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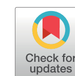
Gene sequence screening for manganese poisoning-susceptible genes and analysis of gene interaction effects



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ABSTRACT

Manganese poisoning is a common occupational disease, studies have found that the susceptibility to manganese poisoning differs in individuals. We adopted genome-wide sequencing methods to screen for susceptibility genes involved in gene-mediated metabolic pathways from the perspective of manganese poisoning. We identified 18,439 genes in this study, including 14,272 known genes and 4398 new genes. We then selected 17 differential genes using p values, of which 7 genes were down-regulated and 10 genes were up-regulated. Possible interaction genes for each differential gene were selected according to the String database. *Sgk1*, *HCRTr1*, *HspB1*, *Rem2*, *Oprd1*, *ATF5*, and *TRHr* identified in this study may be involved in oxidative stress mechanisms, dopamine (DA) synthesis, and neuronal survival during apoptosis and may affect susceptibility to manganese poisoning.

1. Introduction

Manganese (Mn) is an essential trace element in the human body and plays an important role in maintaining life activities in humans (Martinez-Finley et al., 2013) However, excessive manganese exposure is harmful, primarily damaging the central nervous system, thereby leading to nervous system diseases (Chen et al., 2016). Workers of manganese mining, welding, and other industries may be chronically exposed to manganese and experience poisoning, attributable to long-term exposure to manganese dust. The extrapyramidal symptoms of chronic manganese poisoning include tremor, symptoms of low dopamine (DA) syndrome, and excessive deposition of manganese in the striatum of the brain, globus pallidus, and substantia nigra, ultimately leading to basal ganglia degenerative lesions (Racette et al., 2012)

An epidemiological survey found that long-term exposure to an environment where manganese was produced resulted in the occurrence of chronic manganese poisoning with significant differences among individuals, suggesting individual gene susceptibility in the development of chronic manganese poisoning (Cai et al., 2011). Previously, it was shown that individual genetic variability more or less affects the susceptibility of an organism to Mn toxicity, ultimately leading to a differential tolerability to manganese and diverse clinical features (Kim et al., 2015b). For example, studies have shown that polymorphisms in *CYP2D6L* may affect sensitivity to manganese-induced neurotoxicity (Zheng et al., 2002). Therefore, when individuals with hazardous genotypes are exposed to manganese, they are more likely to experience chronic manganese poisoning.

Currently, the mechanisms underlying manganese poisoning are not clear; several possible main mechanisms have been proposed, including oxidative stress, DA depletion, free radical effects, calcium homeostasis, and the activation of cell death pathways, eventually leading to degeneration of dopaminergic neurons (Milatovic et al., 2009; Racette et al., 2012). The targets of manganese poisoning are DA and γ-aminobutyric acid (GABA) energy pathways, leading to neurodegenerative diseases (Yutian and Yongjian, 2017). Characteristic pathological changes in the degeneration of DA neurons include denatured proteins (Lewy bodies), with protein aggregates diffusely distributed in various regions of the brain, most notably in the midbrain. Studies have shown that the protein aggregates include α-synuclein, neurofilaments, and ubiquitin, among which α-synuclein is the major component. Overexpression of α-synuclein can enhance the sensitivity of an individual to manganese (Ashford and Porter, 1962)

Abbreviations: DA, dopamine; SPF, specific pathogen free; SNP, single nucleotide polymorphisms; INDEL, insertions and deletions; GABA, γ-aminobutyric acid; DEG, differentially expressed gene; GO, gene ontology; RIN, RNA integrity number; SE, skipped exons; A5SS, alternative 5' splicing sites; A3SS, alternative 3' splicing sites; MXE, mutually exclusive exons; RI, retained introns

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With the development of genome-wide sequencing technology, we can compare reads obtained by sequencing to the reference genome sequence. We can then reconstruct the transcript of the sample and compare the reconstructed transcript with the reference annotation information to obtain new transcripts and calculate statistics. At the same time, differentially spliced genes (DSGs) can be detected between samples after alignment with reference genomes. For comparison of differentially expressed genes, further gene enrichment analyses can be performed to determine possible metabolic pathways and interactions with proteins (Kim et al., 2015a; Pertea et al., 2015; Trapnell et al., 2012; Shen et al., 2014).

There are no specific treatment methods for chronic manganese poisoning and early preventive diagnoses. We adopted genome-wide sequencing methods to screen for susceptibility genes involved in gene-mediated metabolic pathways from the perspective of manganese poisoning to explore gene therapy or genetic diagnoses related to manganese poisoning.

2. Materials and methods

2.1. Sample acquisition

All experimental animals were specific pathogen free (SPF) grade healthy male Sprague-Dawley (SD) rats maintained in an SPF-class barrier animal room. Conditions included temperatures between 18 °C to 20 °C, humidity at 40%~60%, artificial lighting, and a 12-h light/dark cycle. Six SD rats were randomly divided into control group and exposed group. Rats in the exposed group were given 25 mg/kg $MnCl_2 \cdot 4H_2O$; intraperitoneal injection was performed at 0.2 ml/100 g, once every 48 h, and the control was injected with PBS at the same dose. Rats in both groups were injected for one month. After anesthesia, the rats were sacrificed, and the striata were separated on ice. Striata were placed in liquid nitrogen and stored at an ultra-low temperature of -80 °C.

2.2. RNA preparations

Frozen striata were crushed in a cooled vitreous homogenizer, and total RNA was extracted using TRIzol (Thermo Fisher, USA) reagent following the manufacturer's instructions. Total RNA concentration, RNA integrity number (RIN) 28S/18S values, and fragment size were determined with an Agilent 2100 Bioanalyzer (Agilent RNA 6000 nano kit, Agilent).

2.3. cDNA library construction and sequencing

After the RNA was extracted, total RNA was enriched using oligo (dT) magnetic beads. An appropriate amount of interrupting agent was added to the mRNA and then fragmented under high temperature conditions. Interrupted mRNA was used as a template to synthesize a cDNA chain and configure a two-strand reaction system to synthesize a two-strand cDNA. Then, 200 ng to 1 μg total RNA was taken and purified with 50 μl RNA Purification Beads. Subsequently, 19.5 μl of Elute, Prime, Fragment Mix was added to the Bead combined with mRNA and incubated at 94 °C for 8 min for fragmentation. Then, 17 μl of the above supernatant was taken for cDNA synthesis. Once the reaction was completed, 25 μl of Second Strand Master Mix was added and incubated at 16 °C for 1 h. It was purified using 90 μL of Ampure XP Beads (AGENCOURT) and resuspended in 60 μL of Resuspension Buffer. Then, 40 μL of End Repair Mix was added to 60 μL of ds cDNA for 30 min at 30 °C on a ThermoMixer (Eppendorf, 5355), purified using 160 μL of Ampure XP Beads (AGENCOURT), resuspended in 17.5 μL of Resuspension Buffer, and 12.5 μL of A-tailing Mix. The reaction was incubated for 30 min at 37 °C, then Adapter index was added, and finally PCR was performed to construct a gene library. After controlling for the quality of cDNA using an Agilent 2100 Bioanalyzer (Agilent, G2939AA) and real-time PCR system (ABI, StepOnePlus), the library was sequenced using an HiSeq™ (Illumina, HiSeq 2000).

2.4. Data filtering

Raw reads were treated to generate clean-read datasets according to the following procedure. First, we removed reads with adaptors, i.e., reads in which unknown bases (N) were more than 5%, and low-quality reads (We defined a read with a quality of less than 15 as a low quality read). The clean reads were aligned to the reference genome sequence using HISAT (Kim et al., 2015a); the reference sequence version used was UCSC_rn6. The clean reads were aligned to the reference genome to predict new transcripts and detect differentially spliced genes. Then, each sample was transcribed using String Tie (Pertea et al., 2015), and then the reconstructed transcript was compared with the reference sequence using Cuffcompare (Trapnell et al., 2012) to obtain a new transcript. The coding potential of the new transcript was predicted by CPC software (Kong et al., 2007). We added new transcripts with potential for protein coding to the reference gene sequence to form a complete reference sequence and then calculated the gene expression level. Finally, we conducted an in-depth cluster analysis and functional enrichment analysis using multiple samples and detected differences between the expression of genes.

2.5. Verification of differential genes

Randomly selected 6 rats were exposed to 1, 2, or 3 months as described in 2.1, 6; control rats were also randomly selected. The rats in each group were anesthetized and the striatum was taken on ice. The total RNA of the striatum was extracted for qPCR experiments to verify the expression levels of differential genes. The sequences of primers used are as follows:

GAPDH: 5′-CTGGAGAAACCTGCCAAGTATG-3′, 5′-GGTGGAAGAATGGGAGTTGCT-3″. SGK1: 5′-AGCGAGTCCGTCCTGCTAAG-3′, 5′-GGACCCAGGTTGATTTGTTGAG-3′. hcrtr1: 5′-GAAGCGACCCTCAGAGCAACT-3′, 5′-GGCGAAACATCCCAAACACTC-3′. HSPB1: 5′-ACTGGCAAGCACGAAGAAAGG-3′, 5′-ACCTGGAGGGAGCGTGTATTT-3′. REM2: 5′-CGACCTACCTGTCATCCTTGTTG-3′, 5′-GCAGCCTGATCTGACGCACA-3′. OPRD1: 5′-CGGGATGGAGCAGTGGTATG-3′, 5′-CACACGGTGATGATGAGAATGG-3′. ATF5: 5′-TGGCTCGTAGACTATGGGAAACT-3′, 5′-CGCTCGGTCATCCAATCAG-3′. TRHr: 5′-GAAAAATGACTCAACCCATCAGAAC-3′, 5′-GCTGCACGAAACTTCTGAGACA-3′.

3. Results

3.1. Construction of gene library

We sequenced 6 samples in total using the Illumina HiSeq platform and generated approximately 6.72 Gb per sample. The average genome mapping rate was 88.12%, and the average gene mapping rate was 60.31%. We identified 18,439 genes out of which 14,272 were known genes and 4398 were novel genes. Further, we identified 22,121 novel transcripts out of which 12,704 were previously unknown splicing events for known genes, 4398 were novel coding transcripts without any known feature, and the remaining 5019 were long noncoding RNA (Table 1).

For testing the quality of the gene, we examined the proportion of clean reads Q20 and Q30. Clean Reads Q20 (%) values were greater than 98%, and Clean Reads Q30 (%) values were greater than 95%. Further, distribution of base quality on clean reads showed that the samples were essentially high-quality bases, indicating that the sequencing quality was good (Table 2, Fig. 1).

After filtering the reads, we mapped clean reads to the reference genome. On average, 88.12% of reads were mapped, and the uniformity of the mapping results for each sample suggested that the samples were comparable (mapping details are shown in Table 3).

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