REVIEW ARTICLE

MicroRNAs: The New Challenge for Traumatic Brain Injury Diagnosis

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Abstract: The acronym TBI refers to traumatic brain injury, an alteration of brain function, or an evidence of brain pathology, that is caused by an external force. TBI is estimated to become the third leading cause of permanent disability and mortality worldwide. TBI-related injuries can be classified in many ways, according to the degree of severity or the pathophysiology of brain injury (primary and secondary damage). Numerous cellular pathways act in secondary brain damage: excitotoxicity (mediated by excitatory neurotransmitters), free radical generation (due to mitochondrial impairment), neuroinflammatory response (due to central nervous system and immunoactivation) and apoptosis. In this scenario, microRNAs are implicated in the regulation of almost all genes at the post-transcriptional level. Several microRNAs have been demonstrated to be specifically expressed in particular cerebral areas; moreover, physiological changes in microRNA expression during normal cerebral development upon the establishment of neural networks have been characterized. More importantly, microRNAs show profound alteration in expression in response to brain pathological states, both traumatic or not. This review summarizes the most important molecular networks involved in TBI and examines the most recent and important findings on TBI-related microRNAs, both in animal and clinical studies. The importance of microRNA research holds promise to find biomarkers able to unearth primary and secondary molecular patterns altered upon TBI, to ultimately identify key points of regulation, as a valuable support in forensic pathology and potential therapeutic targets for clinical treatment.

1. INTRODUCTION

The acronym TBI refers to traumatic brain injury, defined as "an alteration of brain function, or an evidence of brain pathology, that is caused by an external force" [1]. TBI is estimated to become the third leading cause of permanent disability and mortality worldwide [2]. Among the causes of TBI, falls, vehicle accidents, firearms, sports and work-related injuries are the most common ones [3]. TBI always implicates neurological dysfunction and often results in long-term damage [4], which hampers patient's self-sufficiency [5]. The pathophysiology of TBI does not alter a single pathway: traumatic alteration triggers a multitude of signalling cascades [6]. MiRNAs represent interesting candidates for the characterization of the principal pathways involved in TBI since they are implicated in almost all cellular regulatory processes [7]. The most important molecular networks involved in TBI will be discussed in the present review. The ultimate aim of research regarding TBI-related miRNAs is the development of new therapeutic strategies to treat TBI patients in the most effective way. Many research works have been performed so far in this direction, comprising experimental and human studies, which will be described in the present review. The importance of miRNA research holds promise to find biomarkers able to disclose primary and secondary molecular patterns altered upon TBI, to ultimately identify key points of regulation as potential therapeutic targets for clinical treatment [8].

2. TBI CLASSIFICATION AND MOLECULAR PATHWAYS INVOLVED

TBI is usually classified according to the site of injury in open-head and closed-head types, their difference being whether dura is damaged or not, respectively [9]. Furthermore, TBI can be typified into three grades: mild, moderate, and severe [10] on the basis of structural imaging information, alteration of consciousness / mental state (AOC) and Glasgow Coma Scale (GCS). Scoring systems are employed

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from over 40 years to standardise the depth and duration of impaired consciousness and coma, that is to say, the measurements of illness severity and to predict outcomes of patients [11]. Among others, GCS is the most used in order to classify TBI in accordance with severity [12], although it has some limitations [13]. TBI-related injuries can be classified into primary and secondary brain damages [14]. The first refers to the local neuronal destruction due to primary insult, whereas the second one is represented by neuronal death and it is induced by many events which include ischemia, brain edema, axonal injury, mitochondrial dysfunction and dysregulation of calcium homeostasis [15-17]. More specifically, primary brain injury consists of those damages immediately occurring at the moment of impact. This implies the disruption of parenchymal continuity and cerebral blood vessel damages. Secondary brain injury starts in the minutes following primary insult, and it is responsible for neurological impairment experienced by TBI patients. At this stage, numerous signalling cascades turn on into the cells causing systemic effects. Death of cells from the central nervous system generally goes through two phases, an early necrotic and a prolonged apoptotic phase [18-20]. Numerous cellular pathways that act in secondary brain damage include excitotoxicity (mediated by excitatory neurotransmitters), free radical generation (due to mitochondrial impairment), neuroinflammatory response (due to central nervous system and immunoactivation) and apoptosis [21, 22]. These processes will be briefly described below.

2.1. Excitotoxicity

One of the main causes of secondary brain damage is the massive release of endotoxins, such as glutamate and aspartate, during primary insult. This event alters the permeability of NMDA glutamate receptor, thus increasing intracellular calcium and sodium cellular uptake and concomitant activation of calcineurin and calmodulin. This last mediator is responsible for axonal destruction [23, 24]. In mammals, glutamate is the most widespread excitatory neurotransmitter. It has been studied since its crucial importance in the onset of acute and chronic neuronal damage became apparent [25]. In normal brain, glutamate neurotoxicity is known since 1983, when the "excitotoxic" hypothesis was made by Rothman and Olney [26]. After this time, many advances have been made to understand the neurotoxicity of others endogenous excitatory amino acid neurotransmitters, including the role of aspartate [27].

In mammalian neurons, four glutamate receptor subtypes have been characterized so far and intense research has been made to clarify the molecular networks triggered by glutamate binding [25, 28-31]. At the physiological level, almost all cortical and hippocampal cellular pathways depend on glutamate [29, 32-34]. However, when an excess of glutamate accumulates in the extracellular space, overactivation of NMDA glutamate receptor takes place, leading to an unproper sodium and calcium cellular intake with concomitant potassium escape. All these events end with neuronal death, similar to that observed upon ischemia, in a process known as "fast excitotoxicity" [35, 36]. On the contrary, when calcium intake decreases, neurons are progressively destroyed in the so-called "delayed neuronal death" [37]. Finally, when

potassium moves out of cells, astrocytes swelled up to absorb it trying to balance ionic alterations [38]. This event causes "cytotoxic edema" perhaps the main factor responsible for posttraumatic raised intracranial pressure (ICP). Studies on animal models confirmed the massive release of glutamate upon neurotrauma and stroke [25, 39]. Pharmacological treatment able to inhibit glutamate effects has been demonstrated to impair ischemic brain damage [40, 41]. Similarly to animal models in which TBI is experimentally induced, also in patients experiencing TBI, an increase in extracellular glutamate occurs [24, 42]. Where does glutamate come from? It may reach the brain upon the disruption of the blood-brain barrier. Intraparenchymal hemorrhage often occurs after trauma, leading to glutamate leaking at the site of cortical impact [43].

Efficiently maintaining low glutamate extracellular concentrations is vital to avoid neurotoxicity. Several evidences suggest that inefficient glutamate transport leads to the accumulation of excessive neurotransmitters in the synapse. Five subtypes of glutamate transporters have been cloned so far: GLAST (EAAT1), GLT-1 (EAAT2), EAAC-1 (EAAT3), EAAT4 and EAAT5 [44], the first two being mainly localized in astrocytes [45], while the others being mainly typical of neurons [46, 47].

2.2. Free Radical Generation

Besides glutamate-related alterations, blood loss due to injury-related hemorrhages causes vessel spasm [48], which is accompanied by increased oxidative stress and increased risk of ischemic events. Under physiological conditions, free radicals are involved in the maintenance of vascular tone and immune system functionality, their action being limited by endogenous scavengers. Brain injury disrupts this equilibrium triggering free radical production and making the action of scavengers insufficient [49]. The degree of oxidative stress strongly influences the pathogenesis of TBI [50]. Reactive oxygen species damage lipids, proteins and nucleic acids. In particular, lipid peroxidation is responsible for the production of free radicals and it is frequently occurring in brain-injured patients. Molecular signalling cascades triggered by reactive oxygen species after TBI cause cytoskeletal damage, alter normal signal transduction [51] and impair mitochondrial function [52]. Mitochondria are hypothesized to produce the vast majority of reactive oxygen species after TBI [53]. In animal models, the pharmacological suppression of free radical generation holds promise to be successfully converted into therapeutic protocols for patients with brain injury, stroke, and subarachnoid hemorrhage [54].

2.3. Neuroinflammatory Response

The first activation of inflammation after brain injury mainly originates from blood products which come out from vessels, reactive oxygen/nitrogen species and products released by microglia and astrocyte resident in the central nervous system which sense perturbation [55]. Inflammatory processes following TBI greatly reinforce secondary damages. This becomes a systemic event, often causing multiple organ dysfunction syndromes. Inflammation processes are triggered by primary insult: this leads to the activation of microglia and astrocyte cells, from one hand, and to the infil-

tration of neutrophils and macrophages in the injured area, from the other. The most important inflammatory mediators are growth factors, catecholamines, cytokines and chemokines [56]. In particular, cytokines released by inflammatory cells induce macrophages and granulocytes to produce iNOS-derived NO. This phenomenon was demonstrated both in rodent models [57] and humans [58, 59].

2.4. Apoptosis

Apoptosis is a programmed cell death responsible for the elimination of cells during development. In normal adults, it represents a physiological mechanism ensuring cell renewal [60]. The first description of apoptosis came from studies on neuronal development of the roundworm [61]. TBI causes the increase in expression of genes encoding for the caspase family of cysteine proteases (among which interleukin-1β converting enzyme and cpp32) and genes homologous to Bcl-2, which presides over caspase-dependent and independent apoptosis [60, 62]. All apoptotic signalling cascades culminate with the disruption of DNA integrity and the formation of membrane-wrapped apoptotic bodies, which are phagocytised by macrophages [63]. Neuronal apoptosis following TBI is hypothesized to exert a protective role for the brain, removing irreparably damaged cells so as not to affect healthy surrounding tissue [64]. Signs of apoptotic processes have been reported at the site of injury in the acute post-traumatic period and distal regions and at a later time [65]. This phenomenon can be explained as follows: after brain injury, the damaged tissue experiences two cycles of neuronal death, early necrosis and consecutive apoptosis [66]. The recent discovery of a protein family able to inhibit apoptosis holds promise for the development of new therapies for TBI [67]. In animal models, a reduction in contusion size and dorsal hippocampal loss was reported after the intracerebral administration of a caspase-3 inhibitor, although this benefit did not translate into an improvement of functional outcome [68].

3. MicroRNAs INVOLVED IN TBI

The pathophysiology of TBI is not restricted to the alteration of a single pathway, being rather concomitantly involved apoptosis, inflammation, neural plasticity and regeneration processes. In fact, traumatic alteration triggers a strong cellular attempt to restore homeostasis. [64, 69]. In this scenario, the involvement of microRNAs (miRNAs) is not surprising, due to their crucial role in the regulation of almost all genes at the post-transcriptional level [70]. Although their functions in pathology remain to be fully elucidated, miRNAs are known to preside over almost all cellular pathways, including cell cycle [71], cell metabolism [72], apoptosis [73] and immune responses [74]. Specifically, miRNAs are hypothesized to regulate over one-third of human genes [75]. From their discovery, miRNAs have attracted scientific interest: in the clinical field, they have soon been considered as promising diagnostic and prognostic biomarkers for human pathological statuses, from cancer [76] to neurodegenerative diseases [77-79]. MiRNA research holds promise to identify key points of regulation which could become therapeutic targets and ameliorate clinical treatment [80]. Last but not least, circulating miRNAs are of particular interest, not only for their stability and intrinsic advantages related to their sampling, but also for their potential role as cell-to-cell messengers [81]. Concerning the central nervous system, several miRNAs have been demonstrated to be specifically expressed in particular cerebral areas: [82-84] physiological changes in miRNA expression occur during normal cerebral development [85], upon the establishment of neural networks [84, 86]. Furthermore, miRNAs show profound alteration in expression in response to brain pathological states, both traumatic or not. The interest in the relationship between miRNA and CNS injury can be deduced from the wealth of scientific studies demonstrating the alteration of miRNAs after different forms of CNS injury [87-93]. Starting from these premises, miRNAs could be promising biomarkers able to unearth the primary and secondary molecular patterns altered upon TBI. Despite these advantages, the number of research studies on TBI-related circulating miRNAs is scant [91, 94, 95]. Table **1** summarizes the most important reports concerning studies on TBI-related miRNA in animal and clinical studies.

3.1. MicroRNAs: Biogenesis and Function

MicroRNAs represent the most recently discovered class of regulatory molecules that modulate almost all known gene expression [96]. MiRNAs are short (from 18 to 25 nucleotides) RNA molecules which target specific mRNAs through complementary base pairing and impair their translation into proteins [97-99]. MiRNAs are transcribed in the nucleus as long transcripts named pri-miRNAs [100]. Later, they are subjected to a nuclear and a cytoplasmic cleavage, the first being carried out by Drosha RNase III endonuclease, which forms an intermediate stem-loop product called pre-miRNA [97]. At this time, active pre-miRNA transfer to the cytoplasm takes place through exportin-5, and a second cleavage carried out by another ribonuclease, named Dicer, leads to the formation of the mature miRNA, which is approximately 22-nucleotide long [101]. The mature miRNA exerts its function binding to 3'-UTRs of target mRNA and thus inhibiting its translation into protein [102].

3.2. MicroRNAs Expression Profiles in Animal Models of TBI

The development of research on TBI would have been impossible without the use of animal models, which are designed to resemble human pathological counterpart as much as a possible. The ultimate aim is to dissect the plethora of molecular changes occurring in brain-injured patients [103]. Different mechanisms lead to a TBI: Holbourn was the first author to classify a TBI as a "localized injury due to scull distortion" distinguishing from an "injury due to rotation" [104].

In light of this complexity, a classification of the most widely used TBI models is provided below.

a) Focal "Impact Loading" which usually results in focal injury

    1. Weight drop model [105, 106].

    2. Fluid percussion injury model [107-109].

    3. Controlled cortical impact model [110, 111].

4. Missile and ballistic injury models [112, 113].

5. Penetrating TBI model [114].

b) Diffuse "Inertial Loading"

1. Impact

1.1 Inertial acceleration model [115].

1.2 Diffuse injury model [116, 117].

1.3 Impact acceleration model [118].

1.4 Central fluid percussion injury model [108].

2. Non impact

2.1 Inertial acceleration models [119, 120].

2.2 Rotational TBI model [121, 122].

2.3 Blast TBI model.

The diversity of human TBI triggering causes need the development of many animal models which could resemble human counterparts as much as possible. In the work made by Lei and colleagues [89], the characterization of miRNA alterations in the post-TBI rat cerebral cortex was performed. On this purpose, a dozen adult Wistar rats were employed. Specifically, two were kept as controls (called sham-operated group), and each remaining couple was subjected to injury and analysed immediately thereafter (as injury severity controls) and 6, 24, 48 and 72 hours later. The upregulation of miR-21 was reported in all experimental points, letting authors hypothesize a role for this miRNA in the molecular networks triggered by TBI.

Another research group made use of a rat controlled cortical impact injury (CCI) model to dissect miRNA alterations 1- and 7-days post-TBI. In this experimental setup, 60 days-old rats were subjected to CCI or sham-operated and then sacrificed 1 and 7 days later to extract RNA from the dorsal hippocampus. Next-generation sequencing platforms coupled to bioinformatic analysis shed light on miRNA signatures altered after CCI and on the molecular pathways regulated by those miRNAs [123]. Prediction analysis tools were carried out to identify mRNA targets of modulated miRNAs. Specifically, miRNAs which showed an alteration in expression during the acute post-injury phase (1 day after CCI) were predicted to target genes involved in apoptosis, protein folding, and aerobic respiration. On the contrary, those miRNAs altered 7 days after trauma are predicted to target genes related to repair processes.

Rat models were also used to delve into the effect of blast overpressure injury on circulating miRNAs, both from the bloodstream and cerebrospinal fluid [87]. On this purpose, rats underwent multiple blast overpressure exposures (each of 120-kPa). After that, blood was withdrawn at specific time points after trauma and miRNA expression analysis was performed. Besides other alterations in the expression of a panel of serum miRNAs, the authors noticed let-7i be abundant both in serum and in cerebrospinal fluid shortly after treatment (3 hours post-injury). Bioinformatic analysis predicted miR-let-7i to target S100B and UCH−L1, which are TBI-related proteins [124, 125], letting researchers hypothe-

size this miRNA to play a crucial role in TBI. Furthermore, the analysis of cerebrospinal fluid confirmed its potential to support in the future clinical strategies to diagnose TBI.

The reliability of circulating miRNAs as diagnostic tools for TBI was also investigated by Nagaraja and colleagues, which analysed miRNA signatures form serum and amygdala of animal models, concentrating their research on post-traumatic stress, in which fear perception is taken under consideration [126]. After identifying differentially expressed miRNAs in serum, a paired analysis of the same panel of candidates was performed in amygdala. A specific miRNA panel turned out to be upregulated both in the serum and amygdala of animal models 2 weeks after traumatic stress. Specifically, miR-142-5p, miR-19b, miR-1928, miR-223-3p, miR-322-3p, miR-324, miR-421-3p, miR-463-5p and miR-674-3p were identified as the most interesting candidates for further research on biomarkers of a physical brain perturbation status.

Besides other miRNAs, miR-21 was reported to significantly increase the expression in the hippocampus of rodent models after TBI, reaching its maximum 3 days after injury and returning at baseline levels (approximately those of sham controls) after 2 weeks [92]. This is a widely studied miRNA, both for its alteration in expression under pathological conditions [127-129] and the plenty of its predicted targets [130, 131].

In the above-cited study made by Redell, for example, 99 genes were predicted to be potential targets of this miRNA because of the presence of miR-21 binding sites in their untranslated regions. Further analysis of molecular functions of these targets revealed them to belong to enzyme-linked receptor signalling networks, transcriptional regulation and developmental processes. Under physiological conditions, miR-21 is homogeneously expressed in the brain, while its increase in response to stress is exacerbated in the cortex and hippocampus.

Microarray platforms are preferred over real-time PCR assays as they represent a more holistic approach to characterize miRNA signatures showing alterations under injury. As an example, Redell and co-workers made use of this technology to characterize the alteration in expression of 444 miRNAs in the hippocampus of rat models after cortical impact injury at fixed time points (3 and 24 hours) [90]. This analysis led to the identification of 50 downregulated and 35 upregulated hippocampal miRNAs after injury. Further analysis of a restricted panel of miRNAs (miR-107, -130a, -223, -292-5p, -433-3p, -451, -541, and -711) was pursued by use of real-time PCR assays. Prediction analysis revealed that the majority of gene targets to preside over signal transduction, transcriptional regulation, proliferation, and differentiation processes, confirming the crucial importance of these gene regulators in modulating the cellular response to injury.

Liu and co-workers made use of microarrays to follow the time course of miRNA signatures in the hippocampus from rat models after TBI. Among 156 detected miRNAs, 10 were deeply altered in expression 1 hour up to 1 week after trauma. Specifically, miR-144, miR-153 and miR-340-5p

Table 1.   Principal findings on animal and human studies on TBI-related miRNAs.

	Methods and Techniques	Main Findings	Model/ Patient Groups	Matrix	Experimental Setup
Animal Studies					
[89]	microarray with 509 mature miRNA sequences from 3 species (rat, mouse, human)	13 upregulated, 14 downregulated (6h post TBI); 4 upregulated, 23 downregulated (24h post TBI); 16 upregulated, 11 downregulated (48h post TBI); 19upregulated, 5 downregulated (72h post TBI); miR-21 always upregulated	Adult Wistar rats	Cerebral cortex	6, 24, 48 and 72 hours post TBI
[88]	next generation sequencing platform	8 upregulated, 13 downregulated (1 day after CCI); 3 upregulated, 7 downregulated (7 days after CCI)	Sprague Dawley male rats subjected to CCI	Hippocampus	1 and 7 days post TBI
[87]	qRT-PCR	serum let-7i, miR-122, miR-340-5p upregulated both in LII and SII; serum miR-874 downregulated both in LII and SII; miR-200b upregulated in SII. Let-7i elevated also in cerebrospinal fluid	Male Spargue-Dawley rats	Blood and cerebrospinal fluid	SII and LII
[126]	qRT-PCR	miR-142-5p, miR-19b, miR-1928, miR-223-3p, miR-322-3p, miR-324, miR-421-3p, miR-463-5p and miR-674-3p, upregulated in both matrixes	Male albino Sprague Dawley rat	serum and amygdala	14 days PTSD
[92]	qRT-PCR	mir-21 upregulated (3 days after CCI)	Male Sprague-Dawley rats	ipsi- and contralateral hippocampal tissue	3h, 24h, 3 days, 15 days after CCI
[90]	microarray with 444 mature miRNA sequences	11 upregulated (3h after CCI); 8 upregulated (24h post CCI); 16 upregulated (3h and 24h post CCI)	Male Sprague-Dawley rats	hippocampus	3 hr and 24 hr after CCI
[132]	microarray	miR-142-3p, mir-144, mir-340-5p, mir-674-5p, miR-153, mir-186, mir-190, mir-132-5p, mir-138-1-3p upregulated (all time points); let-7b downregulated (all time points)	Adult Sprague-Dawley rats	ipsilateral hippocampus	1h, 1 day, 3 days, 5 days and 7 days post injury
Clinical Studies					
[91]	microarray with 875 mature human miRNA sequences	27 upregulated in severe TBI vs HV; miR-765 upregulated in sTBI vs HV; miR-16, miR-92a downregulated in sTBI vs HV; mir-16 and mir-92a upregulated in patients with mTBI vs HV	Non-trauma HV, Orthopedic injury patients, Orthopedic and mTBI injury subjects, sTBI patients	plasma	24 h after TBI
[133]	qRT-PCR	miR-93, miR-191, and miR-499 upregulated in TBI vs controls (persistently elevated)	76 cases and 38 controls	serum	24 h and daily for up to 21 days after TBI
[7]	microarray with 754 mature human miRNA sequences	miR-184, miR-502, miR-505, miR-301b altered (mTBI+EC, day 1; miR-203, miR-654-3p, miR-655 altered (mTBI+EC, day 15); mir-425-5p altered (mTBI only); mir-21, mir-335 upregulated (sTBI+EC)	HV (30), EC injury patients (30), EC + mild TBI patients (30), EC + severe TBI patients (30)	serum	T0-1h, T4-12h, T48-72h and 2 weeks after TBI
[134]	microarray with 1500 human small noncoding RNAs	mir-671-5p downregulated	9 mTBI and 9 control cases	blood mononuclear cells	
[135]	custom miRNA PCR array	mir142-3p and mir423-3p downregulated over time	3 groups of TBI patients classified on the basis of GCS and WPTAS	plasma	arrival, day 5 and day 30

	Methods and Techniques	Main Findings	Model/ Patient Groups	Matrix	Experimental Setup
		**Clinical Studies**			
[95]	384-well TaqMan Low Density Human Mi-croRNA Array Cards	miR-151-5p, miR-195, miR-20a, miR328, miR-362- 3p, miR30d, miR-451, miR-486, miR-505, miR-92a upregulated (mild to moderate TBI and severe TBI)	sTBI (serum, 8; CSF, 8); mild to moderate TBI (serum, 8); orthopedic injury patients (serum, 7); normal controls (serum, 8; CSF, 6)	CSF and serum	2 days after injury
[137]	96.96 Dynamic Array IFC (Proximity Extension Assay); nCounter Human v3 miRNA Expression Assay Kits	21 miRNAs differentially expressed; miR-27b-3p, let-7i-5p, miR-142-3p, miR-107, miR-135b-5p confirmed in validation cohort. Let-7i-5p confirmed as the best biomarker of mTBI	6 concussed and 6 non-concussed athletes; 22 concussed athletes (dis-covery cohort); 10 matched non-concussed athletes (validation co-hort)	saliva	48–72 h after a concussion
[136]	qRT-PCR	miR425-5p was significantly down-regulated in Group A compared with Group B and HV	15 concussed athletes divided into 2 groups: Group A (n=9, assessed within a week post-concussion); Group B (n=6, assessed over 2 weeks); 8 HV	serum	
[138]	36 bp single end reads on an Illumina NextSeq 500	miR-10b-5p, miR-30b-5p, upregulated in both serum and saliva; miR-3678-3p, miR-455-5p, miR-5694, miR-6809-3p, and miR-92a-3p, downregulated in both serum and saliva	MMA fighters	saliva and serum	1 week or 1 hour prefight, immedi-ately post-fight, 2–3 days, 1 week, 3+ weeks
[139]	NEXTflex Small RNA-Seq Kit v3 (Illu-mina HiSeq 2500)	miR182-5p, miR-221-3p, mir-26b-5p, miR-320c, dpwnregulated; miR-29c-3p, miR-30e-5p, upregulated	pediatric patients sampled within 14 days of initial injury; CSF samples collected from 8 children with sTBI and 2 controls; 61 saliva samples from mTBI cohort and 19 from con-trol cohort	salivar and CSF	day 1, day 4-7, and day 8-17 after injury
[140]	NEXTflex Small RNA Sequencing Kit version 3, Illumina HiSeq 2500	14 differentially expressed miRNAs; miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3p, and miR-1307-3p identified patients with prolonged symptoms	children, 22 acute concussion symp-toms group; 30 prolonged concussion symptoms	saliva	
[141]	Affymetrix GCS300 with FlashTag Biotin HSR	miR-451 only detected in cerebrospinal microparticles from TBI patients; miR-9 more prevalent in cerebrospinal micropar-ticles from controls	26 sampling over a period of 2 years	cerebrospinal microparticles	
[142]	GeneChip miRNA 3,0 Array with 5818 human premature and mature miRNAs	miR-141, miR-572, miR-181a, miR-27b, miR-483-5p, miR-30b, miR-1289, miR-431, miR-193b, miR-499-3p upregulated; miR-1297, miR-33b, miR-933, miR-449b downregulated	comatose patients (26); controls (21)	CSF	
[137]	Human miRNA microar-rays from Agilent Tech-nologies in plasma sam-ples; Agilent miRNA microar-ray system; Gene Cluster (version 3.0) and Java Treeview software pro-grams to visualize the miRNAs	65 miRNAs upregulated and 29 miRNAs downregulated in mild TBI; 33 miRNAs upregulated and 27 miRNAs downregu-lated in moderate TBI; 16 miRNAs upregulated and 6 miRNAs downregulated in severe TBI; 13 miRNAs altered in all TBI groups (hsa-miR-1281, hsa-miR-1304-3p, hsa-miR-1825, hsa-miR-2861, hsa-miR-3195, hsa-miR-328-5p, hsa-miR-3665, hsa-miR-4665-3p, hsa-miR-4669, hsa-miR-4725-5p, hsa-miR-6867-5p, hsa-miR-762 and hsa-miR-940)	90 patients with mild, moderate and severe TBI (15 in the microarray group, 75 in the validation group); 30 Healthy Volunteers (5 in the microar-ray group, 25 in the validation group)	plasma	The sampling times in the mild, moderate, and severe TBI groups were 7.54±2.81, 6.08±0.79, and 6.16±1.19, re-spectively.

Abbreviations: CCI, controlled cortical impact injury; SII, short interval injury group; LII, long interval injury group; PTSD, post-traumatic stress disorder; HV, healthy volunteers; mTBI, mild TBI; sTBI, severe TBI patients; EC, extra-cranial; GCS, Glasgow Coma Scale; WPTAS, Westmead post-traumatic Amnesia scale; CSF, cerebrospinal fluid.

progressively increased in expression after TBI. Western Blot assays confirmed the downregulation of target gene products (calcium/calmodulin-dependent serine protein kinase, nuclear factor erythroid 2-related factor 2 and alpha-synuclein), confirming these miRNAs to be potential targets for therapeutic prevention of TBI-related secondary damages [132].

3.3. Clinical Studies of TBI: Do They Really Benefit from Animal Models?

As stated before, the intrinsic difference between animal and human TBI makes use of animal models challenging. Nonetheless, the scientific efforts made in this field try to progressively lower this discrepancy, in order to translate research findings into therapeutic benefits. Research on miRNAs could help to fill the gap, since miRNAs are widely conserved among species and can behave as minimally invasive biomarkers of disease.

Clinical studies are essentially based on the analysis of circulating miRNAs in patients with TBI [7, 91, 95, 133-136]. The basic aim of clinical studies is the discovery of diagnostic biomarkers of TBI within few hours after trauma. The comparison of miRNA expression levels is made with healthy volunteers. Once validated, circulating miRNA biomarkers could be used in combination with current clinical practices for the diagnosis and treatment of TBI (imaging, neurocognitive and motor examinations) to ameliorate patient classification and therapeutic strategies.

In 2010, Redell and coworkers first identified 108 plasmatic miRNAs in healthy controls, and then searched for alterations of their expression in a group of patients with severe TBI [91] 33 of these miRNAs were downregulated in TBI, whereas 19 were upregulated. Furthermore, the authors found 8 miRNAs to be specifically expressed only in TBI group. They also characterized a panel of 3 miRNAs (miR-16, miR-92a, and miR-765) with diagnostic power for severe TBI within the first 24 h after trauma. The combination of these miRNAs in the Receiver Operating Characteristics Analysis provided the maximum level of specificity and sensitivity. For mild TBI, only miR-16 and miR-92a alterations in expression were reported. Qin and colleagues, in another study, identified a cohort of seven plasma miRNAs that can be used as acute biomarkers of TBI: in particular, miR-3195 and miR-328-5p, may be utilized during diagnosis to distinguish mild and moderate TBI from severe TBI [137].

Numerous studies performed the characterization of miRNAs at different times from injury, to find out interesting candidates for early diagnosis. For example, Yang and coworkers collected sera from 76 severe TBI patients and 38 healthy controls to detect eventual differences in miRNA profiles at different sampling points (from 24 hours to 21 days after injury) by means of real-time PCR assays [133]. MiR-93, miR-191 and miR-499 progressively increased in TBI patients 24 hours up to 7 days after trauma; then, a slight decrease was observed in the 8-14 days interval, although remaining higher than control counterparts.

Another study focussed on circulating miRNAs made use of microarray technology to verify if discrimination between

mild and severe TBI through miRNA profiling was possible. On this purpose, the authors analysed serum miRNA expression profiles of 5 mild TBI with patients experiencing extracranial injury, 5 severe TBI patients and 5 healthy controls 1 day and 2 weeks after trauma [7]. From this first screening, 10 differentially expressed miRNAs were selected for further analysis enlarging patient population (120 patients) and time intervals (T0-1h, T4-12h, T48-72h and 2 weeks after injury) by means of real-time PCR assays. miR-425-5p and miR-502 early decreased in expression after mild TBI, while miR-21 and miR-335 markedly increased in expression in severe TBI. Regarding prognostic information, miR-425-5p was able to predict the patient 6-month outcome at T0-1h and T4-12h and the same holds true for miR-21 at T4-12h.

A miRNA profiling study employing microarray technology was also performed starting from blood mononuclear cells of 9 cases of mild TBI compared with 9 non TBI control cases. MiR-671-5p displayed alterations in expression [134].

Mitra and colleagues withdrew venous blood from 35 adult patients experiencing TBI at arrival and after 5- and 30-days post-injury. MiR-142-3p and miR-423-3p were able to distinguish patients with mild TBI from subjects with concussive symptoms [135].

Bohemia and colleagues explored the diagnostic power of 10 miRNAs (miR-151-5p, miR-195, miR-20a, miR328, miR-362- 3p, miR30d, miR-451, miR-486, miR-505 and miR-92a) in the cerebrospinal fluid and sera from patients experiencing different grades of TBI [95]. Bodily fluids were collected within 2 days after injury. These miRNAs were only able to distinguish mild TBI cases from two non-TBI groups (healthy and orthopedic injury controls).

It is interesting to note that individuals playing impact sports can become a source of miRNAs useful for mild TBI-related research through the sampling of their saliva [138], serum [136] or both [139] after a concussion.

An eventual overlap between salivary and cerebrospinal fluid miRNA profiles in TBI was recently explored by means of RNA sequencing by Hicks and colleagues in pediatric patients with severe (n=8) and mild (n=60) TBI [140]. Six salivary miRNAs (miR-182-5p, miR-221-3p, mir-26b-5p, miR-320c, miR-29c-3p, and miR-30e-5p) were found to be altered in mild TBI. Moreover, this panel showed a similar trend in the cerebrospinal fluid of severe TBI patients. These miRNAs are involved in neuronal development.

Other salivary miRNAs (miR-320c-1, miR-133a-5p, miR-769-5p, let-7a-3c, and miR- 1307-3p) were able to predict risk for prolonged concussion symptoms in a cohort of 52 children with mild TBI [141]. Three other miRNAs (miR-320c-1, miR-629 and let-7b-5p) were associated with specific symptoms such as memory difficulty and headaches after a month.

Another study made use of cerebrospinal fluid to discriminate 11 severe TBI cases from 17 healthy controls [142]. The authors reported the differential expression of miR-9 and miR-451 in CSF-derived microparticles. Further

characterization of these microparticles revealed their main content to consist of non-coding RNAs, confirming the active packaging of cellular mediators to carry out crucial functions at distal body districts.

You and colleagues analysed miRNA expression profiles through microarray platforms in cerebrospinal fluid from 26 comatose patients with severe TBI and 21 controls. 10 miRNAs were increased in expression in TBI patients, whilst 4 were decreased compared to controls. Moreover, a single nucleotide polymorphism (SNP) was discovered in the promoter region of miR-431-3p whose potential interference with transcription factor binding needs to be investigated [143].

4. TBI IN FORENSIC PATHOLOGY: CURRENT STATUS AND NEW DIRECTIONS

From a clinical point of view, traumatic brain injury represents a big challenge for the clinicians in terms of survival and recovery and a proper dating owns a great importance for its evaluation.

Immunohistochemistry currently plays the most important role in TBI diagnosis and evaluation, with β-APP (β-amyloid precursor) and anti-glial fibrillary acid protein (GFAP) are first in the list [144, 145]. Despite this situation, there is the need for a better dissection of the molecular mechanisms involved in TBI pathogenesis, to better understand and treat the resulting damage. On this purpose, oxidative stress leading to apoptosis has been demonstrated to play a crucial role and its characterization holds promise to ameliorate TBI diagnosis [127].

Furthermore, great attention is currently devoted to miRNA research. In fact, these stable and ubiquitous markers have greatly attracted the scientific community and numerous clinical studies demonstrated the potential of miRNAs in the diagnosis and prognosis of almost all human pathological statuses [79, 127, 146-148].

Concerning TBI, the vast majority of scientific studies performed so far on miRNAs investigated the expression of these biomarkers both in animal (cerebral cortex, hippocampus) and human (blood, CSF) tissues [8, 89-91, 94, 130, 132, 135, 140]. Despite these scientific efforts, molecular biology assays did not formally enter in the current practice of forensic pathology. On this purpose, miRNAs could become the ideal candidates considering their intrinsic characteristics: these small endogenous non-coding single-stranded RNA molecules exert important regulatory functions and often present a tissue-specific expression.

To establish the truth, forensic histopathology needs to progressively improve its knowledge at the medical, biological, psychological, investigative, legal, criminal and informatic levels. Molecular biology exerts a great attraction, since it is able to provide detailed information about the molecular changes occurring in disease.

Unfortunately, current methods are often inadequate for a proper diagnosis, thus forensic science could benefit from the use of new technologies more than any other disciplines. In fact, there are many cases where the autopsy is performed with the best standard practise and it is accompanied by a valuable macroscopic and a histological investigation, but it could not provide a definite diagnosis.

In these cases, molecular biology may provide valuable support [149]. With this in mind, we recently explored the diagnostic and prognostic power of selected miRNAs (mir-21, mir-16, mir-92) in diffuse axonal damage, to verify DAI diagnosis and severity performed with current antibody assays for DAI (β-APP, IL-1β, GFAP, NFL, Spectrin II, 8OHdG, TUNEL). MiRNAs were revealed as strong predictor of survival and inflammatory response [79]. MiRNA dysregulation has been reported in almost all physio and pathological conditions, including traumatisms. Traumatic brain and spinal cord injuries (SCI) are the most common causes of disability in young adults. Several studies regarding miRNA expression in these multiple traumas have been conducted [17, 150-153].

CONCLUSION

Although many scientific efforts have been made to improve the current clinical treatment of TBI, many powerful results obtained in animal models did not succeed in clinical studies. This fact could be attributed to the complex pathogenesis of TBI, which involves numerous networks and corresponding mediators. Thus, the characterization of specific biomarkers for brain injury is challenging. Furthermore, one must also consider the absence of specific biomarkers for the central nervous system able to monitor the efficacy of therapeutic treatments, which vary depending on the nature of TBI [154, 155].

Appreciable results were instead obtained in dissecting molecular networks associated with TBI, increasing our knowledge of this intricate pathophysiological scenario. Animal models are used as a testbed for many experimental neuroprotective drugs, which have been also tested in human TBI, as documented by clinical trials [125, 156-158].

Surely, research studies on human TBI must be reinforced by larger cohorts of patients, which would make results more reliable. The need for large-scale randomized studies is also mandatory considering the intrinsic heterogeneity of TBI patients, which increases the risk of biases. In this complex panorama, miRNAs are the most interesting candidates for diagnostic, prognostic and therapeutic approaches.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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