Short Communication

Regulation of Dihydropyrimidinase-like 3 Gene Expression by MicroRNAs in PC12 Cells with Induced Ischaemia and Hypothermia

(ischaemia / hypothermia / dihydropyrimidinase like 3 (Dpysl3) / microRNAs / PC12 cells)

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Abstract. Although hypothermic treatment has been reported to have some beneficial effects on ischaemia at the clinical level, the mechanism of ischaemia suppression by hypothermia remains unclear due to a lack of mechanism understanding and insufficient data. The aim of this study was to isolate and characterize microRNAs specifically expressed in ischaemia-hypothermia for the dihydropyrimidinase-like 3 (*Dpysl3*) gene. PC12 cells were induced with $CoCl_2$ for chemical ischaemia and incubated at 32 °C for hypothermia. In ischaemia-hypothermia, four types of microRNAs (miR-106b-5p, miR-194-5p, miR-326-5p, and miR-497-5p) were highly related to the *Dpysl3* gene based on exosomal microRNA analysis. *Dpysl3* gene expression was up-regulated by miR-497-5p but down-regulated by miR-106b-5p, miR-194-5p and miR-326-5p. Our results suggest that these four microRNAs are involved in the regulation of *Dpysl3* gene expression. These findings provide valuable clues that exosomal microRNAs could be used as therapeutic targets for effective treatment of ischaemia.

Introduction

Brain-neurodegenerative diseases such as ischaemia and stroke are mainly caused by high blood pressure, diabetes, hyperlipidaemia, obesity, and eating fatty and high-calorie instant foods (Barthels and Das, 2020). They will increase in the future. In particular, stroke is a common disease that one in six people suffer from worldwide (Feigin et al., 2022). In Korea, 150,000 new stroke patients occur every year, and it is a major cause of death and physical disability (Hong et al., 2013). It is the number one cause of death as a single disease (Hong et al., 2013). Its high incidence of disability and complications are adding to the burden of public medical expenses (Rochmah et al., 2021). Although a definitive treatment method is not available yet for the recovery of ischaemic brain injury, several trials such as high-quality cardiopulmonary resuscitation, targeted temperature management, thrombolytic agents and various neuroprotective drugs are currently being attempted (Cramer, 2018).

Ischaemic brain cells express immediate early genes, heat-shock protein genes, apoptosis and autophagy-associated genes, and inflammation-related genes sequentially or cooperatively according to the specific time interval (Yagita et al., 2008). The expression of one gene in ischaemic brain cells directly or indirectly affects the life and death of neurons or expression of other genes. Ischaemic tolerance is a neural protective mechanism or

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Abbreviations: CRMP – collapsin response mediator protein family, DD-PCR – differential display PCR, Dpysl3 – dihydropyrimidinase-like 3, NGS – next-generation sequencing.

phenomenon that occurs at 24–48 hours after ischaemia from ischaemic stimuli, which is important for regeneration and recovery of function of neurons involved in expression of multiple genes (Kirino, 2002). Although more research is required to understand the pathophysiology of brain-neurodegenerative disease, gene expression of ischaemic tolerance might be an important therapeutic target in ischaemic injury.

Although the mechanism of neuron protection by hypothermic treatment has not been clearly explained, it is generally accepted in clinical practice that hypothermic treatment is advantageous to patients regardless of various controversies (Kurisu and Yenari, 2018). Recent animal models have shown a neuroprotective effect of hypothermic treatment (32 ℃) after ischaemia induction on neural cell death processes related to apoptosis, necrosis and autophagy (Victor et al., 2022). This result suggests that the positive effect of hypothermia has much more complex mechanisms than simple oxygen consumption reduction. In this study, eight genes (*Dpysl3*, *Emp1*, *Nrp1*, *Dck*, *Hmgcs1*, *Rdx*, *Sfrp2*, *Cdca2*) that were up- or down-regulated by hypothermia (32 ℃)-chemical ischaemia ($CoCl_2$) were isolated from PC12 cells. Among these genes, four exosomal microRNAs (miR-106b-5p, miR-194-5p, miR-326-5p and miR-497-5p) involved in the expression of the *Dpysl3* gene reported to be related to ischaemia were tested to determine whether they could positively or negatively affect the expression of the target gene *Dpysl3*. Our results strongly suggest that it will be very helpful to treat ischaemia if we can control the specific microRNA separated from the exome of the ischaemic neuron after hypothermic treatment.

Material and Methods

PC12 cells (derived from rat pheochromocytoma, similar to primary culture of foetal neurons) were cultured in collagen-coated flasks containing 85 % RPMI-1640 medium supplemented with 25 mM HEPES buffer, 10 % heat-inactivated horse serum, 5 % heat-inactivated foetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 g/l D(+)glucose, 25 µg/ml streptomycin, and 25 U/ml penicillin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA). These cells were maintained in a humidified incubator at 37 °C with 5 % CO_2. The medium was changed every 2 days until the following experiments. To induce chemical ischaemia, cobalt (II) chloride hexahydrate ($CoCl_2$ $6H_2O$, C8661, Sigma-Aldrich, Burlington, MA) was used for preventing prolyl and asparaginyl hydroxylase activity and proteasome degradation of the hypoxia inducible factor 1α. $CoCl_2$ was dissolved in a 100 mM stock solution with phosphate-buffered saline (PBS) and stored at –80 °C in a freezer. When PC12 cells showed 70–80 % confluence, $CoCl_2$ at a final concentration of 150 µM was added directly into the medium and mixed well. The ischaemic-hypothermic treatment was performed at 32 °C under normal cell culture conditions. To obtain differentially expressed genes caused by ischaemia-hypothermia, a differential display PCR (DD-PCR) method was used. After total RNA-Seq analysis using 2 µg total RNA of each sample, DD-PCR was performed with Ribo Corp RNA Depletion + NEB Next Ultra II Directional RNA Kit by ebiogen Inc. (Seoul, Korea).

Three total RNA samples (control, 32 °C-ischaemia, 37 °C-ischaemia) were prepared using an SV Total RNA Isolation System (Promega, Madison, WI). The mRNA in the total RNA sample was then reverse-transcribed using a SuperscriptII™ First Strand Kit (Invitrogen, Carslbad, CA). Expression of each gene was mainly determined by RT-PCR as described below. RT-PCR conditions included 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min (10 min in the final cycle) using primers and Taq DNA polymerase (Solgent Co., Ltd., Daejeon, Korea). RT-PCR bands were quantitatively analysed using ImageJ program of NIH. Primer pairs used were *Dpysl3*-F (5'-CTGATTGTCCCTGGAGGTGT-3') and *Dpysl3*-R (5'-GGTGATGTCCACATGCAAAG-3') supplied by Bioneer Co. (Daejeon, Korea).

Exosome purification from the medium supernatant was performed using an Exo-spin Exosome Purification Kit (Cell Guidance Systems LLC, Cambridge, UK) based on the standard protocol. The supernatant was centrifuged at 300 × *g* for 10 min to remove cells. The resulting supernatant was centrifuged at 20,000 × *g* for 30 min to remove the cell debris. The resulting supernatant was then transferred to a new tube and 0.5 volumes of Buffer A were added. The solution was mixed well and incubated at 4 °C for at least 5 min. After incubation, the solution was centrifuged at 20,000 × *g* for 30 min. The supernatant was discarded and the pellet was suspended in 100 µl of PBS. Exosomes in 100 µl of PBS were applied to Exo-spin columns and centrifuged at 50 × *g* for 60 s. The eluted solution was discarded and 200 µl of PBS was applied to each column. Columns were centrifuged at 50 × *g* for 60 s and exosomes were dissolved in eluted PBS. Exosome markers (CD9 and CD63) were expressed in all samples based on Western blotting. After purification, total exosome RNA was stored in a deep freezer. Total microRNA sequencing analysis of purified exosomal RNA was performed by next-generation sequencing (NGS) using a NEBNext Multiplex Small RNA Library Prep Kit by ebiogen Inc. Both programs DESeq2 and TargetScan were applied to screen for differentially expressed microRNAs (DE-miRNAs) that recognized the *Dpysl3* gene as a target.

To achieve stable over-expression of *Dpysl3* in PC12 cells, cells were plated in normal 85 % RPMI-1640 medium for 24 h prior to transfection so that the cells reached 50 % confluence on the day of transfection. Rat Dpysl3 cDNA was constructed from PCR products spliced into pcDNA3.1 (Invitrogen, Carslbad, CA) in the correct orientation. Cells were transfected with a TurboFectin 8.0 Transfection Reagent (OriGene™ Technologies, Inc. Rockville, MD). Clones over-expressing the *Dpysl3* gene were confirmed by RT-PCR.

To determine statistical significance between multiple groups, one-way analysis of variance (ANOVA) was

performed. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc.). Data are presented as mean ± standard error of the mean (N = 6). Significance was indicated by asterisk (*P < 0.05, **P < 0.005, ***P < 0.001).

Results and Discussion

Recently, hypothermic treatment for ischaemia has been attracting attention as it can inhibit both primary injuries (such as cerebral contusion, axonal shearing, blood-brain barrier damage and nerve apoptosis) and secondary injuries (such as hypoxia, cerebral oedema, intracranial pressure elevation and neurotransmitter release) (Kabon et al., 2003). Although hypothermic treatment has shown an increase in favourable neurological recovery compared to normothermia in clinical trials, a study of factors related to advantages of hypothermic treatment and an understanding of sufficient mechanisms are needed (Crossley et al., 2014).

The induction of ischaemia by $CoCl_2$ has already been well demonstrated in several types of cultured cells (Tripathi et al., 2019). Here, chemical ischaemia of PC12 cells was induced by $CoCl_2$. Hypothermia was then induced by culturing cells at 32 °C. As shown in Fig. 1, eight specific genes (*Dpysl3*, dihydropyrimidinase-like 3; *Emp1*, epithelial membrane protein 1; *Nrp1*, neuropilin 1; *Dck*, deoxycytidine kinase; *Hmgcs1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1; *Rdx*, radixin; *Sfrp2*, secreted frizzled-related protein 2; *Cdca2*, cell division cycle associated 2; *PPIA*, peptidylprolyl isomerase A) showed decreased expression in ischaemia. However, their expression levels in ischaemic hypertension were increased differentially to almost control expression levels based on total RNA sequencing and DD-PCR. We first studied the *Dpysl3* gene because its relevance to ischaemia has already been discussed among these eight genes. Dpysl3 is a member of the collapsin response mediator protein family (CRMP) that consists of five cytoplasmic phosphoproteins (CRMPs1-5) (Manivannan et al., 2013). Dpysl3 is also called CRMP4 or turned on after division-64 kDa (TOAD-64) (Kowara et al., 2006). The gene for Dpysl3 is differentially expressed in the brain (Li et al., 2016). It is involved in various neuron-associated physiology states and diseases such as neuronal migration, synapse and axon formation, synaptic plasticity, neuronal development and disease (Ohtani-Kaneko, 2019). It is dysregulated in Huntington's disease and Alzheimer's disease (Nakamura et al., 2018). Current research studies on Dpysl3-related ischaemia have revealed the following: 1) calpain-mediated CRMP4 proteolysis is induced by neurotoxicity and traumatic brain injury in ischaemic brain; 2) CRMP4 expression is induced in the corpus striatum of transient brain ischaemia; and 3) Dpysl3 protein fragmentation is induced by chemical ischaemia in cultured rat cortical neurons (Liu et al., 2003; Seo et al., 2013; Nakamura et al., 2020).

Increasing evidence indicates that selective regulation of gene expression by microRNAs represents transcriptional regulation by complementary base binding of microRNAs to specific target genes. Several types of microRNAs are present in exosomes. Exosome is a membranous extracellular vesicle with a diameter of 40–100 nm (Doyle and Wang, 2019). It was first reported during the reticulocyte formation process (Johnstone et al., 1987). It is present in the blood, cerebrospinal fluid, urine, saliva and sweat (Doyle and Wang, 2019). The exosome contains a variety of physiologically ac-

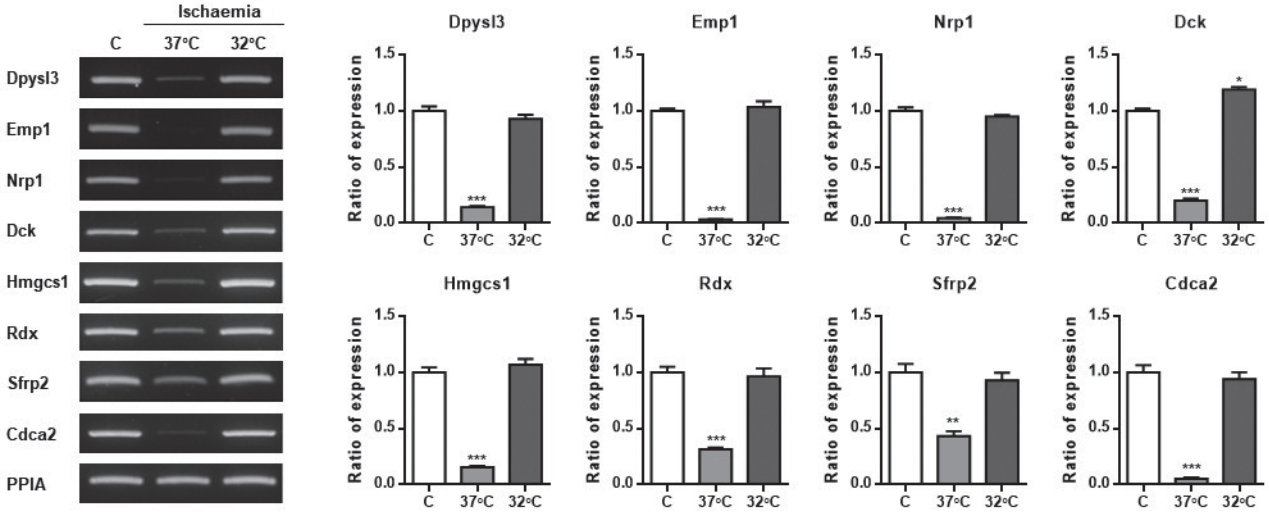

Fig. 1. Expression of genes after ischaemia-hypothermia treatment
Dpysl3, dihydropyrimidinase-like 3 (NM_012934 3); *Emp1*, epithelial membrane protein 1 (NM_012843); *Nrp1*, neuropilin 1 (NM_145098); *Dck*, deoxycytidine kinase (NM_024158); *Hmgcs1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (NM_017268); *Rdx*, radixin (NM_001005889); *Sfrp2*, secreted frizzled-related protein 2 (NM_001100700); *Cdca2*, cell division cycle associated 2 (NM_001107273); *PPIA*, peptidylprolyl isomerase A (NM_008907.1).

tive substances, including some DNA fragments, RNAs (microRNA, snRNA, circRNA, lncRNA piRNA, etc.), lipids and proteins. In particular, microRNAs (21–24 nucleotides) have been used to diagnose various diseases (Doyle and Wang, 2019). They are also used for immune modification (Robbins and Morelli, 2014). They can also be used to search for various disease biomarkers by analysing microRNA sequences because they serve for horizontal transition and intercellular communication between cells (Kalluri and LeBleu, 2020). It has already been reported that microRNA is deeply involved in ischaemia. miR-451, miR-195, miR-7 and miR-143 can down-regulate ischaemia induction (Pignataro, 2021). However, inhibition of miR-19a, miR-124 and miR-210 can avoid ischaemia (Xin et al., 2022).

We first isolated exosomes under the ischaemic-hypothermic conditions and then explored microRNAs that could regulate *Dpysl3* gene expression. Exosomal microRNAs were NGS sequenced. Target gene analysis was performed based on the database using both DESeq2 and TargetScan algorithm. As shown in Fig. 2, four types of microRNAs were found to be involved positively or negatively in the regulation of *Dypsl3* gene expression in ischaemic-hypothermic conditions. These four types of microRNAs are miR-106b-5p, miR-194-5p, miR-326-5p and miR-497-5p. While miR-497-5p up-regulates *Dpysl3* gene expression (Fig. 2D), the other three types, miR-106b-5p, miR-194-5p and miR-326-5p, down-regulate its expression (Fig. 2A–2C). It has been reported that a specific microRNA gene is located in the same chromosomal region as a gene directly related to a disease, suggesting that the expression of a specific microRNA gene can affect the expression of a disease-related gene (Ghorai and Ghosh, 2014). The *Dpysl3* gene is located on chromosome 5q32. However, none of the four types of microRNAs shares the same chromosome region (miR-106b-5p (7q22.1), miR-194-5p (1q41), miR-326-5p (11q13.4) and miR-497-5p (17p13.1), respectively). Among these four types of microRNAs, miR-106b-5p and miR-497-5p are closely related to ischaemia. It has been reported that miR-106b-5p is involved in cerebral ischaemia-reperfusion injury and miR-497-5p is involved in the regulation of liver ischaemia-reperfusion (Xu et al., 2018). The results of this study suggest that it is possible to protect against ischaemia if the expression of the miR-106b-5p gene down-regulated by ischaemia-hypothermia is maintained in the down-regulated state or if the up-regulated miR-497-5p gene is down-regulated. It would be interesting to see results of simultaneous regulation of both genes, miR-106b-5p and miR-497-5p, shortly in future research. Eventually, the results in Fig. 2 (A–D) indicate that *Dpysl3* gene expression is regulated by four types of microRNAs. To demonstrate this result more clearly, each type of microRNA was used to treat PC12 cells in which the *Dpysl3* gene was over-expressed by about

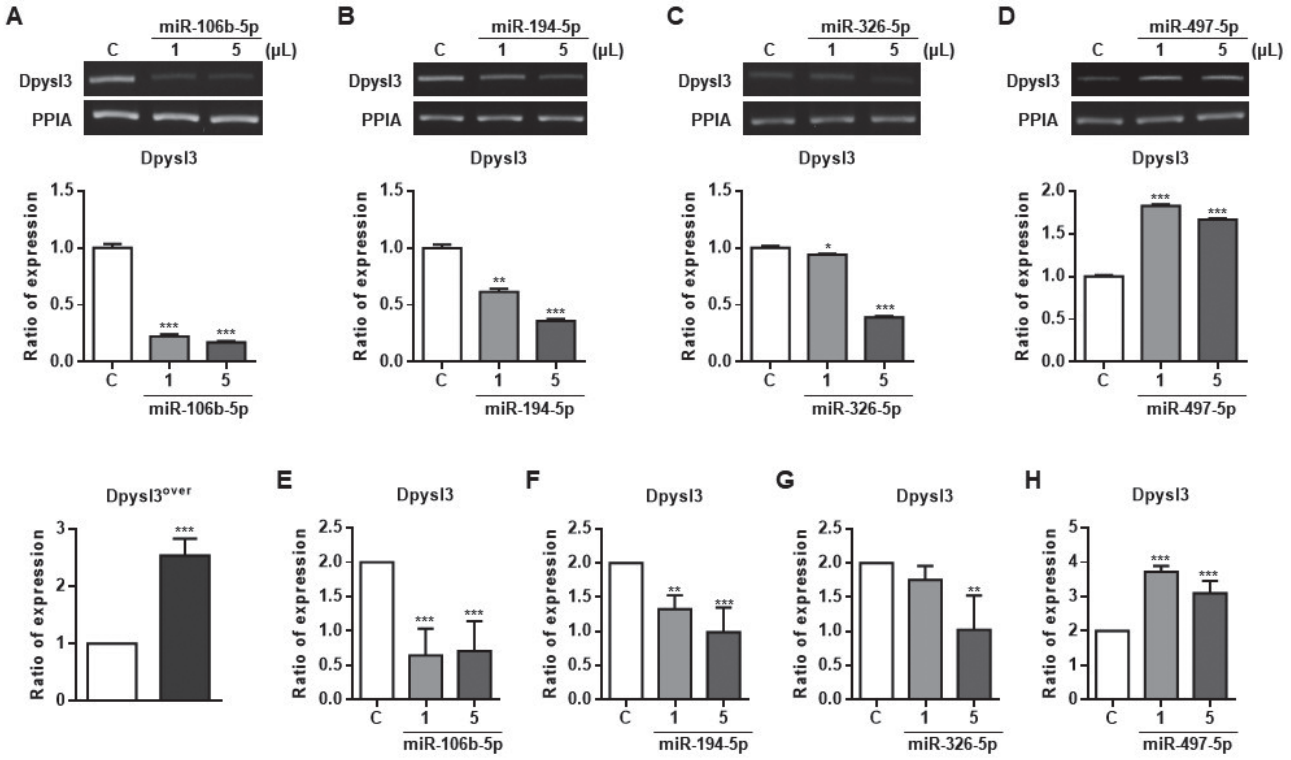

Fig. 2. *Dpysl3* gene expression in PC12 cells and Dpyl3^{over} PC12 cells after treatment with microRNAs
A–D were treated with 1 μl or 5 μl microRNAs for 24 h in PC12 cells. *Dpysl3* expression was then measured by DD-PCR. **E-H** used PC12 cells in which *Dpysl3* was over-expressed by about 2.5 times (Dpysl3^{over}). The experiment was repeated six times and then statistically processed.

2.5-fold (shown as Dpysl3^{over} in Fig. 2). The same pattern of *Dpysl3* gene expression as in Fig. 2A–2D was shown in Fig. 2E–2H. Thus, miR-497-5p could up-regulate the *Dpysl3* gene expression, while miR-106b-5p, miR-194-5p and miR-326-5p could down-regulate this gene expression.

In conclusion, our data showed that four types of microRNAs (miR-497-5p, miR-106b-5p, miR-194-5p and miR-326-5p) specifically expressed in neuronal ischaemia-hypothermia could regulate *Dpysl3* gene expression positively by miR-497-5p and negatively by miR-106b-5p, miR-194-5p and miR-326-5p.

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