



ORIGINAL INVESTIGATION

Downregulation of CSF-derived miRNAs miR-142-3p and miR-17-5p may be associated with post-dural puncture headache in pregnant women upon spinal anesthesia

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KEYWORDS

Spinal anesthesia;
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Abstract

Background: Postdural puncture headache (PDPH) develops due to puncture of the dura mater. The risk factors that influence PDPH incidence are Body Mass Index (BMI), sex, spinal needle type, history of headache, and loss of Cerebrospinal fluid (CSF) volume, yet there is no consensus on these risk factors. The pathophysiology of PDPH is poorly understood. The molecular pathways that may lead to PDPH are unknown. In this study, CSF – derived microRNAs (miRNAs) were investigated for their potential to predispose to PDPH in a population of pregnant women. **Methods:** Pregnant women going under cesarean section via spinal anesthesia were included in the study with the criteria of the subjects presenting American Society of Anesthesiologists (ASA) physical status I. Patients were classified into two groups as with PDPH (n = 10) and without PDPH (n = 12) based on International Headache Society's PDPH definition. CSF-derived microRNAs were investigated for their differential expression levels in PDPH patients compared with the healthy controls using microfluidic gene expression platform.

Results: Out of seventy-six miRNAs, two miRNAs, namely miR-142-3p and miR-17-5p, were significantly downregulated in PDPH patients (Mann-Whitney U test, $p < 0,05$). BMI and age did not influence PDPH occurrence. The mean visual analogue scale (VAS) of the PDPH patients was 6,8 out of 10.

Conclusion: We have shown that downregulation of miR-142-3p and miR-17-5p may predispose pregnant women to PDPH upon spinal anesthesia. However, which genes are targeted by miR-142-3p and miR-17-5p-mediated effect on PDPH remains to be elucidated.

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Introduction

Postdural puncture headache (PDPH) is a condition provoked after spinal anesthesia with varying incidence rates of between 2 to 85%.¹ PDPH is defined as any headache after a lumbar puncture that worsens within 15 minutes of sitting or standing and is relieved within 15 minutes of the patient lying down.² Majority of the PDPHs develop within three days of the procedure and more than half of them start within the first 48 hours.³ The major mechanism postulated for PDPH is the reduced cerebrospinal fluid (CSF) pressure due to CSF leakage through the dura at the puncture site. It is well known that puncture of the dura allows CSF to leak from the subarachnoid space, which results in decreased CSF volume and pressure. BMI, age, sex, spinal needle gauge, chronic headache history and loss of CSF volume are among the risk factors that predispose to PDPH.⁴⁻⁶ However, the molecular factors that predispose to PDPH has not been investigated. One mechanism postulated more than twenty years ago is hypersensitivity to substance P and the up-regulation of neurokinin-1 receptors (NK1R).⁷

Molecular mechanisms of pain processing have been dissected in recent years with roles of miRNAs in nociceptive pathways. microRNAs (miRNAs) are 18–25 nucleotide long, non-coding small RNA molecules which inhibit gene expression post-transcriptionally.⁸ miRNAs have been shown to target molecules which mediate pain processing such as γ -aminobutyric acid- α 1 (GABA α 1), Na⁺ and Ca²⁺ channels and TRPV1 channels.⁹ miRNAs can be carried outside the cells by extracellular vesicles and thus found in bodily fluids such as CSF, urine, and saliva.¹⁰ CSF-derived miRNAs are reflective of brain functions and are used as candidate biomarkers for diagnosis, prognosis and classification of neurodegenerative diseases and neoplastic diseases of the central nervous system.^{11,12} Recently, CSF-derived miRNAs were shown to play a role in fibromyalgia which is a chronic pain condition.¹³ However, due to the difficulty to obtain CSF samples, the studies on CSF and pain-related miRNAs have been limited.

The pathophysiology of postdural puncture headache (PDPH) is poorly understood. Although several risk factors have been identified, there is no consensus on the factors that influence PDPH incidence. Given the recent findings on roles of miRNAs on pain processing and nociception, we hypothesized that CSF-derived miRNAs can be associated with predisposition to PDPH upon spinal anesthesia. Therefore, in this study we aimed to identify differentially expressed miRNAs derived from the CSF of PDPH patients. To our knowledge, this is the first time that CSF-derived miRNAs are interrogated for their potential to predispose to PDPH.

Methods

Study design and subjects

This prospective cohort study was carried out at the Faculty of Medicine with an approval of the medical ethics committee. All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration. Subjects with an informed written consent were enrolled in the study. Pregnant women going under cesarean

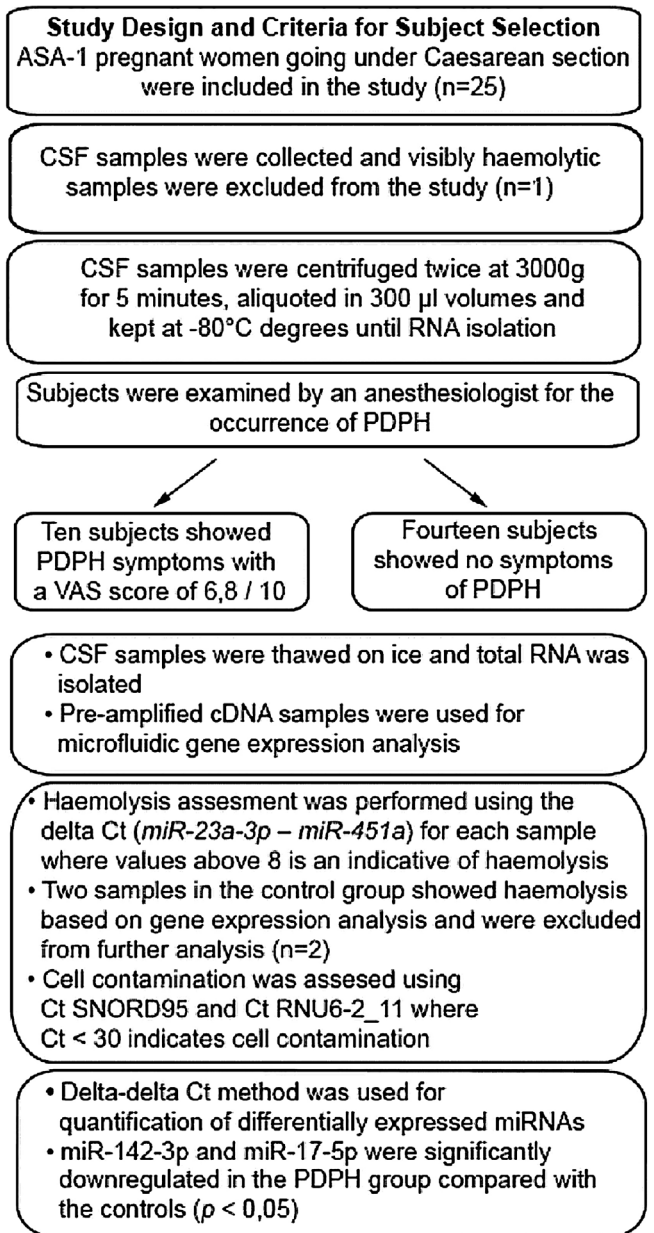


Figure 1 The schematic of the study design and the pipeline for miRNA profiling.

section via spinal anesthesia were included in the study (n = 25) with the criteria of the subjects being physical status I of American Society of Anesthesiologists (ASA) who is defined as a normal healthy patient with characteristics of being non-obese and non-smoking with good exercise tolerance (Fig. 1).¹⁴ Subjects with mild or severe systemic diseases (i. e., ASA II–IV) were not included in the study cohort. In order to induce spinal anesthesia, lumbar puncture was performed using same type of spinal needle for all subjects that is an atraumatic 26G spinal needle with a 20G introducer (Braun Atraucan). After the spinal needle reached the subarachnoid space, the CSF was allowed to drop through the needle tip. CSF drops were collected into sterilized tubes. During the CSF collection, visibly hemolytic samples were discarded (n = 1). After the operation, patients

were hospitalized for five days and PDPH was diagnosed by an anesthesiologist during the hospitalization period where Visual Analogue Scale (VAS) scores of the patients were interrogated. Patients were classified into two groups as with PDPH and without PDPH based on the International Headache Society's (IHS) PDPH definition which is defined as any headache after a lumbar puncture that worsens within 15 minutes of sitting or standing and is relieved within 15 minutes of the patient lying down.² Ten patients had PDPH, and 14 patients did not develop PDPH. According to IHS guidelines, the majority of PDPH symptoms start within three days of the procedure however, it can take up to five days for the symptoms to develop and up to two weeks to resolve spontaneously. To ensure that the PDPH diagnosis was correct, patients were interrogated by the anesthesiologist at two-week time point. None of the patients had persisting symptoms of PDPH at this point. The duration of the PDPH symptoms were also recorded ($5,7 \pm 0,4$ days). The neonatal Apgar scores of the PDPH patients and the controls were between 8–10 therefore there was no significant difference between the groups.

All CSF samples were processed for gene expression analysis and hemolysis assessment was performed where two of the samples from without PDPH group were excluded from further analysis as indicated below. In the end, ten PDPH samples were compared with twelve control patients without PDPH.

CSF sampling

The CSF samples were collected into a microfuge tube with approximate volume of 600 μl and centrifuged twice at 3000 g for 5 minutes. Supernatant was stored at -80°C in 300 μl aliquots until total RNA isolation.

Sample preparation

CSF samples were thawed on ice and total RNA was isolated using miRVANA PARIS RNA Purification Kit (catalog no AM1556, Ambion-ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Briefly, 2X denaturing solution was added 1:1 volume to 200 μl of CSF samples and mixed immediately. After addition of denaturing solution to the CSF samples, this mixture was incubated on ice for 5 minutes, upon which 25 femtomoles of *cel-miR-39* miRNA mimic was added per sample as a spike-in control. Acid-Phenol:Chloroform was added in 1:1 ratio and vortexed for 45 seconds and centrifuged for 5 minutes at 10.000 g at room temperature. After organic extraction, RNA grade glycogen (ThermoFisher) was added to the aqueous phase at a final concentration of 1 $\mu\text{g} \cdot \mu\text{l}^{-1}$ to increase RNA yield. It was added 1,25 volumes of ethanol to the aqueous phase and mixed thoroughly by pipetting. The mixture was loaded on the columns and centrifuged for 30 seconds at 10.000 g. After washing steps, RNA was eluted in a new microfuge tube and stored at -80°C degrees. cDNA synthesis was performed using Qiagen's miScript II RT kit. RNA was thawed on ice. 5X miScript HiSpec Buffer, 10x Nucleic Mix, RNase-free water and miScript reverse transcriptase mix was added to RNA samples in accordance with manufacturer's instructions. The mixture was incubated at 37°C degrees for 60 minutes and at 95°C degrees for 5 minutes. Before

gene expression analysis, cDNA samples were diluted in 1:3 and amplified with miScript Microfluidics PreAMP Kit using 5XPreAmp buffer (Qiagen, Hilden, Germany). Pre-amplified samples were used for microfluidic gene expression analysis.

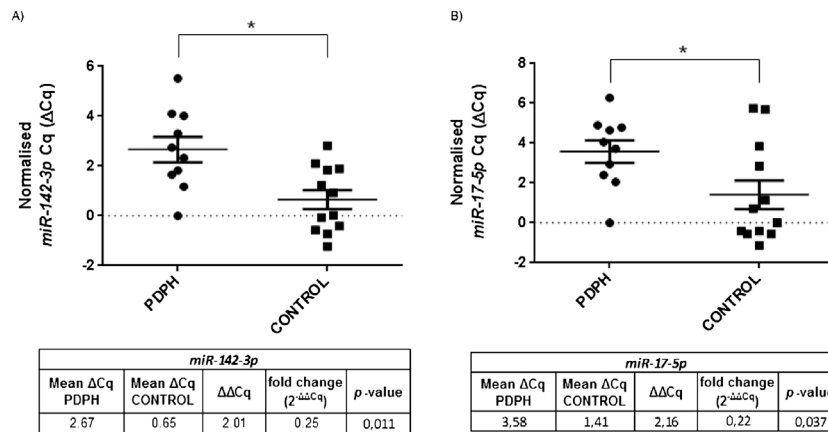
RT-qPCR and normalization

Fluidigm's microfluidic gene expression system was used for RT-qPCR analysis. Dynamic Array (96.96) integrated fluidic circuit (IFC) (Fluidigm, South San Francisco, CA) was injected with Control Line Fluid Kit and primed using the IFC Controller HX (Fluidigm). Mixtures for assay inlets and sample inlets were prepared using miScript Microfluidics PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, primer assays and miScript universal primer were mixed with 2xAssay Loading Reagent (Fluidigm) and dispensed on the assay inlets of the IFC. Pre-amplified cDNA samples were mixed with Microfluidics qPCR master mix provided in the miScript Microfluidics PCR Kit (Qiagen) and 20xDNA binding sample loading reagent (Fluidigm). The sample mixture was dispensed on the sample inlets of the IFC, and the loading process was started using IFC Controller HX (Fluidigm). After completion of priming and loading steps, the IFC was run on the BioMark™ HD System.

The custom miRNA list is comprised of 77 miRNAs and was ordered from Qiagen in the form of primer assays. Candidate miRNAs were selected based on their robust and dominant expression in CSF in various conditions reported by other groups^{15–17} (Supplementary Table 1). Out of 77 candidate miRNAs, 7 of them, namely hsa-miR-124-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-191-5p, hsa-miR-24, hsa-miR-99a-5p and hsa-let-7b were included as potential reference genes based on previous reports.^{16,18,19} hsa-miR-124-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-191-5p were used as reference genes by Murray et al and miR-30b-5p was chosen as the best reference gene by the NormFinder algorithms and used for further analysis by this group.¹⁸ miR-99a-5p, miR-30b-5p and let-7b were used as reference genes by Holm et al. and miR-24 was used as a reference gene by Baraniskin et al.^{16,19} In our experimental set-up, miR-99a-5p was found as the best reference gene by the NormFinder, which uses a model-based approach to determine genes that are stably expressed among a set of candidate normalization genes.²⁰ All custom miRNA candidates were interrogated for their reference performance, that is, for being robustly expressed across all samples and hsa-miR-99a-5p was found as the best reference gene in our experimental design. In addition to no amplification control and no template control as negative controls, absence of two small RNAs, SNORD95 and RNU6-2.11, were tested as an indicator of contaminating cells in the CSF.²¹ Hemolysis assessment was carried out using hsa-miR-23a-3p and hsa-miR-451a. The samples with delta Ct values of hsa-miR-23a-3p - hsa-miR-451a above 8 (Table 1) were excluded from the study due to hemolysis which corresponded to two samples in the control group (without PDPH). After determination of the most suitable reference genes using algorithms of the NormFinder, normalization of samples was performed as described by Murray et al., using hsa-miR-99a-5p as a reference gene and the spike-in control.¹⁸ The expression level of miRNAs was determined using $\Delta\Delta\text{Cq}$ method. Fold changes were calculated

Table 1 The raw ΔCq (*miR-23a-3p* - *miR-451a*) values for hemolysis assessment are indicated.

CSF Sample (PDPH)	ΔCq (<i>miR-23a-3p</i> - <i>miR-451a</i>)	Hemolysis Assessment	CSF Sample (Control)	ΔCq (<i>miR-23a-3p</i> - <i>miR-451a</i>)	Hemolysis Assessment
1	-2,58	Passed	1	-3,72	Passed
2	-0,11	Passed	2	-0,15	Passed
3	-1,75	Passed	3	-1,16	Passed
4	3,21	Passed	4	-2,80	Passed
5	-2,99	Passed	5	-0,21	Passed
6	-1,77	Passed	6	-2,64	Passed
7	-1,44	Passed	7	-1,12	Passed
8	-2,07	Passed	8	-3,20	Passed
9	1,73	Passed	9	-4,13	Passed
10	-2,80	Passed	10	3,28	Passed
			11	-4,41	Passed
			12	-1,98	Passed
			13	8,23	Failed
			14	9,85	Failed

**Figure 2** miR-142-3p and miR-17-5p are downregulated in PDPH patients compared with the healthy controls. Normalized ΔCq values for A) miR-142-3p and B) miR-17-5p in PDPH patients and the healthy controls are shown. The mean ΔCq for each miRNA is indicated along with the fold changes (Mann-Whitney U test, * $p < 0,05$).

using the $2^{-(\Delta Cq_{PDPH} - \Delta Cq_{Control})}$ formula where $\Delta Cq = Cq_{target} - Cq_{reference}$.

Statistical analysis

Differential miRNAs between the PDPH and the controls were determined using Mann-Whitney U test ($p < 0,05$). Continuous characteristics of the study cohort that is weight, height, BMI and age of the PDPH patients and the controls were compared using Shapiro-Wilk normality test for normal distribution and Student's t-test was performed. Graphpad Prism 6.0 (trial version) was used for statistical analysis.

Results

CSF-derived miRNA profiles of PDPH patients were compared with the controls. Out of seventy-six miRNAs (Supplementary Table 1), ten of them were downregulated and fifteen of them were upregulated (cut-off for fold change below 0,5 and above 2). Two out of 10 downregulated miRNAs namely,

miR-142-3p and miR-17-5p, were significantly altered while none of the upregulated miRNAs showed a significant difference ($p < 0,05$) (Fig. 2). Distribution of miR-142-3p and miR-17-5p values across the cohort is indicated in Fig. 2A and 2B, respectively. miR-142-3p was expressed approximately four times less than the controls. Expression of miR-17-5p in the PDPH patients decreased approximately 5-fold in comparison with the controls.

The PDPH patients were compared with the controls in terms of the reported bias factors that is BMI and age (Table 2). There was no significant difference between the BMI and age of the PDPH patients ($p > 0,05$). Given all subjects were female and same type of spinal needle was used for each pregnant women, sex and spinal needle gauge effects were not applicable bias factors for this study. The mean VAS of the PDPH patients was 6,8 out of 10.

Discussion

In this study, we addressed the role of CSF-derived miRNAs in the PDPH pathophysiology and identified two differentially-

Table 2 Characteristics of the female subjects with and without PDPH. Mean \pm SEM are indicated.

	PDPH (n = 10)	CONTROL (n = 12)	p-value ^a
VAS	6,8 \pm 0,6	–	NA
Weight (kg)	72,7 \pm 1,9	74,9 \pm 2,1	0,458
Height (cm)	162,3 \pm 1,9	162,9 \pm 1,9	0,827
BMI (kg m ⁻²)	27,6 \pm 0,7	28,3 \pm 0,7	0,572
Age (years)	31,3 \pm 1,6	30,0 \pm 1,3	0,542

NA, not applicable.

^a Student's t-test.

expressed miRNAs in the CSF which may be risk factors for PDPH occurrence. To our knowledge, there are only two studies investigating the relation between pain conditions and CSF-derived miRNAs in humans. One of them was carried out on fibromyalgia patients where miRNA profiles of these patients were compared with the miRNA profiles of healthy pregnant women and urological patients receiving spinal anesthesia. This study reported nine downregulated miRNAs namely, miR-21-5p, miR-145-5p, miR-29a-3p, miR-99b-5p, miR-125b-5p, miR-23a-3p, miR-23b-3p, miR-195-5p, miR-223-3p, in fibromyalgia patients.¹³ In our study, these nine miRNAs were interrogated for their differential expression in the CSF of PDPH patients; however, no significant results were obtained. Given that fibromyalgia is a chronic pain condition, its molecular mechanisms may differ with acute pain conditions such as PDPH.

In the second study, miR-124 was found downregulated in the CSF of bone cancer pain patients compared with the patients without cancer and chronic pain history.^{22,23} In the light of animal studies where intrathecal injections of miR-124 mimics ameliorated the bone cancer pain, miR-124 was suggested as a potential analgesic drug for cancer pain patients. miR-124 was included in our miRNA list and it was not significantly altered in PDPH patients.

We have found that miR-142-3p and miR-17-5p were downregulated in the CSF of patients with PDPH. Interestingly, miR-142-3p was also downregulated in the serum of fibromyalgia patients.²⁴ CSF-derived miRNA findings from fibromyalgia patients did not overlap with our miRNA findings in the CSF of PDPH patients; however, an overlap was observed in the serum-derived miRNAs of fibromyalgia patients. The lack of significantly altered miR-142-3p in the CSF of FM patients could be due to the small sample size (10 FM patients and 8 patients in the control group). CSF and serum are distinct bodily fluids yet the findings in the serum can sometimes be extended to the CSF and vice versa.¹¹ Besides its putative role in fibromyalgia, miR-142-3p may also be involved in neuroinflammation during multiple sclerosis.²⁵

The miR-142-3p plays a role in inhibition of neuronal differentiation by targeting a double stranded RNA-binding protein Staufen1 (STAU1), which is involved in neuronal morphogenesis and implicated in the nociception with the virtue of its expression in the dorsal root and trigeminal root ganglia (DRG) in rats.²⁶ In a mouse model of neuropathic pain induced via spinal nerve ligation (SNL), miR-142-3p was found downregulated in the dorsal root ganglion of the SNL

mice. In line with this observation, inhibition of neuropathic pain was observed when miR-142-3p was overexpressed.²⁷ Given our findings on the downregulation of miR-142-3p in the CSF of PDPH patients and its down regulation in the aforementioned studies on fibromyalgia patients and animal models, further studies are required to dissect the role of miR-142-3p in pain processing.

The second differentially expressed miRNA identified in our study was miR-17-5p which promotes cell proliferation and functions as an oncogene. miR-17-5p has been reported with roles in nociception recently. In the blood of (natural killer cells) Chronic Fatigue Syndrome patients, miR-17-5p was downregulated compared with non-fatigued controls.²⁸ In a rat model of neuropathic pain, miR-17-5p was upregulated in the DRG neurons upon injury and inhibition of miR-17-5p ameliorated mechanical allodynia.²⁹ Potassium channels were identified as one of the targets of miR-17-5p and it is postulated that miR-17-5p may mediate nociception through modulation of potassium channels. Given the predictive roles of miR-17-5p in cancer prognosis and its profound expression levels in hepatocellular carcinoma, lung cancer and colorectal cancers; miR-17-5p is not a specific marker for PDPH.³⁰ Similarly, miR-142-3p has been reported as a potential diagnostic biomarker for colorectal cancer and prognostic biomarker for cervical cancer.^{31,32} Several studies have used CSF-derived miRNAs for interrogation of biomarkers for diverse range of diseases. Baraniskin et al., identified the first CSF-based biomarkers for detection of glioma comparing the CSF-derived miRNA profiles of glioma patients (n = 10) and patients with various neurologic disorders (n = 10) as control.³³ They found that miR-15b and miR-21 are upregulated in the glioma patients and miR-21 biomarker was further confirmed by Shi et al. where authors compared CSF miRNA profiles of glioma patients (n = 70) and non-tumor controls (n = 25) and found that miR-21 is an important indicator for diagnosis and prognosis of glioma.³⁴ Galimberti et al. showed that miR-125b can be used as an Alzheimer's Disease (AD) biomarker for both serum and CSF comparing 22 AD patients and thirty six non-inflammatory neurological disease controls.²¹ Haghika et al. showed that CSF-derived miRNAs can be used to differentiate the Multiple Sclerosis (MS) disease course.¹²

Similar to the studies indicated above, our findings implicate a role for miR-142-3p and miR-17-5p in PDPH development. Their differential expression suggest that these miRNAs can be used as biomarkers for PDPH. miR-142-3p and miR-17-5p are validated regulators of nociception, therefore it is likely that they play regulatory roles in how individual variability of pain phenotypes are determined at the molecular level. miRNAs can affect the pain induction directly or indirectly. miR-142-3p has been shown as a key molecular mediator of synaptic dysfunction in MS patients by disrupting the glutamate homeostasis and it is implicated with roles in nociception by the virtue of its target gene which functions in the DRG neurons.²⁵ Dysregulated miR-142-3p levels may render subjects more susceptible to developing PDPH due to disrupted glutamate homeostasis similar to the miR-142-3p, miR-17-5p functions in the DRG neurons. Potassium channels were identified as one of the targets of *miR-17-5p*. Lumbar puncture creates an acute tissue insult which can cause release of mediators leading to cell membrane depolarization including potassium ions.

These signals are transmitted to the spinal cord via the DRG neurons. Given their role in the DRG neurons, it is possible that dysregulated levels of miR-142-3p and miR-17-5p may cause sensitivity to pain. Growing body of evidence suggest that a pain phenotype that is specific to an individual may be due to the differential regulation of gene expression, including the findings presented in this study. Our findings await confirmation with larger cohorts and mechanistic approaches.

The roles of miRNA in chronic pain conditions have been elaborated with recent studies. Blood-derived miRNAs and CSF-derived miRNAs were identified in migraine patients and fibromyalgia patients, respectively.¹³ However, little is known with regards to acute pain conditions such as PDPH. It has been reported that in inadvertent puncture of the dura mater cases, only half of the patients develop PDPH. There is conflicting data on the risk factors that affect PDPH incidence. Reports on negative correlation between increased BMI and PDPH incidence is confronted by studies indicating the lack of such a correlation.⁵ It is postulated that loss of CSF volume due to CSF leakage at the puncture site, thus decreased CSF pressure, creates a downward pull on the pain-sensitive brain structures causing PDPH. On the other hand, recent studies showed similar CSF opening pressure between PDPH and controls.^{35,36} History of headache was thought as a predisposing factor but it was shown migraine was not a risk factor for PDPH.³⁷ These conflicting results can be due to the molecular factors that predispose to PDPH which has been overlooked. Here we showed for the first time that miRNAs can influence the PDPH occurrence. In our study, spinal anesthesia was performed using the same type of spinal needle for each pregnant woman by the same anesthesiologist. The BMI and the age of the PDPH and the control group did not show a significant difference. All subjects were female and the subjects with a history of headache and neurological diseases have not been accepted to the study. Therefore, all bias factors for PDPH occurrence were ruled out which highlighted the role of CSF-derived miRNAs that can predispose to PDPH.

The findings on CSF-derived miRNAs and pain conditions have been scarce due to the difficulty to obtain CSF samples from patients with pain conditions and due to the lack of a reliable control group that is CSF obtained from ASA I subjects. For obvious ethical reasons, it is not possible to collect CSF from healthy subjects. Majority of the CSF based studies make use of CSF samples from patients with diseases that are not pertinent to their disease of interest. For this reason, pregnant women going under spinal anesthesia appear to be the best option for obtaining CSF samples from healthy subjects. The characteristics of CSF from ASA I pregnant women is the closest to the characteristics of CSF obtained from healthy subjects. Therefore, our findings can be extended to the other studies as a cross-control group.

The incidence of PDPH has been reported to be higher in obstetrics patients.³⁸ Various factors increase the incidence of PDPH and several studies have shown that young age and female gender are among risk factors.³⁹ Studies which investigate the incidence of PDPH in pregnant women have sample size ranging from 100 to 250,000.^{40,41} PDPH incidence in our study is higher than the ones reported in the literature for atraumatic needles in obstetrics patients. However, number of subjects in our study is low (n = 24) to infer a

PDPH incidence. In terms of sample size, our study design is comparable to the reports on CSF-derived biomarkers where number of subjects ranged from 10 to 25.^{21,33} The biomarkers identified in these studies with small sample size were then confirmed with a larger sample size.³⁴

A limitation of this study is the small sample size, therefore putative PDPH biomarkers identified with this work await further confirmation with larger cohorts and mechanistic approaches. Future studies should replicate the current set-up while including a more comprehensive miRNA library.

In conclusion, we have shown for the first time that downregulation of miR-142-3p and miR-17-5p may predispose pregnant women to PDPH upon spinal anesthesia. Both candidate miRNA biomarkers have roles in pain conditions or pain processing. However, which genes are targeted by miR-142-3p and miR-17-5p-mediated effect on PDPH remains to be elucidated.

Funding

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Conflicts of interest

The author declares no conflicts of interest.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the medical ethics committee (2017/565) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bjane.2021.08.016>.

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