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Forensic application of microRNA-706 as a biomarker for drowning pattern identification

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ABSTRACT

Forensic research using microRNA has been used so far only for the identification of body fluids, but its use in understanding biological processes in post-mortem pathology has not been studied before. Therefore, we performed experiments in mice to compare between freshwater and saltwater drowning models, and miRNA expression was analyzed in the brain through a forward bioinformatics screening approach. In this study, we identified eight specific microRNAs whose expression increased in freshwater and decreased in saltwater. Among them, miR-706 – targeting *HCN1* – was identified as a potent biomarker for the drowning pattern identification. A higher expression of miR-706 was detected in the freshwater drowning compared to the control and saltwater drowning group ($p < 0.05$, and $p < 0.01$, respectively). *HCN1* mRNA expression, a suggested candidate target for miR-706, was lower in the freshwater ($p < 0.01$) than in the saltwater drowning group, and showed statistical difference between freshwater and saltwater drowning ($p < 0.01$). miR-706 was specifically expressed in the hippocampal neurons as detected by *in situ* hybridization. Our data suggest that a specific microRNA may provide clues to understanding some crime scene investigations and pathobiological processes in the dead body.

1. Introduction

Drowning is usually determined on the basis of macropathology and supplementary laboratory findings, including diatoms and chemistry [1]. To determine the drowning pattern, such as freshwater drowning (FD), saltwater drowning (SD), or bathtub drowning, several kinds of studies must be performed [2–8], and there is no single biomarker to determine the drowning pattern. Therefore, the combined use of laboratory techniques is needed for this determination.

Molecular forensic autopsy has been dominated by RNA research over the past decade, leading to the success of RNA technology in criminalistics. After the invention of quantitative or real-time PCR [9], more applications using RNA were explored, among which was the forensic potential of RNA for the identification of body fluids [10–12] and for understanding the process by which the death occurred [13,14]. However, even though the usefulness of mRNA profiling has been established in the forensic field, mRNA stability and its susceptibility to degradation remained a hindrance for mRNA-based gene expression analysis [15].

A process to death is another aspect of the tissue injury. An ideal biomarker of this process should be abundant, be produced in the tissue of interest, and be typically present at low concentrations in the blood and other tissues [16]. MicroRNAs (miRNAs) are a type of small noncoding RNA that are typically 22 nucleotides long. These small regulatory molecules are involved in a variety of biological processes [17]. miRNAs can downregulate gene expression by base-paring with the 3′ untranslated regions (3′UTR) of target mRNAs, and have been identified as key players in producing rapid adaptation to changing environmental conditions [18,19]. miRNA regulation is found to be tissue type-specific and is highly evolutionary conserved. After the first reports of the feasibility of using miRNA profiling in forensic science [20], a few groups reported on the possibility of the use of miRNA profiling in body

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fluid identification [21–23], the estimation of post mortem interval [24], and as a biomarker for traumatic brain injury [25], in the forensic field.

miRNA-based posttranscriptional regulation is extremely sensitive to microenvironment stimuli, such as low oxygen (hypoxia), variations in pH, osmolarity, temperature, and ion concentration [19]. In the case of electrically excitable cells or tissues, intracellular events triggered by ion dynamics can affect the single-cell or even cell cluster behavior [26]. In fact, there are several lines of accumulating evidence indicating that the body microenvironmental factors such as blood serum electrolyte concentrations [1] and oxygen saturations [27] are changed and differ depending on the drowning pattern. The microenvironmental changes associated with the death process might induce different miRNA expression patterns, and specific miRNAs can be used as reliable markers to confirm the drowning pattern. Some miRNAs have a role in regulating genes coding for ion channels leading to channelopathies and water transport [28–30].

We hypothesized that some miRNAs are up- or down-regulated in SD and FD models; one of these miRNAs has a clear ability to differentiate between SD and FD, and can help to elucidate the biological processes underlying the drowning pattern. The primary objective of this study was to examine this hypothesis.

2. Materials and methods

2.1. Animal experiments

2.1.1. Animals

All experiments were conducted on 8-week-old male Imprinting Control Region (ICR) mice, which were obtained from SAMTAKO (Seoul, Korea). All mice were bred and housed in a comfortable environment with a 12-h light/dark cycle. All animal experiments were approved by the Committee on Animal Care and Use of Chonnam National University Medical School.

2.1.2. Experimental drowning model

An experimental drowning model was developed as follows: tap water was used for freshwater drowning and seawater – from the Shinan Island area, Chonnam, Republic of Korea – was used for salt water drowning. In this study, all animal experiments were conducted on 8-week-old male ICR mice (SAMTAKO, Seoul, Korea). The mice were divided into three groups. Each group was killed according to the conditions indicated below, and confirmation of drowning was done according to the classical methods: cardiac arrest, frothing around the mouth, and ballooning of the lungs.

Group I (Control, $n = 3$): anesthetized with ether to induce death by cervical dislocation.
Group II (Fresh water drowning model, $n = 5$): to induce drowning, a box was filled with freshwater and the mice were placed in there.
Group III (Salt water drowning model, $n = 5$): to induce drowning, a box was filled with seawater and the mice were placed in there.

Mice were maintained in freshwater and saltwater for 24 h after complete drowning, and the removed cerebrum from each mouse was immediately frozen in liquid nitrogen and stored at −80 °C until each experiment.

2.2. RNA extraction

Total RNA was extracted and purified using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions and RNA quality was assessed using the RNA Integrity Number (RIN) obtained from an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

2.3. miRNA microarrays

miRNAs in total RNA were labeled with miRNA Complete Labeling and Hyb kit (Agilent Technologies) following the labeling section in the manufacturer's instructions. Each slide was hybridized with 100 ng Cy3-labeled RNA using miRNA Complete Labeling and hybridized onto the Agilent Mouse miRNA 8 × 60 K slides (Agilent Technologies). The hybridized arrays were washed, dried, and scanned using the Agilent DNA Microarray Scanner (part number G2505C). Data were extracted using Agilent Feature Extraction software (version 11.5.1.1). Using GeneSpring GX (version 7.3), a one-way ANOVA ($p < 0.01$, at least 2-fold average difference between any two sample types) was conducted. The resulting data from biological replicates ($n = 3$) were averaged, and median-centered $\log_2$ intensity data was subjected to hierarchical clustering. Target genes regulated by these differentially expressed miRNAs were predicted using TargetScan Mouse, MicroCosm, and miRanda. Microarray experiments were performed by E-Biogen Inc. (Seoul, Korea).

2.4. TaqMan RT-PCR

miRNAs were reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription (RT) Kit (Applied Biosystems, Darmstadt, Germany) including Multiscribe™ Reverse Transcriptase ($n = 3$–5, each group). For each sample, 100 ng of total RNA were transcribed and an RT reaction mix containing dNTPs (100 mM), 3 μl of assay specific stem-loop primers, Multiscribe™ Reverse Transcriptase (50 U/ml) with special buffer, RNase Inhibitor (20 U/ml), and water in a final reaction volume of 15 μl was used. All TaqMan assays were run in triplicates in a 7300 Real-time PCR system (Applied Biosystems) using TaqMan universal PCR master mix without UNG (Applied Biosystems), using 45 PCR cycles. Amplification signals were computed with the SDS v.2.1 software (Applied Biosystems). Mouse small nucleolar RNA-202 (snoRNA-202) was used as a normalization control. Relative expression values from three independent experiments were calculated using the $2^{-\Delta\Delta C_t}$ method. All miRNA assays used in this study are listed in Table 1.

2.5. Reverse transcriptase reaction

Total RNA was extracted from control and experimental drowning model mouse brains with Geneall, according to the

Table 1
qRT-PCR TaqMan assay probe sequences.

miRNA	Mature miRNA-sequence (5′ → 3′)	miRBase	Assay-ID
mmu-miR-706	AGAGAAACCCUGUCUCAAAAAA	MIMAT0003496	001641
mmu-miR-30c-1-3p	CUGGGAGAGGGUUGUUUACUCC	MIMAT0004616	002108
mmu-miR-320	AAAAGCUGGGUUGAGAGGGCGA	MIMAT0017057	002277
mmu-miR-130a	CAGUGCAAUGUUAAAAGGGCAU	MIMAT0016983	000454
snoRNA202	GCTGTACTGACTTGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG		001232

miRBase: mirBase accession number; Assay-ID: assay-ID according to manufacturer's catalog.