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Genome-wide microRNA expression profiling in placentas of fetuses with Down syndrome

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ABSTRACT

Introduction: Down syndrome (DS) is the most common aneuploidy, caused by an extra copy of all or part of chromosome 21 (chr21). Differential microRNA (miRNA) expression is involved in many human diseases including DS. However, the genome-wide changes in miRNA expression in DS fetal placentas have yet to be determined, and the function of these changes is also unclear.
Methods: We profiled genome-wide miRNA expression in placenta samples from euploid or DS fetuses by using microarray technology and predicted the functions of differentially expressed miRNAs using bioinformatics tools.
Results: Thirty-four miRNAs were significantly differentially expressed in the DS placenta compared with the normal placenta (16 up-regulated and 18 down-regulated). However, expression of chr21-derived miRNAs did not change. Predicted target genes included 7434 genes targeted by up-regulated miRNAs and 6071 genes targeted by down-regulated miRNAs. Seventy-six of these target genes were located on chr21 (10 genes controlled by down-regulated miRNAs and 34 genes by up-regulated miRNAs, and 32 genes by both). Target genes on chr21 were significantly associated with DS and DS-related disorders, such as mental retardation, neurobehavioral manifestations, and congenital abnormalities.
Discussion: To our knowledge, this is the first genome-wide study to comprehensively survey placental miRNAs in DS fetuses. Our results provide new insight into miRNA expression in placentas of fetuses with DS. Additionally, our findings indicate that the differentially expressed miRNAs in the DS placenta may potentially affect various pathways related to DS pathogenesis.

1. Introduction

The most common aneuploidy is trisomy 21, referred to as Down syndrome (DS). Most fetuses with aneuploidy are spontaneously terminated during fetal development. DS, however, has a high survival rate and occurs with an incidence of approximately 1 in 800 births in the general population [1]. The incidence of fetal DS increases in a maternal age-dependent manner to 1 in 35 term births for women 45 years old [1]. DS is associated with a number of deleterious phenotypes, including cognitive impairment, immune defects, congenital heart defects, hypotonia, childhood leukemia, mental retardation, dementia, and early-onset Alzheimer's disease (AD) [2]. These complicated and varied phenotypes are generally thought to result from the abnormal gene dose between trisomic genes on human chromosome 21 (chr21) and disomic genes on other chromosomes. Therefore, to date, studies of DS have mainly focused on changes in expression of chr21-derived genes in tissues from subjects with DS.

MicroRNAs (miRNAs) are a class of endogenous RNAs that are 18–25 nucleotides long, single-stranded, and have emerged as key

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post-transcriptional regulators of gene expression [3,4]. miRNAs play a key role in various cell processes, such as cell proliferation, differentiation, apoptosis, and embryonic development [5–7]. miRNAs are transcribed from intergenic and intragenic regions of the genome and inhibit gene expression by perfect complementary binding to target mRNA degradation or imperfect binding in the 3′ untranslated region (UTR) to inhibit translation [3,4]. Therefore, abnormal miRNA expression has been reported to be involved in the occurrence and development of various diseases, such as cancer, cardiovascular disease, mental retardation, fetal growth restriction, AD, and DS [8–15].

Recently, bioinformatic annotations have indicated that chr21 contains 584 genes and 29 miRNAs. These chr21-derived miRNAs have been correlated with the complex and variable phenotypes of DS [14–20]. These previous studies have focused on changes in expression of chr21-derived miRNAs in various tissues from subjects with DS. However, changes in miRNA expression that affect phenotypes can occur in the entire genome, i.e. an miRNA can potentially regulate a large number of protein-coding genes, and multiple miRNAs can regulate a single target gene [21]. Hence, to investigate the genome-wide expression patterns of miRNAs, miRNAs that are differentially expressed according to presence or absence of disease need to be identified. The regulatory mechanisms for these miRNAs in the progression of complex and variable phenotypes should also be investigated. Until now, only one genome-wide study of miRNA expression profiles in fetuses with DS has been reported [15]. This study investigated the genome-wide expression of miRNAs in cord blood mononuclear cells from fetuses with DS and demonstrated that most mRNA targets of differentially expressed miRNAs were associated with immune modulation [15]. However, genome-wide changes in miRNA expression in the placenta of fetuses with DS have yet to be determined, and the functions of these changes in miRNA expression are also unclear.

In this study, we performed a comparative analysis of genome-wide miRNA expression in placentas of euploid and DS fetus using microarray technology to identify miRNAs that were aberrantly expressed in placentas from fetuses with DS. In addition, we applied various bioinformatics tools to predict the target genes of the identified miRNAs and explore their biological function and downstream pathways.

2. Materials and methods

2.1. Study subjects

This study was conducted according to the principles expressed in the Declaration of Helsinki. Pregnant women with normal or DS fetuses who visited the Department of Obstetrics and Gynecology, Cheil General Hospital, Korea were recruited between March 2011 and December 2012. Appropriate institutional review board approval for this study was obtained from the Ethics Committee at Cheil General Hospital (#CGH-IRB-2011-85). All patients provided written informed consent for sample collection and subsequent analysis.

2.2. Sample collection and RNA extraction

All placenta samples were obtained by chorionic villus sampling in the first trimester and stored in liquid nitrogen until analysis. Total RNA was extracted from the placenta samples using mirVana miRNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. The purity and concentration of RNA samples were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of the samples was evaluated by microfluidic electrophoresis using the RNA 6000 Nano kit and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An RNA integrity number (RIN) $\geq$ 7.0 was considered acceptable for the microarray analysis.

2.3. Cytogenetic analysis for detection of T21

Chromosomal analyses of fetal chorionic villus samples were carried out using standard protocols as our previous study [22]. Cells from chorionic villus samples were cultured in the AmnioMAX-C100 culture medium (Invitrogen, Carlsbad, CA, USA). Metaphase chromosomes were stained using the GTG banding method, and 20 metaphases per sample were analyzed. All DS samples used in this study were complete trisomy 21 and all normal samples were complete euploidy.

2.4. MicroRNA microarray

Placental microRNA profiles were generated using Human miRNA Microarray kit, 8 × 60K (based on miRBase release 16.0, Agilent Technologies). The miRNA Complete Labeling and Hyb Kit (Agilent Technologies) was used to label and hybridize 100 ng of total RNA, according to the manufacturer's instructions. Briefly, RNA samples were dephosphorylated and labeled with cyanine 3-pCp by T4 RNA ligase for 2 h at 16 °C. RNA samples were hybridized to the miRNA microarrays for 20 h at 55 °C in a hybridization oven at 20 rpm. After washing, the slides were scanned by an Agilent Microarray Scanner (Agilent Technologies). The raw intensity of the array was scanned and extracted by BeadScan, and the data were corrected by background subtraction in the Genome Studio module.

2.5. MicroRNA microarray data analysis

2.5.1. Expression data analysis

Expression data were extracted from the scanned images using Feature Extraction software, version 10.7 (Agilent Technologies) and analyzed with the R statistical environment (version 2.15.1). The background was adjusted by subtracting the median background values from the median expression values obtained by the Feature Extraction software, followed by log base 2 transformations. The data were quartile normalized by the GeneSpring Gx 12.6.1 software. To calculate sample correlation, a Pearson uncentered correlation was performed with the R package. miRNAs expression levels were considered statistically significant if the difference between the DS and control groups was at least 2.5 fold (P-value < 0.05 and percentage of false prediction (pfp) $\leq$ 5%). To obtain a single expression value for each probe set, the median expression value was calculated for multiple probe sets corresponding to a unique miRNA.

2.5.2. miRNA target prediction and DS candidate gene search

To examine the functions of miRNAs specific to DS placenta, miRNA targets were predicted with the miRWalk database [23], which integrates 7 established target prediction tools: miRWalk (March 2013 release), Diana-microT (version 3.0), miRanda (August 2010 release), miRDB (April 2009 release), PITA (August 2008 release), RNA22 (May 2008 release), and Targetscan (version 5.1). Criteria for analysis were as follows: the gene region was the 3′ UTR of all known human genes in addition to the longest transcript of the gene, the minimum seed length was 7, and a P-value < 0.001. Only miRNA-target interactions identified by at least 4 prediction programs were considered for further analysis. Target prediction was performed separately for up- and down-regulated miRNAs.

2.5.3. Functional annotation analysis of the predicted targets

To investigate whether the differentially expressed miRNAs (P-value < 0.05 at least 2.5-fold change in expression) were regulating candidate genes for DS, the VENNY tool [24], which compares lists using Venn diagrams, was used. The predicted targets of differentially expressed miRNAs were compared with a list of all genes on chr21.

The two lists of predicted targets on chr21 (up-regulated and down-regulated miRNAs) were separately submitted to a functional annotation tool provided by DAVID 6.7 and WebGestalt. Gene ontology (GO) analysis and disease-associated analysis of target genes were performed. The putative targets were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Next, an interactive network of selected target genes was predicted with the Search Tool for the Retrieval of Interacting Genes (STRING v. 9.05) database. The target genes were considered as seed molecules to obtain direct and indirect protein–protein interactions. This database provides information on both experimental and predicted interactions from varied sources based on their neighborhood, gene fusion, co-occurrence, co-expression, experiments, and literature mining. We constructed an interactive network of target genes with a high confidence score of 0.7, which implies that interactions with high level of confidence were extracted from the database.

2.6. Statistical analysis

The clinical characteristics of the study population were analyzed using the Mann–Whitney U-test. In all tests, a P-value <0.05 was used for statistical significance. Statistical analyses were performed using the Statistical Package for Social Sciences 12.0 (SPSS Inc.).

3. Results

Five placenta samples from euploid fetuses and 4 from fetuses with DS were used for genome-wide miRNA expression profiling. Maternal age, gestational age, body mass index, and gender ratio of the fetuses did not differ between DS and normal fetuses ($P > 0.05$ for all, Table 1).