

Original article

Assessing the responses of miRNA-200b and miRNA-429 in cyanide poisoning cases

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Abstract

Background: Cyanide toxicity disrupts cellular respiration by inhibiting mitochondrial cytochrome oxidase a3, resulting in cellular hypoxia. The cell's response to hypoxia is mediated by hypoxia-inducible factors (HIFs) and associated microRNAs (hypoxamiRs). Among these, miR-429 and miR-200b have been implicated in the regulation of HIFs and were found to be upregulated during acute hypoxic conditions. However, their specific roles in cyanide-induced hypoxia remain poorly understood.

Objective: The aim of this study was to evaluate the expression of miR-429 and miR-200b in postmortem peripheral blood collected from cyanide poisoning fatalities.

Methods: Postmortem blood was obtained from five cyanide poisoning cases and five nonasphyxial controls; for all cases, the postmortem interval was < 24 hours. After total RNA was extracted from the blood, 10 ng of RNA was reverse-transcribed into complementary DNA using MultiScribe™ Reverse Transcriptase. The expression of miR-429 and miR-200b was quantified using the QuantStudio™ 5 system and TaqMan™ MicroRNA assays. Relative miRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method and compared using the Mann–Whitney U test. One cyanide case was excluded because of low RNA quality.

Results: Toxicological screening confirmed lethal cyanide concentrations consistent with oral ingestion. Common autopsy findings included pulmonary edema and renal tubular injury, with no definitive cerebral hypoxic changes. The comparative analysis revealed that miR-429 and miR-200b tended to be higher in the cyanide group (n = 4) than in the controls (n = 5), but the differences were not statistically significant (miR-429: $P = 0.31$; miR-200b: $P = 0.22$).

Conclusion: This study identified higher expressions of miR-429 and miR-200b in cyanide poisoning cases compared with controls, which was consistent with previous evidence linking these microRNAs to hypoxic responses. Although the increase in expression did not meet statistical thresholds, the observed patterns indicate that these microRNAs may be involved in cyanide-induced hypoxia. These preliminary findings underscore the need for further investigation of larger, well-defined cohorts to fully elucidate the diagnostic and mechanistic roles of miR-429 and miR-200b in cyanide toxicity.

Keywords: Cyanide, miRNA, sudden death.

Cyanide, a potent and rapidly acting toxin, induces cellular hypoxia by inhibiting mitochondrial respiration. After cyanide enters the body, it binds to cytochrome oxidase at the a3 subunit of complex IV in

mitochondria. This binding interaction inhibits the mitochondrial electron transport chain, preventing cells from utilizing oxygen. ⁽¹⁾ Consequently, death due to cyanide poisoning manifests as acute asphyxia. Acute hypoxia is defined as a state of oxygen deficiency lasting less than 24 hours. ⁽²⁾ A significant challenge in cyanide-related fatalities is the often subtle or non-specific postmortem findings, which are attributed to the rapid mechanism of action. Consequently, cases of cyanide poisoning may be underdiagnosed or lack definitive confirmation through standard autopsy and histopathological examinations.

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Cellular responses to hypoxic or asphyxic conditions involve the activation of the hypoxia-inducible factor transcription factor, which predominantly controls cellular adaptive mechanisms to low-oxygen conditions.⁽³⁾ MicroRNA (miRNAs), short RNA sequences ranging from 18 to 25 nucleotides in length, are key regulators of these genetic responses.⁽⁴⁾ Numerous miRNAs, collectively termed hypoxamiRs, have been identified as contributors to hypoxic responses. For example, miRNA-210 is recognized as a master hypoxamiR because of its significant role during hypoxia,^(3,5) and miRNA-429 and miRNA-200b, have been highlighted as important markers during acute phase hypoxia.⁽⁶⁾

miRNA-429 was first identified in 2015 to target the hypoxia-inducible factor 1 (HIF-1) switch. It is involved in a negative feedback loop with HIF-1, reducing the expression of HIF-1 α -subunit during the early stages of hypoxia.^(7,8) Initial reports describing the upregulation of miRNA-200b in hypoxia were published in 2011 and 2015.^(7,9) However, research in 2018 demonstrated that the hypoxic reduction in endothelial nitric oxide synthase levels was negatively correlated with miR-200b expression and confirmed the upregulation of miRNA-429 and miRNA-200b during hypoxia.⁽¹⁰⁾ Despite the connection of both these miRNAs to acute hypoxic regulation, their specific role in cyanide-induced acute hypoxia has not been investigated. This study aimed to compare the expression of miR-429 and miR-200b in peripheral blood samples from cyanide poisoning fatalities and control cases. The role of these miRNAs as regulators within the HIF pathway during acute hypoxic states suggests that they may serve as potential biomarkers for cyanide-induced hypoxia.

Materials and methods

Study population and sample collection

The study population comprised five cyanide poisoning fatalities (cyanide group) and five control cases of death resulted from causes unrelated to cyanide exposure (control group). All cases had a postmortem interval of less than 24 hours. Individuals with a documented history of significant pulmonary diseases or cancer were excluded from the analysis to minimize confounding miRNA expression. Peripheral blood samples were collected during medico-legal autopsies and stored at -80°C until RNA isolation was performed. All cases were subject to standard autopsy procedures and microscopic examinations.

Cyanide quantification

Cyanide concentrations were quantified in collected peripheral blood and gastric content samples using headspace gas chromatography with flame ionization detection (GC-FID-HS), a standard method for the analysis of volatile substances.

RNA isolation

Aliquots of peripheral blood samples (200 μL) were processed for small RNA isolation using the mirVana™ miRNA Isolation Kit (Invitrogen, Thermo Fisher Scientific). The isolation process was performed in strict accordance with the manufacturer's protocol, and the eluted RNA was quantified and stored at -80°C prior to subsequent analysis.

Reverse transcription and quantitative real-time polymerase chain reaction (PCR)

Complementary DNA (cDNA) was synthesized from 10 ng of isolated total RNA using the MultiScribe™ Reverse Transcriptase enzyme (Invitrogen, Thermo Fisher Scientific) with specific reverse-transcriptase primers for miRNA-200b, miRNA-429, and the endogenous control RNA RNU44 (TaqMan™ MicroRNA Assays, Applied Biosystems, Thermo Fisher Scientific). The relative expression of miR-200b, miR-429, and RNU44 was quantified using the TaqMan™ MicroRNA Assay (Applied Biosystem) in accordance with the manufacturer's instructions. All assays were run on the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). To ensure technical reproducibility, samples were analyzed in triplicate.

Statistical analysis

Relative miRNA expression was calculated using the comparative cycle time method ($2^{-\Delta\Delta\text{CT}}$), in which the expression of target miRNAs (miRNA-429 and miRNA-200b) was normalized to the expression of the endogenous control RNU44.⁽¹¹⁾

Statistical analysis was performed using R software (version 4.4.3). The nonparametric Mann-Whitney U test (also known as the Wilcoxon rank-sum test) was used to compare the relative expression ($2^{-\Delta\Delta\text{CT}}$ values) between the cyanide poisoning and control groups. Differences were considered statistically significant if the calculated two-sided *P*-value was less than 0.05.

Results

Autopsy and microscopic findings of cyanide cases

Standard postmortem examinations were conducted on the five cases of cyanide poisoning. Macroscopic and microscopic findings included pulmonary edema with marked vascular congestion. In renal tissues, there was a loss of nuclei within the proximal tubules in all cases; in three cases (60.0%), this finding was extensive. Notably, microscopic examination revealed no specific hypoxia-related changes within the cerebral tissues in any case. Consistent with the postmortem interval for each case, varying degrees of autolytic changes were observed in the spleen, pancreas, and gastric mucosa. General information on all cases is provided in **Supplementary Table 1**.

Toxicological results

The cyanide concentrations were quantified in peripheral blood (PB) and gastric content (GC) using GC-FID-HS (**Table 1**). The mean peripheral blood cyanide concentration was 17.9 µg/mL (standard deviation (SD) ± 7.0 µg/mL), and concentrations across the five cases ranged from 10.9 to 28.5 µg/mL. The mean cyanide concentration in gastric content was 403.3 µg/mL (SD ± 363.7 µg/mL), and the range was larger (74.6 to 958.5 µg/mL).

In all cases, there was a substantial difference in cyanide concentration between the gastric content and PB, which indicated that exposure occurred via the oral route. The ratio of cyanide concentration between gastric content and PB ranged from 6.9 to 76.1.

Relative expression of selected miRNAs

The expression of miRNA-429 and miRNA-200b was analyzed in PB samples from five control cases and four cases of cyanide poisoning (one cyanide case was excluded owing to poor sample quality, preventing reliable miRNA detection).

The expression of both miRNAs tended to be higher in the cyanide group than the control group (**Table 2**, **Figures 1 and 2**). Specifically, the median of the relative expression ($2^{-\Delta\Delta CT}$) was 20.35 in the cyanide group versus 38.79 in the control group for miRNA-429, and 3.81 versus 0.03, respectively, for miRNA-200b. However, there was substantial inter-individual variability within both groups, particularly for miRNA-429, for which expression was markedly elevated in one cyanide sample (**Figure 1**). Despite the observed trend of upregulation (**Figure 2**), differences between the two groups were not statistically significant ($P > 0.05$) for either miRNA.

Table 1. Cyanide concentrations in peripheral blood and gastric contents of cyanide poisoning cases.

Case no.	Cyanide concentration (µg/mL)		Cyanide concentration ratio
	Gastric content (GC)	Peripheral blood (PB)	GC/PB
1	164.4	20.9	7.9
2	664.2	28.5	23.3
3	74.6	10.9	6.9
4	958.5	12.6	76.1
5	154.5	16.6	9.3
Range	74.6–958.5	10.9–28.5	6.9–76.1
Mean ± SD	403.3 ± 363.7	17.9 ± 7.0	24.7 ± 28.2

SD, standard deviation.

Table 2. Median relative expression ($2^{-\Delta\Delta CT}$) of miRNA-429 and miRNA-200b in control and cyanide groups.

miRNA	Median ($2^{-\Delta\Delta CT}$)		P-value*
	Control group (n = 5)	Cyanide group (n = 4)	
miRNA-429	38.79	20.35	0.31
miRNA-200b	0.03	3.81	0.22

* P-values were determined by the Mann-Whitney U test (two-sided).

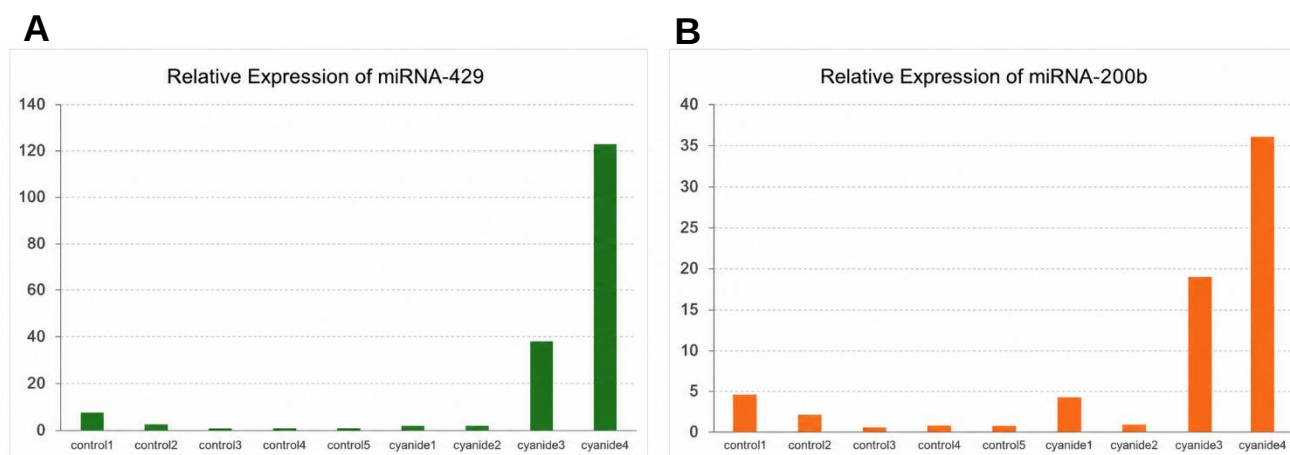


Figure 1. Relative expression ($2^{-\Delta\Delta CT}$) of (A) miRNA-429 and (B) miRNA-200b in peripheral blood from individual control (n = 5) and cyanide poisoning (n = 4) cases. Expression levels show high inter-individual variability, particularly within the cyanide group.

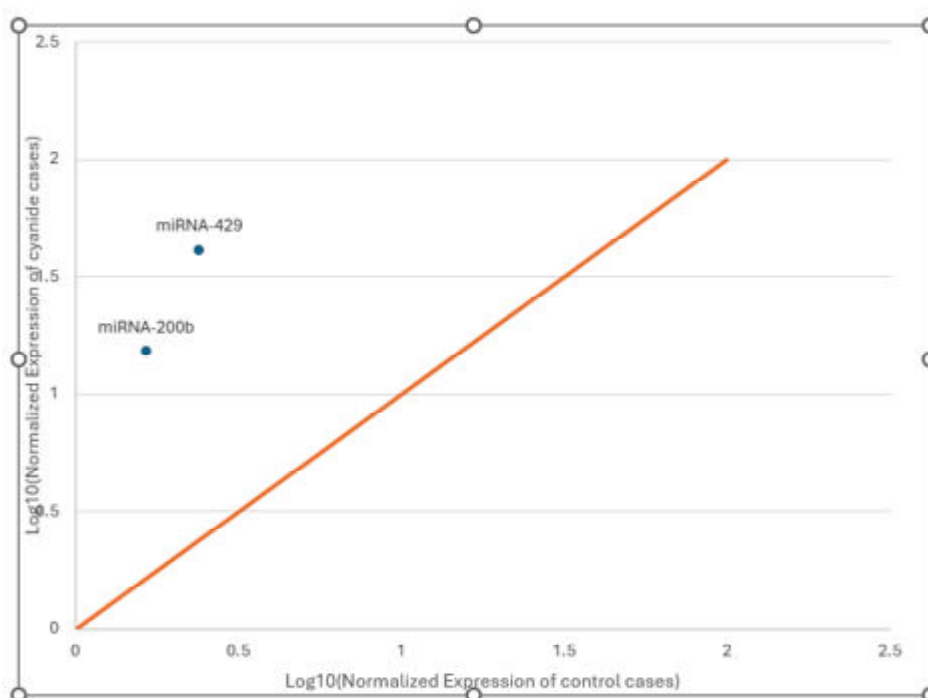


Figure 2. Comparison of average Log10 normalized expression levels for miRNA-429 and miRNA-200b between control cases (X-axis) and cyanide cases (Y-axis). Points above the diagonal line (Y = X) indicate higher average expression in the cyanide group.

Discussion

This study compared postmortem findings and postmortem peripheral blood expression of miRNA-429 and miRNA-200b in four cases of fatal cyanide poisoning and five control cases. The key findings were consistent pulmonary edema and renal tubular changes, the notable absence of cerebral hypoxic changes at autopsy, the confirmation of lethal cyanide levels consistent with oral ingestion, and a statistically nonsignificant trend of upregulation of miRNA-429 and miRNA-200b in the cyanide group.

The autopsy findings in our cases align partially with previous reports on cyanide fatalities.⁽¹²⁾ We observed pulmonary edema and congestion, as well as a loss of nuclei in renal proximal tubules, which are potential indicators of acute tubular necrosis, while renal subcapsular and myocardial hemorrhage were not main features. A notable observation was the absence of classic microscopic hypoxia-related changes in cerebral tissues, despite the mechanism of cyanide involving rapid, potent cellular hypoxia. Rapid death due to the action of cyanide, which usually occurs within minutes, may mean that there is insufficient time for the usual cellular responses to

occur, resulting in the absence of microscopic changes in cerebral tissue. Autolytic changes observed in organs such as the spleen, pancreas, and stomach lining were consistent with the postmortem interval and could therefore potentially obscure some significant findings.

Toxicological analysis confirmed lethal cyanide concentrations in all cyanide poisoning cases. The markedly higher concentrations in gastric content compared with peripheral blood (GC/PB ratio range 6.9–76.1) provide strong support that oral ingestion was the route of exposure.⁽¹³⁾ Although postmortem redistribution (PMR) can sometimes influence drug concentrations, this may have a limited contribution here, as all cyanide cases originated from the same incident and likely had similar postmortem intervals before sampling. The wide variation in blood cyanide levels (10.9–28.5 µg/mL) could potentially be due to differences in survival time after ingestion or individual metabolism rather than PMR effects only.

miRNA-429 and miRNA-200b were upregulated in the peripheral blood samples from cyanide poisoning cases compared with the controls (average fold-change > 1). This trend observed in our study aligns with the literature presented in the introduction, which suggests that both miRNAs are involved in acute hypoxic regulation,^(6–8) but did not reach statistical significance, with *P*-values of 0.30 for miRNA-429 and 0.22 for miRNA-200b.

Therefore, although this upregulation of miRNA-429 and miRNA-200b is consistent overall with the literature regarding their expected response to hypoxia, the lack of statistical significance in this cohort means we cannot confirm that they are reliable biomarkers for cyanide poisoning based on our results. The high variability observed requires cautious interpretation.

The most significant limitation of our study was the small sample size (particularly as a sample was excluded from analysis in the cyanide group) and non-normal distribution of miRNA expression, which markedly limits statistical power in detecting the true differences, especially when there is high inter-individual variability. Other limitations of this study might arise from individual biological differences in response to cyanide. Agonal states or postmortem factors might also influence the variability of RNA detection. Furthermore, although the analysis of peripheral blood reflects a systemic response, it may miss tissue-specific changes that occurred in target organs that are directly affected by hypoxia. Additionally, changes in hypoxamiRs might not exclusively reflect the body's response to hypoxia

arising solely from cyanide but might also be affected by other causes that alter the cellular hypoxic state. Future studies should focus on how these markers change in response to other hypoxic causes.

Conclusion

This study highlights that in fatalities due to acute cyanide poisoning, standard autopsy and microscopic findings can be non-specific, particularly regarding cerebral hypoxic changes. This emphasizes the critical role of toxicological analysis for a definitive diagnosis. In our study, we observed the upregulation of miRNA-429 and miRNA-200b in the peripheral blood of cyanide poisoning victims compared with the control. This observed trend aligns with reports linking these miRNAs to acute hypoxic responses. However, owing to the significant inter-individual variability and small sample size in our study, we did not observe statistically significant differences. Consequently, although these preliminary data are consistent with expected hypoxic responses, further research, involving larger sample sizes and the analysis of specific tissues or comparisons with other forms of acute hypoxia, is essential to determine if miRNA-429 and miRNA-200b can serve as reliable diagnostic or predictive markers for cyanide-induced acute hypoxia.

Author contributions

PV contributed to the conceptualization of the study, acquired the data, reviewed the literature, and drafted the manuscript. NT performed data collection and analysis. WW, TS, and PS contributed to data interpretation and statistical analysis. KV conceptualized and supervised the study, provided resources, and critically reviewed the manuscript for intellectual content. All authors approved the final version submitted for publication and took responsibility for statements made in the published article.

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Conflicts of interest statement

The authors declare no conflict of interest.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for non-commercial purposes from the corresponding author on reasonable request.

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