https://doi.org/10.48047/AFJBS.6.Si4.2024.4195-4210

African Journal of Biological Sciences

Research Paper Open Access

Association of Circulating Circular RNA (Hsa-Circrna-006732) in The Medicolegal Assessment of Traumatic Brain Injury Patients: Case-Control Study

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Article History
Volume 6, Issue Si4, 2024
Received: 5 July 2024
Accepted: 20 July 2024
doi:
10.48047/AFJBS.6.Si4.2024.4195-4210

Abstract:

INTRODUCTION: For medical-legal matters, cases related to traumatic injuries in specific brain regions hold considerable significance .a newly recognized class of noncoding RNAs(ncRNA) has been implicated in various diseases' progression. Yet, their connection with (TBI) remains largely, unexplored. This study aimed to establish the role of the plasma expression levels of hsa-circ- 006732 and Wingless-related integration site Inhibitory factor (WNT IF), as novel biomarkers in the medicolegal assessment of Traumatic Brain Injuries and in dating these injuries.

METHODS: This case-control study comprises 40 participants, Group I:30 TBI patients who were divided according to the Glasgow Coma Scale into three equal groups (mild, moderate & severe). TBI cases were evaluated for age, sex, etiology, manner of trauma, and outcome. and group II: 10 healthy volunteers representing the control group matched for age and sex. Venous Blood samples were withdrawn within the first 24 hours, after 5 days and 30 days from injured patients at the emergency, the neurosurgery department, and the outpatient clinic. RT-PCR was used to evaluate the expression level of

circular RNA (hsa-circ-0067322), The Elisa technique was used to determine the expression level of WNT IF in serum.

RESULTS: The majority of patients were males (90%). The main cause of trauma was violence. The commonest manner of trauma was accidental, in moderate and severe cases, (60%) and (80%) respectively, regarding outcomes 100% recovered in mild cases, death occurred in 20% in severe cases, and 60% were with permanent infirmity, TBI patients showed Significant downregulated expression level of WNTIF with ($p < 0.0001$) and non-significant expression levels of hsa-circ-006732 compared to controls,

CONCLUSION: WNT IF is an excellent biomarker for the dating and diagnosis of TBI injuries, while hsa- circ-006732, can be used as a fair novel diagnostic biomarker for TBI, It is recommended that the current results verify the conflicting age of TBI on a molecular basis for medicolegal purposes, especially the proper dating of crimes.

Keywords: hsa-circ-006732, WNT IF, biomarker, Traumatic brain injury

1. Introduction

TBI is sometimes referred to as the "silent epidemic," and among all trauma-related injuries, It is among the leading causes of both disability and death. It is also becoming a bigger public health issue.(1)

There are three distinct TBI severity categories: mild, moderate, and severe. A long-standing clinical tool (GCS) that relies on physical examination results without the requirement for specialized tools to measure mild (13–15), moderate (9–12), and severe (1-3). Regretfully, there is little association with milder forms of illness. A perfect score of 15 does not rule out the possibility of developing post-concussive syndrome (2), nor does it indicate the absence of TBI.

Computed tomography (CT) is the most prevalent radiographic approach for diagnosing TBI-related intracranial abnormalities. The cost and radiation load involved with neuroimaging, particularly in individuals with moderate damage, has led to increased research of blood biomarkers to assist in identifying neuro-worsening and prognostication (3).

Recent research has revealed distinct circular RNA expression patterns between brains affected by (TBI) and healthy brains, within cells and in the extracellular environment.This suggests that circular RNAs may play a role in the development of TBI and potentially act as regulatory factors (4). Circular RNAs (circRNAs) are a type of non-coding RNA distinguished by their unique circular structure They are formed when the 3' and 5' ends of messenger RNA (mRNA) molecules join together, primarily through a process called back-splicing or by lassoing introns (5)

Circular RNAs encased in exosomes have increased stability, This permits them to pass past the blood-brain barrier (BBB)and into the circulatory system while retaining their original, brain-specific patterns. (6).

A comprehensive investigation verified that circRNAs were unique to tissues and that the brain expressed 20% of them. In other words, circRNAs are expressed more dynamically and in greater abundance in the tissue of the mammalian brain than in any other organ. Exosomes containing different circular RNAS may be released from traumatized brain tissues into the cerebral extracellular space (7).

WNT proteins are cell-specific ligands that regulate a variety of biological processes throughout development and maturation. They are mostly generated by neurons and astrocytes in the brain. Recent research has connected traumatic and vascular brain injuries to dysregulation of the WNT pathway, indicating a direct and major effect on the mechanisms that cause damage (8).

The KEGG pathway study revealed that Wnt signaling is one of the routes that promote brain regeneration and repair, and it may be implicated in the process of (TBI). This is because it promotes neural stem cell formation and differentiation by synthesizing stem cell regulatory factors. Even though various paths have been linked to the pathophysiology of TBI, it is unknown whether a specific circular RNA-miRNA-mRNA-protein pathway network is implicated, and additional study is needed. (9).

Referrals to hospital neurosurgery departments can involve a wide range of injuries, and classifying a case as violent or non-violent is a challenge that cannot be completed by evaluating the injury alone. Therefore, to determine the origin of the injury and help with the initial assessment and care of the injuries involved, a multidisciplinary approach is required (10).

2. Materials and Methods:

2.1. Study participants :

This case-control study comprises 30 TBI cases and 10 healthy controls, TBI cases were recruited from the neurosurgery, emergency departments, and outpatient clinic at Kasr Alainy Hospital, Cairo University, between July 2022 and January 2023. This study was done under the ethical guidelines set in the Declaration of Helsinki and with ethical permission at Kasr Alainy Hospital (MD-109-2021). Written informed permission was acquired by each subject. Based on their GCS scores, TBI cases were divided into three groups: mild TBI (13–15), moderate TBI (9–12), and severe TBI (3-5) (11).

Additionally, 10 healthy controls matched for age- and sex-seeking routine health check-ups were recruited from the same hospital.The inclusion criteria comprised: Adults aged 18-45. who had recent head trauma within 24 hours met the inclusion criteria.

pregnant or nursing women, individuals with diabetes, hypertension, or other significant cardiovascular diseases, and those with debilitating neurological conditions of pathological origin, concurrent spinal cord injuries, or injuries resulting from substance abuse or alcoholism, all have been excluded.

2.2.Sample collection:

Venous Blood samples (5 mL) were taken from both the case and control groups. They were then kept at -80°C until the RNA extraction and ELISA.

2.3.RNA extraction:

Using the Qiagen miRNeasy kit, RNA was extracted from the plasma samples.An instrument made by NanoDrop Technologies, Utilizing the ND-1000 spectrophotometer, The content and purity of RNA were assessed.

2.4.Reverse transcription (RT) and real-time quantitative PCR (qPCR)

hsa-circ-006732 and Gene Expression: ELK Green One-Step qRT-PCR Super Mix kit (Cat. No. EQ007-02) was used to quantify the expression of circular RNA ,This kit utilizes a three-step cycling protocol:

- Reverse transcription: 1 cycle at 50°C for 15 minutes
- Predenaturation: 1 cycle at 95°C for 2 minutes
- Amplification: 40 cycles of:

- Denaturation: 95°C for 10 seconds
- Annealing: 50-60°C for 30 seconds
- Extension: 72°C for 30 seconds

Real-Time PCR Setup: a 10 µL reaction volume per well was prepared. The real-time PCR program used the following steps:

- Initial activation: 95°C for 2 minutes

- Cycling:

- Denaturation: 95°C for 10 seconds
- Combined annealing and extension: 56°C for 60 seconds (40 cycles)

The content and purity of RNA were assessed. Quantitative reverse transcription PCR was used to determine gene expression. (RT-qPCR) on an Applied Biosystems StepOne™ equipment (version 3.1, USA). GAPDH was utilized as a reference gene to normalize the expression of hsa-circ-006732. The 2-ΔΔCt technique was used to analyze RT-qPCR findings.

To assess the efficiency, a 10-fold dilution of our target was prepared, and a regular real-time PCR run was conducted. The Ct (threshold cycle) values obtained from the qRT-PCR experiment were plotted on the y-axis (logarithmic scale) against the corresponding known concentrations of the starting material (e.g., RNA) on the x-axis using GraphPad software, A linear regression analysis was performed on the plotted data points to generate a best-fit trend line. The slope of the generated trend line was then calculated. Finally, the PCR efficiency (E) was determined using the equation: E = -1 + 10^(-1/slope).

2.5. An ELISA method to measure WNT protein levels
Sun Long Biotech Co., LTD provided the kit (Catalogue Number# SL1870Hu) that was utilized for the quantitative detection of WNT protein in serum, plasma, bodily fluids, cell culture supernatants, and tissue homogenate. The sandwich-ELISA approach is what this kit uses. An antibody specific to WNT protein has been pre-coated on the Micro Elisa strip-plate included in this kit. From the standard curve, the samples' WNT IF concentration was extrapolated.

Statistical Analysis:

The sample size was calculated using the (G power software) (31). Microsoft Excel 2019 was used for tabulation and the statistical analysis of the results was done via the SPSS software, version 25 for Microsoft Windows 10 The information was encoded and entered into SPSS (version 28), a statistical analysis tool created by IBM Corp. located in Armonk, New York, USA. Depending on the kind of data, we summarized it using various techniques. We used the mean (average) and standard deviation (spread) for numerical data (e.g., height). For non-numerical data (such as eye color), we merely tallied the frequency—the number of times each category appeared. as well as relative frequencies (shown as percentages) (which show the number of cases). For regularly distributed quantitative variables Comparisons between groups were conducted with unpaired t-tests or analysis of variance (ANOVA). For quantitative data that did not follow a normal distribution, field professionals recommended employing Mann-Whitney and Kruskal-Wallis nonparametric tests [12].

To analyze categorical data, the Chi-squared ($\chi 2$) test was used. For anticipated frequencies less than five, the precise test, as recommended by (13), was used. Spearman's correlation coefficient was employed to investigate the link between quantitative variables.adhering to the approach outlined by (14). To assess the marker's performance in determining the best cutoff values for significant markers in case detection, a ROC curve was constructed. Next, to measure this performance, We examined the area under the curve (AUC). Serial measurements within each group were analyzed using the ANOVA and Friedman tests. The non-parametric Friedman test was used as recommended by (15) for quantitative variables that were not normally distributed, while ANOVA was used to represent quantitative variables with a regular distribution.

3. RESULTS:

3.1. Demographic and Medicolegal data of TBI patients

Table 1 shows the characteristics of the study participants. Patients were older than 18 years, and Age and gender distribution were comparable between the 3 groups (no significant differences ($P > 0.05$). Patients were subdivided depending on severity.: mild, moderate, and severe according to their GCS scores with a median of 15,12, and 6, respectively. 100% of the severely injured patients had hemorrhage of which 40% died.

Table 1. Demographic and injury characteristics of TBI patients.

Variable	Control (n = 10)	TBI (n = 30)			P-Value
		Mild (n= 10)	Moderate (n= 10)	Severe (n= 10)	
Age	26.4 ± 7.1	30.1 ± 11.4	31 ± 8.9	29.9 ± 8.2	0.68
Sex					
Male	6 (60%)	9 (90%)	9 (90%)	9 (90%)	0.19
Female	4 (40%)	1 (10%)	1 (10%)	1 (10%)	
Residence					
Urban		5 (50%)	5 (50%)	7 (70%)	0.58
Rural		5 (50%)	5 (50%)	3 (30%)	
Mechanism of Injury		2 (20%)	3 (30%)	6 (60%)	0.02
Road traffic injury		01(10%)	2 (20%)	1 (10%)	
Fall from height		7(70%)	5(50%)	3(30%)	
Violence					

Manner of injury **Accidental** **Homicidal**		6 (60%) 4 (40%)	6 (60%) 4 (40%)	8 (80%) 2 (20%)	0.36
CT changes **Contusion** **Hemorrhage** **Fracture** **Fracture and hemorrhage**		2(20%) 0 (0%) 3 (0%) 5(50%)	0 (0%) 2 (20%) (0%) 8(80%)	0 (0%) 1 (100%) 3 (30%) 6(60%)	<0.001
Outcome **Recovered** **Permanent infirmity** **Died**		10 (100%) 0 (0%) 0 (0%)	7 (70%) 3(30%) 0 (0%)	2 (20%) 6(60%) 2 (20%)	<0.0001

GCS: Glasgow coma scale, Chi-square test, the p-value was considered statistically significant, * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.2. Comparison of hsa-circ-006732 and WNT IF biomarker between TBI cases and control

Figure 1A showed The expression level of WNT IF with significant downregulation in mild, moderate, and severe TBI cases in comparison to the control (P-value <0.001), while no significant changes were detected regarding the expression level of hsa-circ-006732 in different TBI groups with different severities in comparison to control Figure 1B.

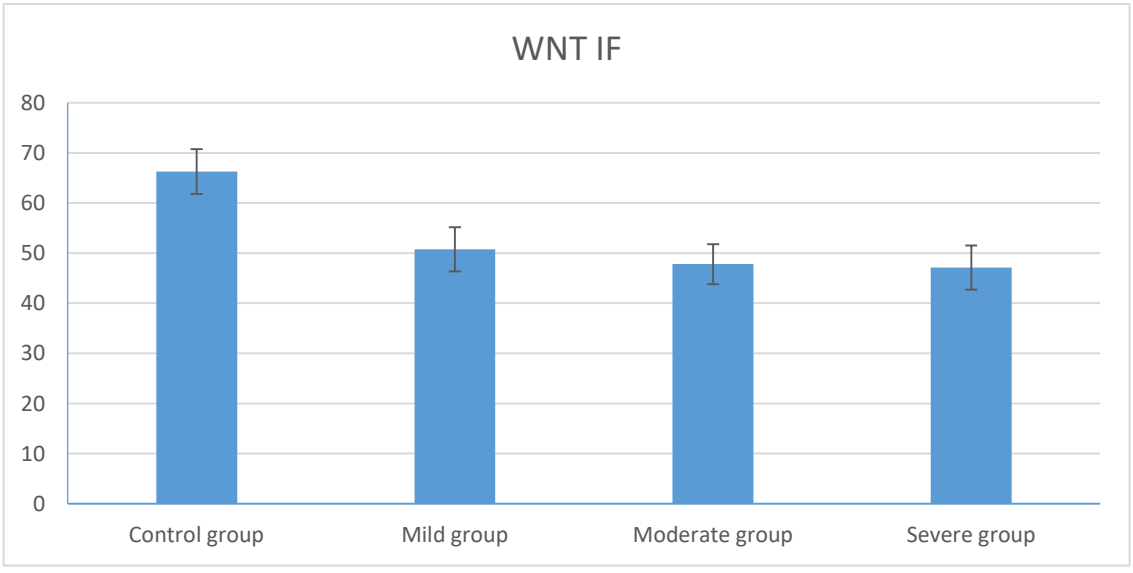

Figure1A

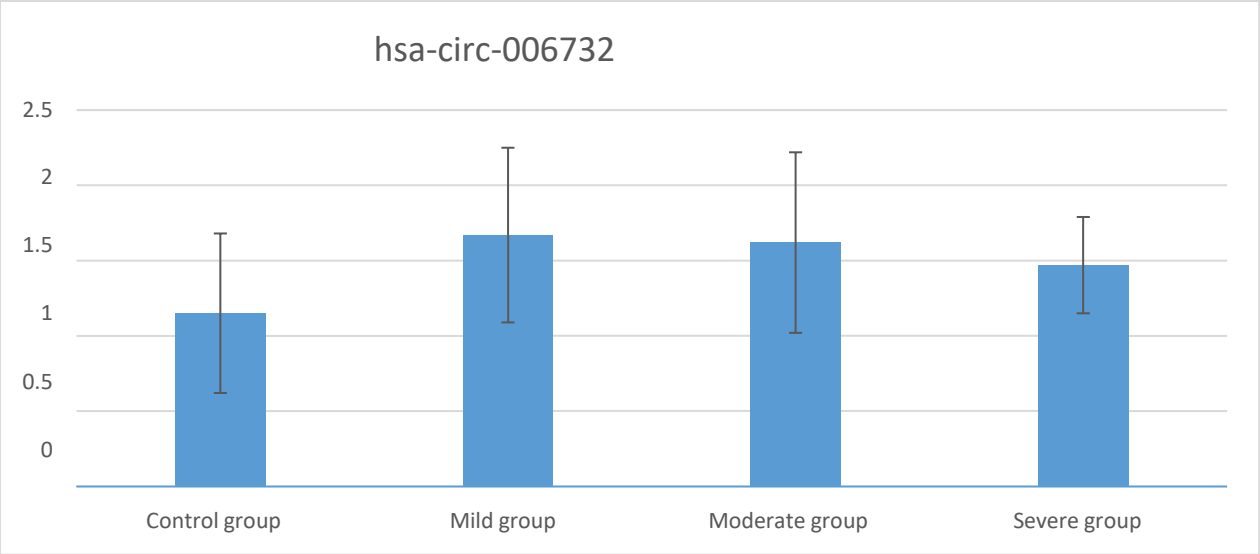

Figure 1B

**P is highly significant if < 0.001, Box PLOT showing , Figure2 A) expression level of WNTIF B) expression level of hsa-circ-006732 in TBI cases (Mild, moderate, severe) in comparison to control

3.3 The expression level of hsa-circ-006732 and WNT IF biomarker for dating of TBI injuries in different timings

Table 2 indicates a statistically significant downregulation in the expression level of WNTIF after the fifth day of damage compared to the initial day of injury in instances of mild, moderate, and severe TBI.

with p-values (0.010,<0.001, <0.001) respectively, also it was significantly downregulated after 30 days from injury in cases of moderate TBI with (p-value 0.025). no statistically significant change was observed for the expression level of hsa-circ-006732 in different timing in different TBI severities.

Table 2. Expression level of circular RNA and WNT IF For dating of TBI injuries in different TBI severities

	Mild			MODERATE			SEVERE		
	mean	SD	PVALUE	mean	SD	PVALUE	mean	SD	PVALUE compared to first 24 hours
WNT IF(24 hours)	50.76	4.43	----	47.79	47.79	----	47.12	4.42	----
WNT IF 5 th days	43.63	2.60	0.010	37.11	37.11	< 0.001	32.88	4.65	< 0.001
WNT IF 30 th days	45.31	6.11	0.082	38.34	38.34	0.025	46.45	5.28	1.000
hsa-circ-006732 (24 hours)	1.64	0.59	----	1.59	1.59	----	1.48	0.31	----
hsa-circ-006732 at 5 th days	1.32	0.57	0.499	1.46	1.46	1.000	1.50	0.53	1.000
hsa-circ-006732 at 30 th days	1.27	0.46	0.217	1.26	1.26	0.826	1.40	0.59	1.000

Chi square test, p-value was considered statistically significant, * $p < 0.05$, ** $p < 0.01$, *** ***$p < 0.001$,

3.4 Relation between hsa-circ-006732 and WNT IF markers and symptoms of TBI

Table 3 shows that There was a statistically significant relation between the measured expression level of circular RNA at the first 24 hours of injury and WNT expression level at the fifth day from injury with the symptoms presented by TBI patients (Disturbed conscious level)

or others (vomiting ,headache, conjunctival hemorrhage ,Racoons eye and blurring of vision) with (pvalue = 0.011and 0.006 respectively)

Table 3. Association of expression level of hsa-circ-006732 and WNT IF with symptoms of TBI cases

	Symptoms				P value
	Disturbed conscious level		Other symptoms		
	Mean	Standard deviation	Mean	Standard deviation	
WNT IF (24 hours)	48.22	4.36	50.24	4.95	0.361
WNT IF 5 th days	36.65	5.38	43.99	2.38	0.006
WNT IF 30 th days	43.15	6.65	44.43	7.49	0.703
hsa-circ-006732 (24 hours)	1.47	0.51	2.06	0.33	0.011
hsa-circ-006732 at 5 th days	1.41	0.51	1.50	0.74	0.957
hsa-circ-006732 at 30 th days	1.31	0.67	1.33	0.48	0.872

Chi square test, p-value was considered statistically significant, * $p < 0.05$, ** $p < 0.01$, *** ***$p < 0.001$

3.5 ROC curve analysis for evaluation of plasma levels of hsa-circ-006732 and WNT IF for diagnosis, severity detection, dating

Table 4 shows the ROC curve analysis :

- WNT IF was an excellent diagnostic biomarker and circular RNA was a fair diagnostic biomarker for differentiation between TBI cases and control with an area under the curve (AUC) of:
- 0.7 For circular RNA ($p < 0.0001$), 1 for WNT IF ($p< 0.0001$).
- WNT IF biomarker was an excellent indicator for dating of traumatic brain injuries with area under the curve (AUC) of:
- 0.9 For WNT IF($p<0.001$).
- WNT IF biomarker has a fair ability to detect TBI severity and distinguish between mild and moderate to severe instances.

- with (AUC) of: 0.7 For WNT IF($p<0.05$).

Table 4.ROC curve analysis of circular RNA and WNT IF for diagnosis, severity detection, and dating of TBI cases

	AUC	95% CI	P-Value	Sensitivity (%)	Specificity (%)
	Control vs TBI patients				
hsa-circ-006732 (first 24 hours)	0.727	0.5-0.9	0.020	76.7	70
WNT IF (first 24 hours)	1	1-1	<0.0001	100	100
	Mild vs Moderate and Severe				
hsa-circ-006732	0.42	0.18-0.65	<0.48	75	30
WNT IF	0.712	0.52 -0.91	<0.05	80	60
	Dating of TBI INJURY				
WNT IF	0.9	42.3	<0.0001	77	100%

DISCUSSION :

The examination of TBI patients holds great importance in the field of forensic medicine due to their potential for fatalities, even when there are limited observable symptoms. Additionally, the timing of the injury can have significant relevance in forensic investigations as it can influence the stage of injury progression and healing processes (16). Moreover, the biomarkers associated with inflammation, tissue damage, and recovery pathways vary depending on the time elapsed since the injury occurred. Nonetheless, the complicated molecular mechanisms underlying traumatic processes and their effect on the brain remain largely unknown. The prospect of discovering novel biomarkers that can predict the severity and timing of an injury presents an exciting frontier in the field of forensic medicine

There has been increasing development in high-throughput RNA- sequencing bioinformatics analysis tools in monitoring gene expression, which has risen dramatically in recent decades. In addition, there has been greater research interest in circular RNAs(17) . The

potential biological roles of the parent gene of these differentially expressed genetic biomarkers were examined using Kyoto Encyclopaedia of Genes and Genomes (KEGG analysis) and Gene Ontology (GO). The purpose of this investigation was to determine the significance of the novel biomarker (hsa_circ_0067322) and WNTIF in TBI patients, as To the best of our knowledge, no previous research has looked at the role of hsa_circ_0067322 in TBI.

In the present study, the expression level of hsa_circ_0067322 and WNTIF was compared between TBI patients in different groups, (mild, moderate, and severe) and healthy volunteers as control in the human model, WNT IF showed significant downregulation in TBI cases in comparison to control with ($P < 0.0001$), while hsa_circ_0067322 showed no statistically significant difference between different TBI cases and control, Previous studies ((18); (19); (20); (21) , similarly have focused on other circular RNAs expression profiles after TBI but in the rodent model cortex, and the study of (23)was performed on another circular RNA in ~~human~~human but in another ~~another~~ischaemic brain injury~~also the study of ~~ , as regards WNT proteins,(22) adopted the role of expression level of WNT IF in TBI but in the rodent model.

The activation of the Wnt pathway following a traumatic brain injury (TBI) actively controls neurogenesis, angiogenesis, and inflammation, which explains why this occurs. Current research in this field tries to target the route that promotes structural and functional rehabilitation.

In the present study, blood samples were withdrawn at first 24 hours , 5, and 30 days from TBI injury in the human model and it was observed that no statistically significant variations existed. in the expression level of circular RNAs (hsa_circ_0067322) over different timing, on the other hand, there was a statistically significant down-regulation of WNT IF in mild and moderate TBI and significant upregulation of WNT IF in severe TBI, similarly, the study of (24) adopted the measurment of the expression level of WNT protein on different timing but in the rodent model .the down regulation of WNT IF over different timing could be explained that WNT Proteins help in promoting healing post TBI .

In the present study, we assessed the diagnostic value of hsa-circ- 0067322, and WNTIF in traumatic brain injury. We performed a ROC curve analysis to differentiate between patients with TBI and healthy controls. ROC curve analysis revealed that WNTIF could differentiate between TBI patients and healthy controls with excellent accuracy, While hsa_circ_0067322 has fair diagnostic value. Moreover, the fact that the expression level of different biomarkers changed over times makes them helpful in evaluating the period after post-trauma. They also can be used for the evaluation of prognosis and detection of the severity of TBI. Similarly the study of (25) he performed ROC curve analysis on another circular RNA to assess the diagnostic performance in ischaemic brain injury in humans.

The amounts of three combined circular RNAs may have greater diagnostic efficiency than that of each circular RNA, according to a study by (25) on circular RNA as a biomarker in another neurological injury. This was because the sensitivity was 71.5%, specificity was 91%, and AUC was 0.875. As a result, future research using hsa_circ_0067322 in conjunction with other identified ones would lead to more precise diagnostic performance.

Conclusion :

hsa_circ_0067322 and WNTIF can be utilized for the diagnosis of TBI. WNT IF can be used for the dating of TBI injuries, which has a high medicolegal value, and in identifying varied TBI severity.
Future studies must incorporate coupled circular RNAs to have more diagnostic efficacy than that of individual circular RNA, and it must be on a wider scale in another age range. There are also several drawbacks to this study, such as the small sample size and the restricted period.

Ethical statement:

The study was authorized by the Ethics Committee and the Review Board of Cairo University's Faculty of Medicine (IRB approval number: MD-109-2021). All study subjects provided written informed consent prior to their participation.

Conflict of interest

The authors disclose no competing interests.

Acknowledgments

None

Funding

Faculty of Medicine, Cairo University

Authors' contributions

practical work prepared the original draft: Yasmin Kamal Abd Rabou; conceptualization: Abeer Ahmed Zayed, revision; Sally A. Fahim: bioinformatics and statistical analysis, writing and final revision; Marwa Abdelgwad: practical work and methodology; Ahmed El Fiki: clinical samples and data collection; Nermin Nabil Fayed: reviewed the manuscript. All authors read and approved the final manuscript.

Data availability statement

Data are available upon reasonable request from the corresponding author

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