Identification of a potential exosomal biomarker in spinocerebellar ataxia Type 3/Machado–Joseph disease

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Aim: To identify spinocerebellar ataxia Type 3 (SCA3)-related exosomal biomarkers and the underlying mechanisms. **Materials & methods:** Exosomal RNAs from plasma and cerebrospinal fluid (CSF) were extracted from 24 SCA3 patients and 22 controls, respectively. Small RNA sequencing and quantitative PCR verification were performed. Gene ontology annotation and Kyoto Encyclopedia of Genes and Genomes pathway analyses of the results were carried out. **Results:** One novel miRNA is notably downregulated in plasma-derived exosomes, while upregulated in CSF-derived exosomes of SCA3 patients. Besides, it is successively upregulated in CSF-derived exosomes from Type 1, Type 2 and Type 3 groups. The downstream target genes were enriched in protein processing in endoplasmic reticulum and axon guidance. **Conclusion:** One exosomal biomarker was identified in SCA3, and this is the first time to report an exosomal miRNA as a biomarker in SCA3 internationally.

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Spinocerebellar ataxia Type 3, also known as Machado–Joseph disease (SCA3/MJD), is a group of highly heterogeneous neurodegenerative disorders with autosomal dominant inheritance, and usually appears as progressive ataxia and spasticity [1]. Some scholars have classified SCA3/MJD into the following subtypes: Type 1 begins early in life normally before age 20, which may progress more quickly with prominent pyramidal signs (rigidity and spasticity) and extrapyramidal features (bradykinesia and dystonia) as well as ataxia; Type 2 has a moderate age of onset (20–50 years of age) with cerebellar ataxia, progressive external ophthalmoplegia and pyramidal signs; Type 3 has a later onset (40–75 years of age) and appears as peripheral signs such as motor neuropathy and muscle atrophy together with ataxia; Type 4 is characterized by parkinsonism associated with other core clinical features; and afterward, a Type 5 SCA3/MJD was recently proposed for pure spastic paraplegia [2]. Among these five subtypes of SCA3/MJD, Type 2 is the most frequent, followed by Type 3, Type 1 and then the rare Types of 4 and 5 [3].

SCA3/MJD is one of nine polyglutamine diseases (polyQ diseases), which is characterized by polyglutamine encoding CAG sequences. Healthy individuals have 12–40 CAG repeats, which is expanded to 51–86 repeats in SCA3/MJD patients [4–6]. *ATXN3*, the gene causatively associated with SCA3/MJD, is located on chromosome 14 [7,8] and encodes a poly-Q-containing protein named ataxin-3 [7]. Ataxin-3 is a deubiquitinating enzyme present in the cytoplasm and nucleus that appears to function in cellular pathways regulating protein homeostasis as well as transcription [9–14]. Expansion of the polyglutamine domain destabilizes the mutant protein, leading to increased misfolding, altered interactions between proteins and neuronal toxicity and death. A feature of polyglutamine

diseases is the formation of neuronal inclusions, which consist of misfolded disease proteins, ubiquitin, molecular chaperones, proteasomes and amounts of other proteins, generally in the nucleus [15–17]. Studies with cell [18–20] and transgenic mice [21–23] models showed that pathogenic ataxin-3 was more toxic when in the nucleus than in the cytoplasm. However, the molecular biomarkers and their accurate mechanism remain to be elucidated in SCA3/MJD.

Exosomes are tiny vesicles (40–150 nm) containing RNAs, DNAs and proteins that secreted by all living cells, which could be detected in various body fluids. Once attached to a target cell, exosomes can be internalized by the target cell's membrane to deliver their content, thereby modifying the physiological state of the recipient cell [24].

Although genetic diagnosis of SCA3/MJD is the gold standard for diagnosis, it cannot be used to estimate other aspects such as clinical clarification, the disease duration or the severity of ataxia [25]. In the past few years, there were only a few studies about the functional roles of exosomes in neurodegenerative diseases. First, biomarker research: Saman *et al.* [26] identified a variety of misfolded tau proteins in CSF-derived exosomes in Alzheimer's disease (AD); Mullins *et al.* [27] found that upregulated pSer312-IRS-1 and downregulated p-panTyr-IRS-1 in exosomes from AD patients were related to its brain atrophy degree, and the above two indicators were consistent with the regional expression of IRS-1. They both indicated that exosomes could be used as biomarkers for diagnosis and assessment for AD progression. Second, pathogenesis research: Stuendl *et al.* [28] found α -synuclein in CSF-derived exosomes in Parkinson's disease (PD) patients differs from that in dementia with Lewy bodies patients, and was associated with cognitive level. Through co-culturing normal mice neural stem cells with fibroblasts from huntington patients, Jeon *et al.* [29] found that exosomes could transfer mutant huntington proteins across species boundaries and then introduce relative performance of behavior and pathology. Third, potential therapeutic research: exosomes could pass through the blood–brain barrier easily due to their small size. With the addition of immune privilege character, exosomes are becoming a new promising therapeutic vector for neurodegenerative diseases increasingly [30]. Unfortunately, the roles of exosomes in SCAs have not been reported so far.

The idea that miRNAs (one group of noncoding RNAs with 21–25 nucleotides in length) secreted in exosomes can be functionally transferred to living cells, resulting in modulation of their target genes, has become one of the most actively explored hypotheses in recent years [24]. As exosomes isolated from extracellular fluids such as CSF and blood represent an enriched source of biomolecules, they are hypothesized to provide potential biomarkers for neurodegenerative diseases. The nervous system is a rich source of miRNAs [31], which may reflect clinical classification, the onset or stages of neurodegenerative disease [32]. While most studies utilize free miRNAs, so as our former research, to investigate the role of miR-25 in cell models of SCA3/MJD [33], exosomes provide a protective environment for miRNAs [34], which demonstrates the feasibility of using exosomal miRNA as an alternative biomarker screening tool for neurodegenerative diseases. However, profiling of exosomal miRNAs in SCA3/MJD patients' blood and CSF has never been illustrated.

In the present study, we explored the profile of exosomal miRNAs derived from the plasma and CSF of SCA3/MJD patients to identify the disease-related biomarker and investigate its potential molecular mechanism.

Material & methods

Recruitment of SCA3/MJD subjects & SCA3/MJD-free controls

Plasma and CSF samples were collected from the Department of Neurology and Neurosurgery in Xiangya Hospital, Central South University (China) from 8 August 2017 to 1 March 2018. All SCA3/MJD patients were under at least two experienced neurologists' clinical diagnosis in accordance with Harding diagnosis criteria and incorporated into this study based on the following criteria. First, the ages range from 18 to 75 years old; second, without other accompanying diseases such as hypertension, diabetes, etc. Third, without fever or traumatic brain injury within 1 month; and fourth, without intaking disease-related drug in the past 6 months. Besides, the neurological analyzing results of the subjects were settled using the mean calculated value of that obtained from these two neurologists. Only the first three relatively common subtypes of type 1, 2, 3 SCA3/MJD diseases were recruited and assigned to the corresponding groups on account of their age and clinical manifestations. Referred to SCA3/MJD-free controls, plasma samples were recruited from healthy volunteers and CSF samples were recruited from aneurysm or migraine patients with chronic headache without intracranial hypertension, whose indexes of routine biochemical and cytology in CSF were basically normal. The SCA3-free controls had a similar range of age and life conditions with SCA3/MJD patients. Both SCA3/MJD patients and SCA3/MJD-free controls had signed the informed consent. All samples were obtained in accordance with the ethical standards in the Declaration of Helsinki and the research was approved by Medical Ethics Committee of Xiangya Hospital, Central South University, China.

As for plasma samples, plasma-derived exosomal RNAs from 12 SCA3/MJD patients (three cases of Type 1, six cases of Type 2 and three cases of Type 3), and 12 healthy controls with matched age and gender were extracted for small RNA sequencing. In addition, plasma-derived exosomal RNAs from 18 other patients (one case of Type 1 and 17 cases of Type 2) and 16 controls were extracted as the validation cohort for further real-time quantitative PCR (qPCR) verification. With respect to CSF samples, CSF-derived exosomal RNAs from 12 SCA3/MJD patients (three cases of Type 1, six cases of Type 2 and three cases of Type 3) and ten controls with matched age and gender were isolated for small RNA sequencing.

Exosomes/exosomal RNA isolation & identification

About 4 ml plasma or 15 ml CSF was applied to separate exosomes and extract exosomal RNAs, respectively, according to the protocols of exoEasy Kit and exoRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). The exosomes were identified by nanoparticle tracking (NTA) under a ZetaView Nanoparticles Trace Analyzer (Particle Metrix, Munich, Germany), a transmission electron microscope (Tecnai G2 Spirit, FEI, Hillsboro, Oregon) and by detection of exosomal marker (CD9) using western blot with antibodies purchased from Santa Cruz Biotechnology (CA, USA) [35,36]. The RNA quality testing of isolated exosomal RNAs was carried out using Agilent 2100 (CA, USA).

Small RNA sequencing

Small RNA sequencing was carried out to discover the profile of exosomal miRNAs derived from plasma and CSF samples of SCA3/MJD patients and SCA3/MJD-free controls. First, following RNAs quality control after exosomal RNA extraction, exosomal RNAs were sequenced using BGISEQ-500 technology (BGI, Shenzhen, Guangdong, China) with 20M reads. The raw data received a subsequent bioinformatics analysis (Supplementary File 1). Second, data filtering: the data were processed using the following steps: remove low quality tags; remove tags with 5' primer contaminants; remove tags without 3' primer; remove tags without insertion; remove tags with poly A; remove tags shorter than 18 nt; summarize the length distribution of the clean tags. Third, reads mapping: the software of Bowtie [37] was used to map clean reads to the reference genome and to other small RNA databases. Fourth, miRNAs prediction: miRDeep2 [38] was employed to predict novel miRNAs by exploring the characteristic hairpin structure of miRNA precursors. Fifth, miRNA expression: the levels of miRNAs expression were calculated by using Transcripts Per Kilobase Million, which was able to eliminate the influence of sequencing discrepancy on the calculation of small RNA expression [39]. Sixth, target prediction: miRanda [40] and TargetScan [39] were applied to predict target genes of miRNAs. Seventh, screening differentially expressed miRNAs: small RNA sequencing could be modeled as a random sampling process, each read was sampled independently and uniformly from every possible nucleotide in the samples [41]. Under this assumption, the number of reads coming from a transcript isoform followed a binomial distribution and could be approximated by a Poisson distribution. DEG seq (differentially expressed gene sequencing) analysis [42] using MA-plot, which was a statistical analysis tool having been widely used to detect and visualize intensity-dependent ratio of microarray data, was further carried out for screening of differentially expressed genes [43]: we defined $M = \log_2 C_1 - \log_2 C_2$ and $A = (\log_2 C_1 + \log_2 C_2)/2$, where C_1 and C_2 were defined by C_i approximately binomial (n_i, p_i ; $i = 1, 2$, n_i denoted the total number of mapped reads and p_i denoted the probability of a read coming from that gene) to denote the counts of reads mapped to a specific gene obtained from two samples. Under the random sampling assumption, the conditional distribution of M given that $A = a$ (a is an observation of A) was proved to follow an approximate normal distribution. For each gene on the MA-plot, we did the hypothesis test of $H_0: p_1 = p_2$ versus $H_1: p_1 \neq p_2$. Then a p-value was assigned based on the conditional normal distribution. The p-values calculated for each gene was adjusted to Q-values for multiple testing corrections by two alternative strategies [44]. To improve the accuracy of DEGs result, we defined the genes whose fold change of reading number ≥ 2 and Q-value ≤ 0.001 as DEGs [44,45]. To improve the accuracy of these results, a miRNA was defined as a differentially expressed miRNA when reads number fold change ≥ 2 and Q-value ≤ 0.001 . Eighth, hierarchical clustering analysis: hierarchical clustering for differentially expressed miRNAs was performed using heatmap, a function of R program. Ninth, gene ontology (GO) enrichment analysis: GO enrichment analysis was used to recognize the main biological functions that differentially expressed miRNA targets exercise: to perform this analysis, all genes were mapped initially to GO-terms in the database (www.geneontology.org/) to calculate the gene numbers for every term.

The hypergeometric test basing on ‘GO::Term Finder’ (www.yeastgenome.org/help/analyze/go-term-finder) was then used to find significantly enriched GO-terms in the input gene list. The algorithm of $P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$ (N represented the number of all genes with GO annotation, n represented the number of differentially expressed miRNAs’ target genes in N, M represented the number of all genes that are annotated to a specific GO term and m represented the number of differentially expressed miRNAs’ target genes in M) was used to perform this analysis. After that, the p-value was corrected by the Bonferroni method [46], where a corrected p-value ≤ 0.05 was taken as a threshold. GO terms fulfilling this condition were defined as significantly enriched GO terms. Tenth, pathway enrichment analysis: pathway-based analysis helped to further understand the biological functions of differentially expressed miRNA-targeting genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) [47] (the major public pathway-related database) was used to identify significantly enriched metabolic pathways or signal transduction pathways in differentially expressed miRNA-targeting genes comparing with the whole genome background. The calculating formula was the same as that in GO analysis, in which N was the number of all genes with KEGG annotation, n was the number of differentially expressed miRNAs’ target genes in N, M was the number of all genes that are annotated to a specific pathway and m was the number of differentially expressed miRNAs’ target genes in M. The p-value was also corrected by the Bonferroni method and a corrected p-value ≤ 0.05 was taken as a threshold. KEGG terms fulfilling this condition were defined as significantly enriched KEGG terms.

Verification of miRNAs by qPCR

Further verification of the differential expression of the miRNAs detected above was carried out among plasma-derived exosomal RNAs from 18 other SCA3/MJD patients and 16 SCA3-free controls using qPCR method. Among the differentially expressed miRNAs, those had differential expression trends in more comparing groups and with relatively higher fold change were chosen for the verification preferentially. Combining with our study, we chose 27 miRNAs for further qPCR verification among the plasma-derived exosomal RNAs in the validation cohort. The specific PCR primers were designed and synthesized by TSING KE Biological Technology (Beijing, China). First, the reverse transcription was carried out using miScript II RT kit (Qiagen). The qPCR amplification for individual miRNA expression was carried out with $2 \times$ T5 Fast qPCR Mix (SYBR Green I) on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, MA, USA) using 384-well plates (the primers of the miRNAs for verification are provided in [Supplementary File 2](#)). Cycle threshold (Ct) values calculated as $\Delta Ct = Ct(\text{miRNA}) - Ct(\text{U6})$ method were obtained for further data analysis.

Statistical analysis

Relative expression levels of miRNAs were evaluated by comparing $-\Delta Ct$ values. Box plots were generated using the software of R Studio. The differences between two groups fitting the normal distribution were analyzed using Student’s t-test. Depending on the frequency histogram of data distribution which did not fit the normal distribution completely, nonparametric test was chosen for statistical analysis using SPSS20.0 (IL, USA). All statistical tests in the study were two sided and statistical significance was set at $p < 0.05$. Besides, linear-regression, Random Forest and LASSO analyses were carried out for the correlation of miRNAs and clinical indexes such as age at onset, CAG repeats, SARA scales and disease duration.

Results

Clinical & genetical characters of SCA3/MJD patients & SCA3/MJD-free controls

Clinical and genetical information of the subjects are detailed in [Table 1](#): Among all the SCA3/MJD patients, there were 20 male patients (55.56%) and 16 female patients (44.44%); among all the SCA3/MJD-free controls, there were 19 males (50%) and 19 females (50%). Through subtype classification, the ages of subtype one patients were 22.8 ± 4.3 years old, while the ages at onset were 17.0 ± 1.4 years old; the CAG repeats of which range among 77.4 ± 2.5 repeats. As for subtype two, the ages of patients were at 40.4 ± 8.5 years old, the ages at onset were at 32.9 ± 8.3 years old; while the CAG repeats fluctuated among 71.8 ± 3.8 repeats. The age of subtype three were at 56.5 ± 4.7 years old; the ages at onset were 47.8 ± 6.8 years old; the CAG repeats ranged at 62.5 ± 5.7 repeats. The clinical characteristics were also obvious in accordance with the classification criteria of the three subtypes, in other words, Type 1 manifests prominent pyramidal signs (rigidity and spasticity) and extrapyramidal features (bradykinesia and dystonia) combined with ataxia, Type 2 has progressive external ophthalmoplegia and pyramidal signs with ataxia. Type 3 appears as peripheral signs together with ataxia.

Table 1. Summary of clinical and genetical information of the subjects.

SCA3 /MJD patients								SCA3 /MJD-free controls				
Tag	Subtype	Age	Gender	CAG repeats	Age at onset	Duration	SARA	Clinical characteristics		Tag	Age	Gender
Samples for small RNA sequencing (plasma & CSF, i.e., P&C)												
P1 (P & C)	1	18	M	80	15	3	12	Gait instability, alalia, dysphagia and choke		C1 (P)	18	M
P2 (P & C)	1	25	F	80	18	7	8	Gait instability, alalia, choke and clumsy hands		C2 (P)	24	F
P3 (P)	1	29	M	77	16	13	24.5	Gait instability, choke, clumsy hands and disharmony		C3 (P)	25	M
P4 (P & C)	2	32	M	76	24	8	17.5	Gait instability, alalia, dysphagia and choke		C4 (P)	36	M
P5 (P)	2	38	F	73	33	13	5	Gait instability, alalia and choke		C5 (P)	41	F
P6 (P)	2	45	F	75	23	22	29	Gait instability, alalia and blurred vision		C6 (P)	43	F
P7 (P & C)	2	49	F	69	42	7	10	Drunk gait, alalia and diplopia		C7 (P)	49	F
P8 (P)	2	47	M	71	46	1	8	Walking instability, dizziness, diminution of vision and diplopia		C8 (P)	47	M
P9 (P)	2	49	M	66	40	9	9	Drunk gait, alalia and diplopia		C9 (P)	48	M
P10 (P & C)	3	51	M	60	40	11	22	Walking instability, alalia and dizziness		C10 (P)	54	M
P11 (P & C)	3	55	F	59	45	10	16	Progressive walking instability, blurred vision, alalia, choke, Lower extremity convulsion and numbness, paroxysmal dyspnea		C11 (P)	54	F
P12 (P)	3	62	M	71	50	12	15	Walking instability and lower extremity numbness		C12 (P)	58	M
P13 (C)	1	20	M	75	18	2	11.5	Drunk gait, alalia, dysphagia choke and slight diplopia		C13 (C)	21	M
P14 (C)	2	35	F	67	31	4	11.5	Gait instability, choke and inflexible hand activity		C14 (C)	28	M
P15 (C)	2	45	M	76	31	12	20	Gait instability, knee weakness and choke		C15 (C)	38	F
P16 (C)	2	51	M	70	42	9	14.5	Drunk gait, alalia, twitching of muscle and diplopia		C16 (C)	44	M
P17 (C)	2	51	F	70	44	7	8	Drunk gait, alalia and diplopia		C17 (C)	47	F
P18 (C)	3	58	F	60	56	2	8	Progressive walking instability, blurred vision and slight choke		C18 (C)	51	F
										C19 (C)	51	M
										C20 (C)	49	M
										C21 (C)	59	F
										C22 (C)	61	F

CSF: Cerebrospinal fluid; F: Female; M: Male; MJD: Machado–Joseph disease; P&C: Plasma & CSF; SCA3: Spinocerebellar ataxia Type 3.

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Table 1. Summary of clinical and genetical information of the subjects (cont.).											
SCA3 /MJD patients								SCA3 /MJD-free controls			
Tag	Subtype	Age	Gender	CAG repeats	Age at onset	Duration	SARA	Clinical characteristics	Tag	Age	Gender
Samples for independent validation (plasma)											
P19	2	42	F	64	40	2	11.5	Gait instability and alalia	C23	42	F
P20	2	51	F	70	42	9	14.5	Drunk gait, alalia, choke, blurred vision	C24	53	F
P21	2	51	M	70	44	7	8	Drunk gait, alalia, twitching of muscle and diplopia	C25	48	M
P22	1	22	M	75	18	4	14	Drunk gait, alalia, dysphagia choke and slight diplopia	C26	43	M
P23	2	36	F	71	23	13	6	Gait instability, choke and clumsy hands	C27	34	F
P24	2	45	M	76	31	14	20	Gait instability, knee weakness and choke	C28	49	F
P25	2	35	F	67	31	4	10.5	Gait instability, choke and inflexible hand activity	C29	41	M
P26	2	36	M	74	26	10	13.5	Gait instability, involuntary jitter of hands and head	C30	54	F
P27	2	42	M	68	32	10	6	Drunk gait, alalia, choke, blurred vision	C31	68	M
P28	2	36	F	76	28	8	13	Drunk gait, alalia, choke, blurred vision and constipation	C32	40	F
P29	2	29	M	79	21	8	10	Drunk gait and alalia	C33	30	M
P30	2	32	F	76	27	5	11.5	Drunk gait and alalia	C34	51	M
P31	2	48	M	69	41	7	8.5	Drunk gait, alalia and diplopia	C35	36	M
P32	2	28	M	74	26	2	4.5	Gait instability	C36	23	F
P33	2	26	F	76	22	4	10.5	Drunk gait, alalia and choke	C37	36	F
P34	2	28	F	73	23	5	8.5	Gait instability and alalia	C38	47	F
P35	2	33	M	72	31	2	7	Drunk gait and alalia			
P36	2	52	M	70	45	7	18	Drunk gait, dysphagia choke and slight diplopia			
CSF: Cerebrospinal fluid; F: Female; M: Male; MJD: Machado–Joseph disease; P&C: Plasma & CSF; SCA3: Spinocerebellar ataxia Type 3.											

Identification of isolated exosomes/exosomal RNAs

Exosome numbers and scales shown in Figure 1 indicated that the particle size distribution was as follows: 10% of the particles were under 81.2 nm, 50% were under 135.4 nm and 90% were under 202.5 nm. The exosomes were observed under the transmission electron microscope as round- or oval-like particles with uneven sizes, which distributed between 40–150 nm in diameter. Normally, they had complete membrane structures and encapsulated low density material inside. Besides, it was confirmed that there was an expression of exosome marker CD9 in the isolated particle proteins, which further identified the extracts as exosomes (Figure 1A–C).

Differential expression of miRNAs in plasma-derived exosomes

Through preliminary RNA sequencing analysis, 53 miRNAs (30 novel miRNAs and 23 known miRNAs) were found to be upregulated, 20 miRNAs (15 novel miRNAs and five known miRNAs) were found to be downregulated significantly (p -value = 0, Q -value = 0) in exosomes derived from SCA3/MJD patients' plasma, as being compared with that from SCA3/MJD-free controls (as shown in Table 2 and Figure 1D of hierarchical clustering analysis heatmap for details).

Differential expression of miRNAs in CSF-derived exosomes

As for CSF samples, 69 miRNAs (41 novel miRNAs and 28 known miRNAs) were detected to be upregulated, and 47 miRNAs (21 novel miRNAs and 26 known miRNAs) be downregulated significantly in exosomes derived from SCA3/MJD patients' CSF, as being compared with that from SCA3/MJD-free controls (p -value = 0, Q -value = 0, shown in Table 2 and Figure 1D of hierarchical clustering analysis heatmap).

Comparison between exosomal RNAs derived from plasma & CSF samples

Based on a comparison of the differentially expressed miRNAs between plasma- and CSF-derived exosomes in SCA3/MJD subjects, the expression trend of 15 miRNAs reached consensus between them, while ten miRNAs showed opposite trend. Among the miRNAs mentioned above, 18 miRNAs showed significant differentiation between plasma- and CSF-derived exosomal miRNAs (listed in Table 3).

Differentially expressed miRNAs of plasma-derived exosomes among different SCA3/MJD subtypes

On the basis of the 69 upregulated miRNAs and 47 downregulated miRNAs detected from CSF-derived exosomes, these miRNA expression levels were further analyzed through the pairwise comparison among 1, 2 and 3 SCA3/MJD subtypes of their plasma-derived exosomes (Table 4).

Differentially expressed miRNAs of CSF-derived exosomes among different SCA3/MJD subtypes

Similar to above, the pairwise comparison of miRNAs expression in the CSF-derived exosomes among Type 1, 2 and 3 SCA3/MJD subtypes are listed in Table 4.

The differential expression of exosomal miRNAs with adjusted p -values mentioned above have been displayed in Supplementary Table 4.

Relative expression levels of miRNAs chosen for further verification in the validation cohort

After a verification by qPCR detection (primers are listed in Supplementary Table 3), the expression of mir-7014 was found to be significantly downregulated in patients' plasma-derived exosomes ($p < 0.05$; Supplementary Table 1–2), which was in accordance with small RNA sequencing results. And after linear-regression analysis with clinical indexes, mir-7014 was found to be correlated with age at onset with a coefficient of 0.01493091 and an intercept of 26.890955696793025 (shown in Supplemental File 2). Besides, the trends of other miRNAs except mir-7014 were either un conspicuous or contrary to the small RNA sequencing results (Figure 1G). Moreover, Random Forest or LASSO seemed to be not that applicable for the present study, the evaluation results of which have been listed in the Supplementary File 4.

miRNAs & downstream target genes' prediction

mir-7014 was predicted to be on Chromosome12 from 56908536 to 56908583 with a mature sequence 'cugugu-uccugagccuug', a complementary sequence 'gucaguggcacagcc' and a precursor sequence 'gucaguggcacagccacugccu-gagggugcuguguuccugagccuug'. Characteristic hairpin structures of the detected miRNAs precursor were explored using MiRDeep2, and its structure predicted by mfold software (website: <http://unafold.rna.albany.edu/?q=mfold>)

Figure 1. Screening and identification of differentially expressed exosomal miRNAs. (A) A representative depicting vesicle size and number of plasma- and CSF-derived exosomes from SCA3/MJD and control subjects. (B) A typical image of plasma- and CSF-derived exosomes screened by a transmission electron microscope. (C) Detection for expression of exosome marker CD9. (D) Hierarchical clustering among different comparing groups. The abscissa represents each comparing group and the ordinate means differentially expressed miRNAs. Colors indicate fold change (upregulated: red, downregulated: blue). (E) Venn statistics of the target predictors of the detected miRNAs. (F) Venn statistics of the filtered target predictors of the detected miRNAs. (G) Expression of miRNAs chosen for qPCR verification in the validation cohort. Gray box denotes relative expression of patients' plasma-derived exosomal miRNAs and dark gray box stands for that of the controls group. CSF: Cerebrospinal fluid; SCA3/MJD: Spinocerebellar ataxia Type 3/Machado–Joseph disease; qPCR: Quantitative polymerase chain reaction.

Table 2. Differential expression of miRNAs in plasma- and cerebrospinal fluid-derived exosomes.

Plasma-derived exosomal miRNAs				CSF-derived exosomal miRNAs			
Upregulated known miRNAs	Downregulated known miRNAs	Upregulated novel miRNAs	Downregulated novel miRNAs	Upregulated known miRNAs	Downregulated known miRNAs	Upregulated novel miRNAs	Downregulated novel miRNAs
hsa-miR-204-5p	hsa-miR-122-5p	novel_mir2738	novel_mir2848	hsa-miR-21-5p	hsa-miR-219a-2-3p	novel_mir6820	novel_mir5
hsa-miR-24-3p	hsa-miR-192-5p	novel_mir9034	novel_mir398	hsa-miR-200c-3p	hsa-miR-181a-5p	novel_mir2256	novel_mir769
hsa-miR-3074-5p	hsa-miR-122-3p	novel_mir1643	novel_mir7227	hsa-miR-191-5p	hsa-miR-1-3p	novel_mir2738	novel_mir1
hsa-miR-328-3p	hsa-miR-194-5p	novel_mir8421	novel_mir769	hsa-miR-223-3p	hsa-miR-378b	novel_mir7857	novel_mir2
hsa-let-7d-3p	hsa-miR-3184-3p	novel_mir6628	novel_mir2256	hsa-miR-16-5p	hsa-miR-181b-5p	novel_mir4358	novel_mir2415
hsa-miR-484		novel_mir7660	novel_mir6438	hsa-miR-27a-3p	hsa-miR-1298-5p	novel_mir9034	novel_mir4884
hsa-miR-486-3p		novel_mir5219	novel_mir5054	hsa-miR-126-3p	hsa-miR-204-3p	novel_mir2446	novel_mir4676
hsa-miR-206		novel_mir4559	novel_mir5077	hsa-miR-150-5p	hsa-miR-7-5p	novel_mir4987	novel_mir201
hsa-miR-375		novel_mir3426	novel_mir7857	hsa-miR-142-3p	hsa-miR-34a-5p	novel_mir8156	novel_mir1706
hsa-miR-146b-5p		novel_mir3836	novel_mir3529	hsa-miR-222-3p	hsa-miR-34c-3p	novel_mir364	novel_mir1364
hsa-miR-1290		novel_mir6555	novel_mir2698	hsa-miR-26b-5p	hsa-miR-4516	novel_mir822	novel_mir6603
hsa-miR-142-3p		novel_mir3209	novel_mir4987	hsa-miR-182-5p	hsa-miR-133a-3p	novel_mir5407	novel_mir371
hsa-miR-92b-3p		novel_mir7862	novel_mir769	hsa-miR-183-5p	hsa-miR-219b-5p	novel_mir1117	novel_mir180
hsa-miR-378g		novel_mir6820	novel_mir4682	hsa-miR-93-5p	hsa-miR-4488	novel_mir2017	novel_mir2003
hsa-miR-1268a		novel_mir4476	novel_mir7014	hsa-miR-31-5p	hsa-miR-9-5p	novel_mir355	novel_mir229
hsa-miR-151a-3p		novel_mir1117		hsa-miR-142-5p	hsa-miR-124-3p	novel_mir412	novel_mir327
hsa-miR-34a-5p		novel_mir364		hsa-miR-31-3p	hsa-miR-4532	novel_mir2539	novel_mir4020
hsa-miR-196b-5p		novel_mir1686		hsa-miR-15b-5p	hsa-miR-3656	novel_mir2297	novel_mir6740
hsa-miR-128-3p		novel_mir6297		hsa-miR-96-5p	hsa-miR-138-5p	novel_mir7014	novel_mir5653
hsa-miR-21-5p		novel_mir4056		hsa-miR-205-5p	hsa-miR-30d-5p	novel_mir3602	novel_mir31
hsa-miR-200c-3p		novel_mir2722		hsa-miR-429	hsa-miR-6087	novel_mir6551	novel_mir356
hsa-miR-200a-3p		novel_mir6749		hsa-miR-1260a	hsa-miR-3960	novel_mir5378	
hsa-miR-340-5p		novel_mir355		hsa-miR-425-5p	hsa-miR-139-3p	novel_mir3529	
		novel_mir6740		hsa-miR-19b-3p	hsa-miR-1246	novel_mir6214	
		novel_mir2446		hsa-miR-340-5p	hsa-miR-135a-5p	novel_mir5077	
		novel_mir2359		hsa-miR-7977	hsa-miR-320c	novel_mir3472	
		novel_mir14		hsa-miR-20a-5p		novel_mir18	
		novel_mir5		hsa-miR-200a-3p		novel_mir2332	
		novel_mir4884				novel_mir302	
		novel_mir7747				novel_mir1550	
						novel_mir7171	
						novel_mir1643	
						novel_mir1178	
						novel_mir3010	
						novel_mir2010	
						novel_mir8983	
						novel_mir1183	
						novel_mir1686	
						novel_mir20	
						novel_mir6417	
						novel_mir3509	

CSF: Cerebrospinal fluid.

is displayed in [Supplementary File 3](#). With regard to the target genes of all the detected miRNAs, 1687,177 common spots between TargetScan and RNA hybrid databases were identified as shown in [Figure 1E](#). In order to minimize the possible interested downstream targets of these miRNAs, appropriate filter conditions including MFE and score evaluation were applied for the further analysis ([Figure 1F](#)).

Table 3. Comparison between differentially expressed exosomal miRNAs derived from plasma and cerebrospinal fluid samples in spinocerebellar ataxia Type 3/Machado–Joseph disease patients.

Plasma↑ CSF↑	Plasma↓ CSF↓	Plasma↑ CSF↓	Plasma↓ CSF↑	Plasma/CSF ↑	Plasma/CSF↓
hsa-miR-21-5p	novel_mir769	hsa-miR-34a-5p	novel_mir2256	hsa-miR-34a-5p	novel_mir2256
hsa-miR-200c-3p		novel_mir5	novel_mir7857	novel_mir4884	hsa-miR-200c-3p
hsa-miR-142-3p		novel_mir4884	novel_mir4987	novel_mir6740	novel_mir2738
hsa-miR-340-5p		novel_mir6740	novel_mir7014		novel_mir7857
hsa-miR-200a-3p			novel_mir3529		novel_mir9034
novel_mir6820			novel_mir5077		novel_mir2446
novel_mir2738					novel_mir4987
novel_mir9034					novel_mir364
novel_mir2446					novel_mir1117
novel_mir364					novel_mir355
novel_mir1117					novel_mir7014
novel_mir355					novel_mir3529
novel_mir1643					novel_mir5077
novel_mir1686					novel_mir1643
					novel_mir769

Plasma/CSF represents differentially expressed exosomal miRNAs with significant difference between plasma and CSF groups.
↑ stands for upregulated; ↓ stands for downregulated exosomal miRNAs expression.
CSF: Cerebrospinal fluid.

GO enrichment & KEGG pathway analysis of mir-7014

GO-based analysis was performed to further investigate the potential roles of mir-7014 involved in SCA3/MJD disease. A total of 4112 potential mir-7014 downstream targets were classified into biological processes, 3018 into cellular components and 1149 into molecular functions. The common biological processes terms were cellular process, single-organism process, metabolic process, biological regulation, regulation of biological process and response to stimulus. Some of the significant cellular components with the highest target numbers were cell, cell part, organelle and membrane. Binding and catalytic activity were among the most abundant classes in molecular function category (Figure 2A).

The KEGG classification system categorized the target genes of mir-7014 into different groups. The top 15 enriched groups among KEGG categories included signal transduction, overview of cancers, transport and catabolism, global and overview maps, immune system, folding sorting and degradation, cellular community-eukaryotes, endocrine system, translation, cancers: specific types, signal molecules and interaction, nervous system, infectious diseases (Figure 2B).

Moreover, KEGG pathway enrichment analysis of these target genes was carried out to identify potential pathways regulated by mir-7014 (Figure 2C). The results exhibited that target genes were enriched in protein processing in endoplasmic reticulum (Figure 3A) and axon guidance (Figure 3B).

Discussion

In view of all the results achieved from the present study, exosomal mir-7014 was found to be downregulated in SCA3/MJD patients’ plasma, while upregulated in patients’ CSF. Moreover, mir-7014 in CSF-derived exosomes from Type 1, 2 and 3 groups were successively upregulated with a statistical significance basing on the present study. Further, after analyzing the correlation of mir-7014 with clinical related indexes such as age at onset, SARA scores, CAG repeats and duration, we found that there was a correlation of it with age at onset, while no obvious correlation with other aspects in our present study, which may need more exploration in the future work. Consequently, exosomal mir-7014 is probably a significant biomarker candidate in SCA3/MJD, which indicates the clinical classification and age at onset. As one kind of important transport medium for noncoding RNAs, exosomes are more stable than their parent cells and could cross the blood–brain barrier, allowing communication between periphery and brain both in normal and disease conditions [48–50]. Exosomes could be released from all types of cells and bidirectionally transported through the blood–brain communication interfaces, blood–brain and blood–cerebrospinal fluid barriers [51]. As to our results, there is an opposite trend between plasma- and CSF-derived exosomal mir-7014 expression levels, how this trend formed deserves to be discussed. According to

Table 4. Pairwise comparison of plasma- and cerebrospinal fluid-derived exosomal miRNAs among 1, 2 and 3 spinocerebellar ataxia Type 3/Machado–Joseph disease subtypes.

Plasma-derived exosomal miRNAs						CSF-derived exosomal miRNAs					
Type1/Type2 up	Type1/Type2 down	Type1/Type3 up	Type1/Type3 down	Type2/Type3 up	Type2/Type3 down	Type1/Type2 up	Type1/Type2 down	Type1/Type3 up	Type1/Type3 down	Type2/Type3 up	Type2/Type3 down
		hsa-miR-21-5p	hsa-miR-21-5p	hsa-miR-21-5p	hsa-miR-21-5p	hsa-miR-21-5p	hsa-miR-21-5p	hsa-miR-200c-3p	hsa-miR-200c-3p	hsa-miR-21-5p	hsa-miR-21-5p
		hsa-miR-200c-3p	hsa-miR-200c-3p	hsa-miR-200c-3p	hsa-miR-200c-3p	hsa-miR-200c-3p	hsa-miR-200c-3p	hsa-miR-200c-3p	hsa-miR-200c-3p		
		hsa-miR-142-3p	hsa-miR-142-3p	hsa-miR-142-3p	hsa-miR-142-3p	hsa-miR-142-3p	hsa-miR-142-3p	hsa-miR-142-3p	hsa-miR-142-3p		
		hsa-miR-340-5p	hsa-miR-340-5p	hsa-miR-340-5p	hsa-miR-340-5p	hsa-miR-340-5p	hsa-miR-340-5p				
		hsa-miR-200a-3p	hsa-miR-200a-3p	hsa-miR-200a-3p	hsa-miR-200a-3p	hsa-miR-200a-3p	hsa-miR-200a-3p				
	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820	novel_mir6820
		novel_mir2256	novel_mir2256	novel_mir2256	novel_mir2256	novel_mir2256	novel_mir2256	novel_mir2256	novel_mir2256	novel_mir2256	novel_mir2256
		novel_mir2738	novel_mir2738	novel_mir2738	novel_mir2738	novel_mir2738	novel_mir2738	novel_mir2738	novel_mir2738	novel_mir2738	novel_mir2738
		novel_mir9034	novel_mir9034	novel_mir9034	novel_mir9034	novel_mir9034	novel_mir9034	novel_mir9034	novel_mir9034	novel_mir9034	novel_mir9034
	hsa-miR-34a-5p	hsa-miR-34a-5p	hsa-miR-34a-5p	hsa-miR-34a-5p	hsa-miR-34a-5p	hsa-miR-34a-5p	hsa-miR-34a-5p				
novel_mir5											
novel_mir4884											
	novel_mir6740	novel_mir6740	novel_mir6740	novel_mir6740	novel_mir6740	novel_mir6740	novel_mir6740				
						novel_mir7857	novel_mir7857	novel_mir2446	novel_mir2446	novel_mir7857	novel_mir2446
						novel_mir2446	novel_mir2446	novel_mir4987	novel_mir4987	novel_mir2446	novel_mir4987
						novel_mir4987	novel_mir4987	novel_mir364	novel_mir364	novel_mir4987	novel_mir364
						novel_mir1117	novel_mir1117	novel_mir1117	novel_mir1117	novel_mir1117	novel_mir1117
						novel_mir355	novel_mir355				
						novel_mir7014	novel_mir7014	novel_mir7014	novel_mir7014	novel_mir7014	novel_mir7014
						novel_mir3529	novel_mir3529	novel_mir3529	novel_mir3529	novel_mir3529	novel_mir3529
								novel_mir5077	novel_mir5077	novel_mir5077	novel_mir5077
								novel_mir1643	novel_mir1643	novel_mir1643	novel_mir1643
						novel_mir769	novel_mir769			novel_mir769	novel_mir769

Type1/Type2 represents the comparison of differentially expressed plasma- or CSF-derived miRNAs between Type 1 and 2 SCA3/MJD diseases. Type1/Type3 represents the comparison of differentially expressed miRNAs between Type 1 and 3 SCA3/MJD. Similarly, Type2/Type3 represents a comparison of that between Type 2 and 3 SCA3/MJD.

CSF: Cerebrospinal fluid; MJD: Machado–Joseph disease; SCA3: Spinocerebellar ataxia Type 3.

Figure 2. GO and KEGG analyses of target genes and downstream pathways of mir-7014. (A) GO annotation of downstream target genes of mir-7014. The abscissa is the target gene number and the ordinate left is the GO annotation of mir-7014. **(B)** KEGG classification of the target genes of mir-7014. The abscissa is the target gene number and the ordinate left is the KEGG classification of mir-7014. **(C)** KEGG pathway enrichment analysis of the target genes of mir-7014. The abscissa is the rich factor and the ordinate left is the pathway classification of mir-7014. Rich factor represents the frequency of differentially expressed genes with pathway annotation in all the genes with annotation. The size of dots indicates gene number for enrichment and the color shade of them prompt the Q value. The smaller the Q value is, the better the enrichment evaluation is. GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Figure 2. GO and KEGG analyses of target genes and downstream pathways of mir-7014 (cont.). (A) GO annotation of downstream target genes of mir-7014. The abscissa is the target gene number and the ordinate left is the GO annotation of mir-7014. (B) KEGG classification of the target genes of mir-7014. The abscissa is the target gene number and the ordinate left is the KEGG classification of mir-7014. (C) KEGG pathway enrichment analysis of the target genes of mir-7014. The abscissa is the rich factor and the ordinate left is the pathway classification of mir-7014. Rich factor represents the frequency of differentially expressed genes with pathway annotation in all the genes with annotation. The size of dots indicates gene number for enrichment and the color shade of them prompt the Q value. The smaller the Q value is, the better the enrichment evaluation is. GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

the communication between CSF and plasma, some interflow between them may occur, for example, what if plasma-derived exosomal mir-7014 could be transferred from peripheral blood into CSF through blood–brain barrier? All the above assumption makes it interesting for us to carry out further exploration.

To better understand the functions of novel mir-7014, all suspected downstream genes with differential expression levels were classified into different groups according to their function annotation and pathways enrichment. It is of interest that KEGG pathway annotation of target genes resulted in the identification of two highly enriched pathways in our results: protein processing in endoplasmic reticulum and axon guidance, both of which have been reported to be involved in the pathogenesis of SCA3/MJD [52–54]. Endoplasmic reticulum-associated degradation is the system that mediates the ubiquitination of misfolded proteins or unassembled complex constituents present in the secretory pathway and their exportation to the cytosol for degradation by the proteasome [12,13,55–57]. In the pathway of protein processing in endoplasmic reticulum (Figure 3A), protein processing is divided into two pathways: the correctly folded protein would be transported into the Golgi body via the ERManI-mediated pathway and released into the cytoplasm; while the misfolded protein would be degraded through EDEM and ERManI, in which the family of heat shock proteins such as HSP40/HSP70 could participate. While ataxin3 had been documented to be associated with the endoplasmic reticulum-associated degradation, there were different points of view whether ataxin3 promoted or decreased degradation through this pathway [13,58]. A modeling study by Brown *et al.* [52] demonstrated that IP3-mediated calcium release in the Purkinje neuron could activate voltage-gated KCa channels, namely BK and IK, and provided insight into the interplay between smooth endoplasmic reticulum calcium channel of IP3R1 sensitivity and abundance in the function and dysfunction of the Purkinje cell. Reina

Figure 3. KEGG pathway maps for protein processing in endoplasmic reticulum and axon guidance pathways. (A) KEGG pathway map for protein processing in endoplasmic reticulum pathway. The red boxes indicate the potential target genes of mir-7014 in this particular pathway. **(B)** KEGG pathway map for axon guidance pathway. The red boxes indicate the potential target genes of mir-7014 in this particular pathway.
KEGG: Kyoto Encyclopedia of Genes and Genomes.

et al. [53] pointed out that ER stress could increase misfolded and aberrant proteins without inducing nuclear accumulation of ataxin3, which might reflect a primarily cytoplasmic function for ataxin3 following proteotoxic stresses. In the axon guidance pathway (Figure 3B), genes such as *PAK*, *SEMA5* and *EPHA* play important roles in physiological processes such as axon outgrowth and repulsion. There also have been other SCA3 studies related with this pathway, for example, transcriptional changes in brain tissue of the SCA3/MJD mice were observed by Toonen *et al.* [54], most transcriptional changes occurred in brainstem and striatum, while cerebellum and cortex were only modestly affected. When examining individual brain regions, axon guidance and synaptic transmission pathway were most strongly altered in striatum. All the above threw light on many interesting clues for us, which may have an important potential for further exploration.

Combining the pathway annotations and all the above findings with the previous studies, mir-7014 could probably be a significant regulator of some SCA3/MJD-related genes involved in the pathogenesis process of SCA3/MJD. Moreover, if the exosomal mir-7014 is transferred from peripheral vessels to CNS, exosomes could be served as a potential therapeutic carrier for SCA3/MJD. The exosomes encapsulated with mir-7014 may be delivered from the patients' peripheral vessels to CSF so as to prevent the disease progression. As a result, further studies about the biological functions and the regulation mechanisms of mir-7014 will provide us with more prospective application of exosomal miRNAs in SCA3/MJD diagnosis and treatment.

Up to now, there is not a complete system related to exosomes research in neurodegenerative diseases formed yet, the exploration about how exosomal miRNAs function is not profound enough, and even without any relevant reports in SCAs. However, several studies about the roles of free miRNAs in SCAs have existed: first of all, in our former study, we found that miR-25, miR-125b, miR-29a and miR-34b could be potential biomarkers for SCA3/MJD. Intersected with that, miR-29a was also found to be downregulated in a cellular model of SCA17 [59]. Further, there also have been some miRNA studies in cell models or animal models from SCA3/MJD: in human neuronal cultures derived from SCA3 patient-specific induced pluripotent stem cell (iPSC) lines, miR-370 and miR-543 levels were found to be upregulated, while *DNAJB1* expression was concurrently reduced. Based on these findings, Evert *et al.* [60] proposed that downregulation of *DNAJB1* by miR-370 and miR-543 could be an early event contributing to SCA3 pathogenesis, therefore, inhibition of these two miRNAs in turn could stabilize *DNAJB1* and thereby be beneficial in SCA3 disease. Three other miRNAs, mir-9, mir-181a, and mir-494, have been reported to interact with the *ATXN3*-3' UTR, whose expression was found to be dysregulated in human MJD neurons and in other MJD cell and animal models. Overexpression of these miRNAs in mice could reduce mut-*ATXN3* levels, aggregate counts, and neuronal dysfunction [61]. Bilen *et al.* [62] put forward that miRNA pathways could modulate poly-Q-induced neurodegeneration in *Drosophila* and human cells. Some also carried out research in SCA3-lymphoblastoid cells and proposed that miR-25 and miR-181 family members could bind to the 3'UTR cooperatively to suppress the expression of *ATXN3*. Besides, McCann *et al.* [63] found that in the *Drosophila* model of SCA2, a direct homolog of the disease-associated protein Atx2 interacted with the important effector protein ago1 of miRNAs, whereas the latter was responsible for the effectiveness of miRNAs-mediated gene translation inhibition. However, the interaction of Atx2-ago1 has not been verified in mouse or human models, and the role of the extended mutant ataxin-2 protein needs to be further elucidated.

Above all, there are a number of similar designed studies using exosomal miRNAs as biomarkers in other neurodegenerative diseases: Yang *et al.* [64] found that both serum exosomal miR-135a and miR-384 were upregulated while miR-193b was downregulated in serum of AD patients compared with that of normal controls; Furthermore, they declared that exosome miR-384 was the best among the three miRNAs to discriminate AD, VaD and PDD, and the combination of miR-135a, -193b and -384 was better than a particular one for early AD diagnosis. Some [65] other researchers developed a miRNA profiling strategy for exosomal miRNAs isolated from cerebrospinal fluid (CSF) in PD and AD and found that 16 exosomal miRNAs were upregulated and 11 miRNAs were downregulated significantly in PD-CSF when compared with those in healthy controls (relative fold >2, $p < 0.05$). MiR-1 and miR-19b-3p were significantly reduced, while miR-153, miR-409-3p, miR-10a-5p and let-7g-3p were significantly overexpressed in PD-CSF exosomes. Further bioinformatic analysis by DIANA-mirPath demonstrated that neurotrophin signaling, mTOR signaling, ubiquitin mediated proteolysis, dopaminergic synapse and glutamatergic synapse were the most prominent pathways enriched in quantiles with PD miRNA patterns; Moreover, after analyzing a panel of 760 miRNAs in the CSF of ten AD patients and ten healthy subjects and validation in a larger group by individual qPCR assays, some scholars proposed miR-9-5p and miR-598 as potential biomarkers for AD.

More interestingly, we found that in animal models of HD, which had similar pathogenesis with SCA3/MJD, some researchers [60] ever developed an exosome-based delivery method to treat this HD as follows: exosomes

were harvested from a stable cell line stably overexpressed with miR-124, which exhibited a high level of miR-124 expression. After injecting the above Exo-miR-124 into the striatum of R6/2 transgenic HD mice, expression of the target gene, RE1-Silencing Transcription Factor, was reduced. As we can see, all the above explorations prove that exosomal miRNAs will have a tremendous potential in the field of neurodegenerative disease, in the meantime, more and more scholars are devoting their effort in this field.

On the other hand, with respect to the other miRNAs except mir-7014 that we chose for qPCR verification, we found that the trends of miRNAs expression levels in plasma-derived exosomes were not in consistent with that obtained from the small RNA sequencing (either without significant difference or being opposite to that). One rational reason to explain this interesting discrepancy may be the scale of the collected sample size not large enough for an integrated illustration. Besides, some analysis deviation may be generated due to platform differences for verification. Moreover, some other differentially expressed miRNAs obtained from small RNA sequencing results without our verification remain to be further investigated in SCA3/MJD disease.

It is summarized that exosomal mir-7014 may be a potential biomarker of SCA3/MJD disease suggesting the clinical classification and age at onset of SCA3/MJD. Above all, this is the first research of exosomal miRNAs in SCAs internationally.

Future perspective

For all this, there are still some limitations or deficiency to be improved in our future work over the next few years. For instance, as it is really hard, at least in this study, to control the sample collecting time strictly, the variance of mir-7014 expression measures at different times could hardly be avoided. With respect to exosomes, more detailed indexes such as the polydispersity index of exosomes could not be covered by ZetaView Nanoparticles Trace Analyzer, which will be improved in our future exosome research. Moreover, as a preliminary study, further tests about the functional regulation of exosomal mir-7014 in cell models and mouse models are necessary, which would have important value for preclinical studies of therapeutic intervention.

Summary points

- Exosomes in spinocerebellar ataxia Type 3 (SCAs) were explored for the first time internationally.
- Both plasma and cerebrospinal fluid (CSF) were chosen for detection in this study.
- Apart from comparing exosomal miRNAs between SCA3/Machado–Joseph disease (MJD) patients and controls, their expression levels in three subtypes of SCA3/MJD were also intercompared.
- Total 53 miRNAs (30 novel miRNAs and 23 known miRNAs) were upregulated and 20 miRNAs (15 novel miRNAs and five known miRNAs) were downregulated significantly in exosomes derived from SCA3/MJD patients' plasma compared with that from SCA3/MJD-free controls.
- Total 69 miRNAs (41 novel miRNAs and 28 known miRNAs) were upregulated and 47 miRNAs (21 novel miRNAs and 26 known miRNAs) were downregulated significantly in exosomes derived from SCA3/MJD patients' CSF compared with that from SCA3/MJD-free controls.
- One novel miRNA of mir-7014 is notably downregulated in plasma-derived exosomes, while upregulated in CSF-derived exosomes of SCA3 patients.
- Mir-7014 is successively upregulated in CSF-derived exosomes from Type 1, Type 2 and Type 3 groups.
- Two highly enriched pathways among mir-7014's target genes, which are probably involved in the pathogenesis of SCA3/MJD, were identified in this study: protein processing in endoplasmic reticulum and axon guidance.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at:
www.futuremedicine.com/doi/full/10.2217/epi-2019-0081

Author contributions

X Hou, L Zhang and H Jiang conceived and designed the experiments. X Hou, X Gong, L Zhang, T Li and Y Xie collected the samples and performed the experiments. X Hou and L Zhang analyzed the data. H Yuan, Y Peng, R Qiu, K Xia, B Tang and H Jiang contributed equipment, materials and analysis tools.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This research was approved by Medical Ethics Committee of Xiangya Hospital, Central South University in China, which was in accordance with the ethical standards in the Declaration of Helsinki. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Data sharing statement

The data and materials generated during the study are available from the corresponding author on reasonable request. The datasets generated during the current study are available at the Sequence Read Archive (SRA) repository under accession code PRJNA522863. Confidential patient data have not been shared.

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