

Correlation of miRNA and mRNA expression in normal and tumor tissues

The correlation analysis results showed that 23 miRNAs had a negative correlation between miRNA and mRNA expression for 14 pharmacogenes (Table 3, Fig. 4), resulting in 33 combinations of miRNAs and mRNAs. Hsa-miR-429 decreased 3 mRNA expression levels, including ADH1B (p=2.48×10^{-4}), AHR (p=1.63×10^{-3}), and ALDH1A1 (p=1.44×10^{-3}). Meanwhile, hsa-miR-181d decreased the expression levels of AHR (p=2.88×10^{-3}), BCR (p=6.25×10^{-3}), and CYB5R4 (p=6.30×10^{-3}), whereas hsa-miR-152 decreased the expression levels of ABL2 (p=1.48×10^{-4}), AHR (p=7.44×10^{-4}), and CYB5R4 (p=2.24×10^{-4}). Hsa-miR-98 decreased the expression levels of ADRB2 (p=9.13×10^{-3}).

The correlation analysis results showed that 19 miRNAs had a negative correlation between miRNA and expression levels of 15 pharmacogene mRNAs (Table 4, Fig. 5) to yield 24 combinations between miRNAs and mRNAs. Hsa-miR-520b (1.59×10^{-3}) decreased ADRB1 mRNA expression levels, whereas hsa-miR-152 decreased the expression levels of ABL2 (p=1.49×10^{-4}), AHR (p=4.06×10^{-4}), and CYB5R4 mRNA (p=1.42×10^{-4}). Hsa-miR-98 decreased the expression levels of ADRB2 mRNA (p=1.23×10^{-4}).

Evaluation using GEO datasets

Through evaluation using GSE29623 and GSE57555 datasets, Hsa-miR-520b decreased mRNA expression of ADRB1, while hsa-miR-98 decreased mRNA expression of ADRB2 (p<0.05). Additionally, hsa-miR-152 decreased mRNA expression levels of ABL2 and CYB5R4 (p<0.05).

DISCUSSION

In the present study, we used the integrative analysis to identify miRNAs that contribute to altered expression of pharmacogenes in different tissues and tumors. The integrative analysis of mRNA and miRNA expressions is a powerful
Table 3. miRNA expression negatively correlated with pharmacogene expression in different normal solid tissues derived from cancer patients ($r^2>0.3$)

| Classification | Gene | miRNA | FDR: $p$-value | Adjusted $R^2$ | Pearson correlation coefficient ($r^2$) |
|----------------|------|-------|----------------|----------------|-------------------------------------|
| Metabolizing enzymes | AHR | hsa-miR-429 | $2.48 \times 10^{-24}$ | 0.538 |
| | | hsa-miR-577 | $2.15 \times 10^{-20}$ | 0.468 |
| | CYB5R4 | hsa-miR-152 | $2.24 \times 10^{-9}$ | 0.812 |
| | | hsa-miR-758 | $1.21 \times 10^{-17}$ | 0.437 |
| | | hsa-miR-181d | $6.30 \times 10^{-13}$ | 0.361 |
| Receptors | ADRB1 | hsa-miR-let-7c | $2.39 \times 10^{-15}$ | 0.538 |
| | ADRB2 | hsa-miR-98 | $9.13 \times 10^{-13}$ | 0.738 |
| Targets | ABL1 | hsa-miR-378g | $1.08 \times 10^{-9}$ | 0.377 |
| | ABL2 | hsa-miR-152 | $1.48 \times 10^{-7}$ | 0.800 |
| | | hsa-miR-107 | $6.30 \times 10^{-12}$ | 0.452 |
| | | hsa-miR-217 | $1.96 \times 10^{-9}$ | 0.323 |
| | | hsa-miR-410 | $4.19 \times 10^{-8}$ | 0.317 |
| | ALOX5 | hsa-miR-134 | $1.93 \times 10^{-9}$ | 0.636 |
| Transcription factors | ACE | hsa-miR-511 | $3.92 \times 10^{-9}$ | 0.529 |
| | AHR | hsa-miR-152 | $7.44 \times 10^{-10}$ | 0.597 |
| | | hsa-miR-181d | $2.88 \times 10^{-11}$ | 0.561 |
| | | hsa-miR-429 | $1.63 \times 10^{-3}$ | 0.643 |
| | | hsa-miR-520b | $1.22 \times 10^{-9}$ | 0.558 |
| | | hsa-miR-653 | $6.27 \times 10^{-13}$ | 0.630 |

FDR: false discovery rate.

Fig. 4. Correlation of RNA expression and miRNA changes across normal colon, bile duct, kidney, liver, and lung tissues derived from cancer patients. Line is fitted to the points. Open circle, bile duct; closed circle, kidney; open square, liver; closed square, lung; open triangle, colon (A) correlation of hsa-miR-152 with ABL2 ($p=1.48 \times 10^{-7}$); (B) correlation of hsa-miR-429 with ADRB1 ($p=2.48 \times 10^{-15}$); (C) correlation of hsa-miR-98 with ADRB2 ($p=9.13 \times 10^{-13}$).

The United States has announced a research initiative that aims to accelerate progress toward a new era of precision medicine that is tailored to individuals (http://www.whitehouse.gov/precisionmedicine/). Genetic variations and epigenetic changes between individuals may be related to differences in drug responses (Dluzen and Lazarus, 2015). Most previous studies of miRNA in pharmacogenes examined only a limited number of genes with small sample sizes using traditional methods (Rieger et al., 2013), such that few global miRNA analyses of pharmacogene expression have been performed (Kim et al., 2014). Our results showed that the number of miRNAs expressed at lower levels in primary solid tumors was higher than that seen for normal solid tissues, while the number of miRNA expression levels of pharmacogenes varied in tumor tissues compared to normal tissues. These results indicate that there are considerable differences in the level and distribution of miRNAs across normal and tumorigenic tissues. However, as expected, our results showed that miRNA and miRNA expression levels were similar between liver and bile duct tissues.

https://doi.org/10.4062/biomolther.2017.122
The expression of several drug-metabolizing enzymes and transporter genes was regulated by miRNAs. For example, miR-27a and miR-548a repressed mRNA expression levels of ABCB1 and CYP3A4, respectively (Wei et al., 2014; Messingerova et al., 2016). Although we found negative correlations of the expression of these miRNAs and mRNAs in our study, they were excluded because their relationships did not occur in more than three miRNA target prediction databases.

Nevertheless, we could use the integrative analysis of massive miRNA-mRNA expression data to identify new various miRNAs for various drug-metabolizing enzyme (ADH1B, CYB5R4), receptor (ADRB1, ADRB2), target (ABL1, ABL2, ALOX5) genes, and transcription factor (ACE, AHR) that contribute to their differential expression in bile duct, colon, kidney, liver, and lung tissues. Expression of hsa-miR-148 and hsa-miR-152 was reported to be downregulated in gastrointestinal cancer tissues, suggesting that these two miRNAs may be involved during the early stage of gastric carcinogenesis (Chen et al., 2010). The hsa-miR-520 was also decreased in colorectal carcinoma when compared with normal colorectal tissues (Bahar et al., 2017). Associations between these miRNAs and pharmacogenes have not been previously reported.

Even with targeted therapy, the response to cancer drugs is not solely dependent on tumor epigenetics (Nasr et al., 2016). Moreover, germ line epigenetics can play a role in drug effects. Therefore, understanding and considering the contribution of both somatic and germ line epigenetics is important when predicting drug response and toxicity.

Recently, there has been a rapid increase in knowledge of how pharmacogenes are regulated by epigenetic mechanisms and methods to analyze this regulation (Koturbash et al., 2015). Although we examined a limited set of genes known to be involved in drug responses, the methodology described herein can be easily applied to future studies. One limitation of our study is that we did not stratify the data for age, gender, or racial/ethnic backgrounds, although miRNAs have been shown to exhibit differences related to these parameters (Huang et al., 2011; Kwekel et al., 2015). miRNAs regulate gene expression by repressing translation and/or by mRNA deadenylation and decay (Djuricovic et al., 2012). Several groups demonstrated that protein repression can occur in the absence of mRNA degradation (Wilczynska and Bushell, 2015), but we did not analyze protein expression levels of the pharmacogenes targeted in our study. Although there are further challenges to defining the role of miRNA in drug responses, here we identified miRNA-mediated changes in pharmacogene expression that may influence therapeutic responses.

Table 4. miRNA expression negatively correlated with pharmacogene expression in different normal and tumor solid tissues derived from cancer patients ($r^2>0.3$)

| Classification | Gene     | miRNA             | FDR  | Adjusted p-value | Pearson correlation coefficient ($r^2$) |
|---------------|----------|-------------------|------|-----------------|--------------------------------------|
| Receptors     | ADRB1    | hsa-miR-520b      | 1.59e-03 | 0.450           | 0.450                                |
|               | ADRB2    | hsa-miR-98        | 1.23e-04 | 0.450           | 0.450                                |
| Targets       | ABL2     | hsa-miR-152       | 1.49e-04 | 0.482           | 0.510                                |
| Metabolizing   | CYB5R4   | hsa-miR-152       | 1.42e-09 | 0.510           | 0.510                                |

FDR, false discovery rate.
In conclusion, epigenomic changes, including miRNA-induced regulation of expression of genes encoding drug-metabolizing enzymes, transporters, or targets, can potentially lead to changes in drug activity that may contribute to drug sensitivity, resistance, and toxicity. Here we investigated miRNA using publicly available epigenomic and transcriptomic databases in an effort to advance pharmacogenomics research. We believe the current analysis will lead to more rapid identification of functional miRNAs that are relevant to understanding variability in drug responses of cancer patients.

ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014-R1A1A2055734) and NRF grant funded by the Korea government Ministry of Science, ICT and Future Planning (NRF-2014M3C1B3064644). We gratefully acknowledge the TCGA Consortium and all its members for the TCGA Project initiative, for providing samples, tissues, data processing and making data and results available.

DISCLAIMER

The opinions expressed by Dr. Gilbert J. Burckart do not represent the position of the US Food and Drug Administration.

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