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Short Communication

Identification of dysregulated microRNAs in lymphocytes from children with Down syndrome

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ABSTRACT

Given the important roles of miRNAs in post-transcriptional regulation and its implications for the development of immune tissues and cells, characterization of miRNAs promotes us to uncover the molecular mechanisms underlying the pathway of trisomic chromosome 21 that disrupts the disomic genes expression and immunological defects related to Down syndrome (DS). In the present study, we analyzed global changes and chromosome distribution characteristics of miRNAs expression in lymphocytes from children with trisomy 21 by means of the Illumina high-throughput sequencing technology. Two small libraries were constructed using pool RNA of normal and DS children. The results have been further validated by stem-loop quantitative RT-PCR. Comparison between DS and normal profiles revealed that most of identified miRNAs were expressed at similar levels. The chromosome 21 that contributes to the abundantly expressed miRNAs was small, and not all Hsa21-derived miRNAs were over-expressed with ratios significantly $\geq$1.5 in Down syndrome children lymphocytes. Based on the deep sequencing technology, 108 novel candidate miRNAs have been identified, and 2 of them were derived from human chromosome 21. For the 114 significantly differentially expressed miRNAs, function annotation of target genes indicated that a set of highly abundantly and significantly differentially expressed miRNAs were involved in hematopoietic or lymphoid organ development, thymus development, and T/B cell differentiation and activation. Our results indicated that these abnormally expressed miRNAs might be associated with the mechanisms that trisomy 21 results in dysregulation of disomic genes and involved in the immunological defects seen in DS.

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1. Introduction

Down syndrome (DS) is the most frequent form of mental retardation due to a chromosomal aberration; the incidence is approximately 1 in 750 live births (Hassold and Jacobs, 1984). The clinical presentation of DS is complex and variable. Apart from the mental retardation presented in virtually every DS individual, a constellation of over 80 clinical traits have been described in DS patients, including congenital heart defects, craniofacial abnormalities, gastrointestinal anomalies, childhood leukemia and altered immune responses (Elton et al., 2010). Compared with non-DS subjects, the hematological malignancies, autoimmune diseases and infection occur at higher frequency in DS (Burgio et al., 1975, 1978; Kusters et al., 2009; Levin et al., 1979; Ram and Chinen, 2011), and the infectious diseases is one of the major causes of death in DS (Garrison et al., 2005). Such situation might be associated with the abnormalities of immune system of DS, such as the abnormal maturation of thymus, and the impaired T/B-cell function, and the decrease of the number of T/B-cell and NK-cell in circulation system (Dehingh et al., 2005; Murphy et al., 1995; Philip et al., 1986; Ram and Chinen, 2011). However, the basis of the immune defects remains unclear, and has been considered to be associated with the dosage imbalance of human chromosome 21 (Hsa21) genes and the subsequent global genes deregulation observed overall the genome (Li et al., 2006; Malago et al., 2005; Sommer et al., 2008). While the molecular mechanisms by which trisomic genes disrupt expression of disomic genes and normal immune system development are not well understood.

MicroRNAs (miRNAs) are newly discovered, 18–25 nucleotides (nt) long, non-coding ribonucleic acids (RNAs) that have been proved to play critical roles in the regulation of host genome expression at the post-transcriptional level and various biological processes, such as immune cell lineage commitment, differentiation, maturation, and maintenance of immune homeostasis and normal function (Dai and Ahmed, 2011).

Abbreviations: DS, Down syndrome; Hsa21, human chromosome 21; sRNA, small RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; RISC, miRNA-induced silencing complexes; GO, Gene Ontology; PAGE, polyacrylamide gel electrophoresis; Nt, nucleotides; TPM, transcripts parts per million; HSCs, hematopoietic stem cells; DCs, Dendritic cells.

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MiRNAs are transcribed from the intra- and inter-genetic regions of the genome by RNA polymerase II (Bartel, 2004). Following their processing, mature miRNAs are assembled into ribonucleoprotein complexes that are called miRNA-induced silencing complexes (RISC). RISC subsequently inhibit gene expression by either perfect complementary binding, for mRNA degradation, or imperfect binding at the 3′UTR region, to inhibit translation (Bandiera et al., 2010). Its abnormal expression has been proved to be involved in the happening and development of diseases (Bandiera et al., 2010; Havelange and Garzon, 2010; O'Connell et al., 2008; Szulwach et al., 2009).

In the last several years, to identify the contribution of miRNAs on DS phenotypes and phenotypic variability, several research groups have studied the role of Hsa21-derived miRNAs (i.e., let-7c, miR-99a, miR-125b, miR-155, and miR-802) in the development of DS variable deleterious phenotypes (Kuhn et al., 2010; Li et al., 2012; Sethupathy et al., 2007), which was reviewed in Elton et al. (2010). Nonetheless, very little is known about the molecular pathogenesis of Down syndrome in terms of the contributions of single miRNA to the DS phenotype. To date, only two studies attempting to analyze the consequences of trisomy in DS and its mouse models with miRNA arrays have been reported, one in fetal tissue (Kuhn et al., 2008), and one in tissues and whole blood of the DS mouse model (Keck-Wherley et al., 2011). The expression profile characteristics and the role of miRNAs in the human whole blood with trisomy 21 remain unknown. Considering the important role of miRNAs in the development of immune system, it would be of great significance to identify the miRNAs expression profiles of immune cells for investigating the mechanisms of immune defect linked to DS.

In this work, we employed the Illumina high-throughput sequencing technology to study the miRNAs expression profile characteristics in lymphocytes from children with trisomy 21. To our knowledge, we present the first characterization of a genome-wide miRNAs expression profile in children lymphocytes with trisomy 21. Comparison between DS and normal profiles revealed that most of identified miRNAs were expressed at similar level. The chromosome 21 that contributes to the abundantly expressed miRNAs was small, and not all Hsa21-derived miRNAs were over-expressed with ratios significantly $\geq$1.5 in DS children lymphocytes. Base on the deep sequencing technology, 108 novel candidate miRNAs have been identified, and 2 of them were derived from human chromosome 21. Notably, the unique expression patterns in children lymphocytes with trisomy 21 and GO analysis showed that a set of highly abundantly and significantly differentially expressed miRNAs may be associated with immunological defects seen in DS.

2. Materials and methods

2.1. Samples and ethics statement

Peripheral blood samples got from 6 normal and 6 karyotypically confirmed full trisomy 21 children. This study protocols and consent forms were approved by the Second Clinical Medical College of Jinan University (Shenzhen People's Hospital). Written informed consent was obtained from the families of individuals involved in this study according to the principles expressed in the Declaration of Helsinki. Both DS and normal children included in this study ranged from 5 to 12 years, and were self-reported as free of chronic and acute infections, and fell within normal ranges for the major cell populations (% neutrophils, % monocytes, % lymphocytes, % eosinophils, % basophils), as determined by an automatic counter.

Lymphocytes were prepared from peripheral blood using Lympholyte®-H (Cedarlane, Burlington, USA) and their total RNA was isolated with TRIzol® Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The small RNA libraries were constructed using pooled RNA from 3 DS children (DS group) and 3 normal children (control group), respectively, to eliminate any individual variation. The remaining samples were used as the validation set to confirm the miRNA differential expression patterns by stem-loop quantitative RT-PCR.

2.2. Small RNA library preparation and sequencing

Small RNA library preparation and sequencing were performed using Illumina Sequencing Technology (BGI, Shenzhen, China). Briefly, the small RNA (sRNA) population was isolated by separating 10 μg of total RNA using denaturing polyacrylamide gel electrophoresis (PAGE) and excising the portion of the gel corresponding to the appropriate size (18–30 nt) based on standard oligonucleotide markers. The sRNA was ligated with 3′ (5′-pUCGUAUGCCGUCUUCUGCUUGidT-3′) and 5′ (5′-GUUCAGAGUUCUACAGUCCGACGAUC-3′) adapters using T4 RNA ligase and purified on a 15% tris-borate-EDTA (TBE) urea PAGE. Next, the modified sRNA was reverse-transcribed using Illumina's small RNA RT-Primer (5′-CAAGCAGAAGACGCATACGA-3′). The cDNA was used as the template for PCR amplification (15 cycles) using Illumina's small RNA primer set (5′-CAAGCAGAAGACGGCATACGA-3′ and 5′-AATGATACGG CGACCACCGACAGGTTCAGAGTTCTACAGTCCGA-3′). Finally, the amplified cDNAs were purified by 6% TBE PAGE and sequenced on the Illumina Hi-seq 2000, according to the manufacturer's protocols.

2.3. Read filter and small RNA annotation

Two sRNA sequencing data sets comprised by lymphocytes of the DS and normal individuals were obtained from Illumina fast track sequencing services. The low quality reads were filtered out to exclude that most likely represent sequencing errors, and the 3′/5′ adaptor sequences were subsequently trimmed to clean full-length reads and formatted into a non-redundant FASTQ format. The high-quality clean reads larger than 18 nt were mapped to the reference human genome (hg19, NCBI build 37.1) using the SOAP (version 2.0) program with at most two mismatches (R. Li et al., 2009). Sequences that perfectly matched the genome along their entire length were considered for next analyses. First, discarding rRNA, scRNA, snoRNA, snRNA, and tRNA deposited at NCBI Genbank and Rfam 10.0 database. Repeat overlapping sequences were annotated as repeat-associated small RNAs, and the sequences overlapping with predicted exons and introns were also filtered. The remaining unique small RNAs sequences were aligned with miRBase 17.0 database. Only perfectly matched sequences were considered to be miRNAs molecules. The unannotated sequences served as a source of potential novel miRNAs (Bandiera et al., 2010).

2.4. Normalization of the calculation of transcripts parts per million (TPM)

A normalization step was performed because the total number of reads from different experiments was not the same and variations in the number of reads of individual miRNAs can be due to sequencing depth. The number of reads from a unique sequence (representing a miRNA) was divided by the total clone count of the sample and multiplied by 10^6. The total clone count is the sum of the frequencies of all remaining unique sequences after filtering. Table S1 contains a list of the known miRNAs found in both sequencing samples along with their respective frequencies and TPM values.

2.5. Known miRNAs expression analysis

Differentially expressed miRNAs between the two libraries were identified by the fold-change method as previous study described (Vaz et al., 2010). The statistical significance (p-value) was inferred based on the Bayesian method developed by Audic and Claverie (1997). A specific miRNA was deemed to be significantly differentially regulated if the p-value was $\leq$0.001 and there was at least a 2-fold change in the normalized sequence counts between the two libraries. MiRNAs frequently exhibited variation from their “reference” sequences. We referred to these multiple mature variants as isomiRs.

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