Current State and Prospects of Development of Blood-based Biomarkers for Mild Traumatic Brain Injury

Hyun Haeng Lee, Woo Hyung Lee, Han Gil Seo, Dohyun Han, Youngsoo Kim, Byung-Mo Oh

Highlights

• There are many unresolved clinical problems about mild traumatic brain injury (mTBI) such as a low sensitivity of standard neuroimaging studies, and absence of reliable predicting models.
• It is very difficult to diagnose mTBI in symptomatic patients in the absence of witnesses, clear signs of head trauma, and abnormalities on neuroimaging.
• Blood proteins have great potential as diagnostic and prognostic biomarkers of mTBI.
• Technological advances in the targeted proteomics are expected to realize the clinical potential of blood-based protein biomarkers.
ABSTRACT

The current understanding of the pathophysiology of mild traumatic brain injury (mTBI) is, without doubt, incomplete. Nevertheless, we tried to summarize the state-of-the-art explanation of how the brain is continuously injured even after a single impact. We also reviewed the real struggle of diagnosing mTBI, which culminated in showing the potential of blood-based biomarkers as an alternative or complementary way to overcome this difficulty. Pathophysiology of mTBI is subdivided into primary and secondary injuries. Primary injury is caused by a direct impact on the head and brain. Secondary injury refers to the changes in energy metabolism and protein synthesis/degradation resulting from the biochemical cascades as follows; calcium influx, mitochondrial dysfunction, fractured microtubules, and Wallerian degeneration, neuroinflammation, and toxic proteinopathy. Since the diagnosis of mTBI is made through the initial clinical information, it is difficult and inaccurate to diagnose mTBI without the absence of a witness or sign of head trauma. Blood-based biomarkers are expected to play an important role in diagnosing mTBI and predicting functional outcomes, due to their feasibility and the recent progress of targeted proteomics techniques (i.e., liquid chromatography tandem mass spectrometry [LC-MS/MS]).

Keywords: Biomarkers; Brain Concussion; Blood; Proteomics

INTRODUCTION

Traumatic brain injury (TBI), which is known as a “silent epidemic,” is no longer silent. The report to Congress on TBI in the United States estimated that > 2 million people with TBI in the United States visit the emergency department each year [1]. Although there is no corresponding national statistic yet, according to the “2009 Trauma Statistics” published by the Centers for Disease Control and Prevention (CDC) of Korea, approximately 160,000 people experience TBI in South Korea annually. Mild TBI (mTBI), which accounts for approximately 80%–90% of all the traumatic brain damage, is also called concussion and is reported to occur in approximately 100–300 per million population worldwide [2]. However, considering that many patients with mTBI do not seek medical attention [3], the actual incidence of mTBI should be much more
than 300 per million each year [2], even greater than 3 times the incidence of breast cancer [4]. Although the after-effects of mTBI are often mild, it has tremendous significance in public health. Some researchers report that approximately 44% of the medical expenditures associated with TBI are due to mTBI. The incidence of TBI, especially mTBI, is on the rise owing to an increase in fall cases among the elderly, increased accidents secondary to an increase in leisure sports, and an increase in regional tension and terrorism worldwide [5].

However, studies on the pathophysiology and long-term complications of mTBI are still at the infancy stage. The reasons are varied, but above all, most patients with mTBI appear almost completely recovered within a few months. A considerable number of persons with mTBI experience persistent symptoms after trauma for months, or even years. Patients with sequelae lasting > 1 year account for approximately 15% of all mTBI cases [6]; however, there is no reliable model to predict which patients will continue to have symptoms. Moreover, recent studies have shown that mTBI could be a risk factor for neurodegenerative diseases in the long term; hence, there is an increasing need for a prediction model [7-9]. The fact that it is difficult to assess functional decline and recovery in patients with mTBI also hampers research [10]. There is also a lack of sensitive and feasible tools for the measurements of mild cognitive impairment, decrease in thought speed, and change in behavior, which are the frequent complaints of patients with mTBI. Repeated measurements with typical neurocognitive tests provide the most sensitive results; however, these tests are expensive and require much effort.

In this article, we reviewed the pathophysiology of adult mTBI and the unresolved issues that arise from the clinical course of this condition, and subsequently explored the potentials of blood-based biomarkers in contributing to coping with these challenges. In children, mTBI is very common and involves unique clinical problems that differ from those in adults; however, they are beyond the scope of this review.

**DEFINITION AND DIAGNOSTIC CRITERIA FOR MILD TBI**

Clinical criteria should be used to diagnose mTBI because objective tests such as computed tomography (CT) and blood tests usually do not show any abnormal findings. Although the definition of mTBI is still controversial, the most widely accepted diagnostic criteria are those proposed by the American Congress of Rehabilitation Medicine (ACRM) [11] (Table 1). In short, mTBI is defined in terms of the duration of the initial Glasgow coma

<table>
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<tr>
<th>Diagnostic criteria</th>
<th>A patient with mTBI is a person who has had a traumatically induced physiological disruption of the brain function, as manifested by one or more of the following conditions:</th>
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<td>- Any period of loss of consciousness for up to 30 min</td>
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<td>- Any loss of memory for events immediately before or after the accident for up to 24 hr</td>
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<td>- Any alteration of the mental state at the time of the accident (e.g., feeling dazed, disoriented, or confused)</td>
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<td>- Focal neurological deficit(s) that may or may not be transient</td>
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<th>Exclusion criteria</th>
<th>However, the severity of the injury should not exceed the following conditions:</th>
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<td>- Loss of consciousness exceeding 30 min</td>
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<td>- PTA longer than 24 hr</td>
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<td>- A GCS score that falls below 13 after 30 min</td>
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<td>- Such anomalies should not be due to alcohol, recreational drugs, medications, systemic diseases, or extracranial damage</td>
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<td>- There should be no abnormality on imaging modalities such as CT or MRI (DVA and DoD guidelines)</td>
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mTBI, mild traumatic brain injury; ACRM, American Congress of Rehabilitation Medicine; PTA, post-traumatic amnesia; GCS, Glasgow coma scale; CT, computed tomography; MRI, magnetic resonance imaging; DVA, Department of Veterans Affairs; DoD, Department of Defense.
scale (GCS) score, duration of loss of consciousness, and post-traumatic amnesia (PTA). The
WHO task force team added the exclusion criteria that these abnormalities should not be due
to alcohol or other recreational drugs, medications, systemic diseases, or extracranial injuries
[12]. According to the US Department of Veterans Affairs (DVA) and the Department of Defense
(DoD) guidelines, lesions identified by using structural imaging modalities such as magnetic
resonance imaging (MRI) or CT suggest that the condition is more severe than mild [13].

PATHOPHYSIOLOGY

In mTBI, neurological abnormalities, such as changes in consciousness or memory loss, appear
immediately after the injury and improve within a relatively short period. Symptoms such as
dizziness and impaired concentration mostly disappear within 1–2 weeks [14]. Clinically, the
symptoms improve within 1–2 weeks, but 30–45 days are required for a complete recovery based
on neuropsychological tests [15]. Nevertheless, it does not necessarily mean that the brain
physiology recovers to normal. Many studies on the pathophysiology of brain damage have been
conducted in animal models; however, they considered moderate to severe brain injury, and
hence, not much is known about the pathophysiology of mTBI.

In general, the pathophysiology of brain damage is usually distinguished based on whether
the injury is primary or secondary. A primary injury results from a direct impact to the
head. Primary injuries consist of contact loading and inertial loading, characterized by the
acceleration and rotation of the brain parenchyma in the cranium. Occasionally, an inertial
load alone can cause brain damage without a contact load. A typical example is that of a
passenger wearing a seatbelt, who sustains brain damage during an automobile rollover
without direct impact to the head. Secondary injury is a result of the biochemical cascade
following the primary injury in TBI, due to changes in the energy metabolism and protein
synthesis/degradation [16].

From a neurophysiological point of view, mechanical load can induce a stretching load on the
axons that constitute the white matter, leading to diffuse axonal injury. The pathophysiology
of secondary injury to the axons caused by an excessive stretching load has been described in
many studies (Fig. 1).

Calcium influx
A physical load on the axons is known to cause the elevation of intracellular Ca\(^{2+}\) ions
via multiple pathways. One such pathway is through the inflow of Na\(^+\) via voltage-gated
channels and the subsequent reversal of the Na\(^+/Ca\(^{2+}\) exchanger that results in an increase
in the intracellular Ca\(^{2+}\) concentration [17]. Another pathway of calcium influx is via
“mechanoporation.” Mechanoporation is the term used to describe an opening in the
axolemma caused by a stretch load on the axon [16]. The increase in intracellular calcium
concentration is known to activate calpain, a Ca\(^{2+}\)-activated protease, leading to proteolysis
of structural proteins such as neurofilaments [18]. Calpain is also known to be involved in the
later stages of Wallerian degeneration in the peripheral and central nervous systems [18].

Mitochondrial dysfunction
To compensate for the disturbance in intracellular ionic currents, various membrane ion
pumps are recruited. During this process, glucose metabolism increases, resulting in the
depletion of intracellular adenosine triphosphate (ATP) and the introduction of calcium into
the mitochondria, leading to increased oxidative stress [19,20]. In addition, the influx of calcium triggers the formation of mitochondrial permeability pores on the inner mitochondrial membranes, promoting the migration of molecules (< 1.5 kDa) into the mitochondria. This process ultimately leads to the swelling and death of mitochondria [21,22].

**Fractured microtubules and Wallerian degeneration**

Increased intracellular oxidative stress increases intracellular lactate levels and causes cell edema [23]. Axon swelling is known to cause neurofilament accumulation and microtubule dysfunction and fracture [24]. This results in impaired axonal transport leading to the accumulation of synthesized proteins. Consequently, axonal bulbs are formed as they accumulate at specific sites, and in severe cases, the axons are cut at these sites [25]. This is mainly observed at the cortical-subcortical boundary. Fractured microtubules will be unable to carry nicotinamide mononucleotide adenylyltransferase-2 (NMNAT2), resulting in Wallerian degeneration, mediated by sterile alpha and toll/interleukin-1 receptor (TIR) motif-containing protein 1 (SARM1) proteins via the intracellular signaling pathways [26,27].

**Phagocytosis and neuroinflammation**

Following axonal damage, phosphatidylserine residues on the cell membrane translocate out of the cell. Activated microglia recognize the corresponding residue, the so-called “eat-me” signal, and proceed with phagocytosis [28]. Activated microglia were found to become activated even after the acute phase [29,30]. Ongoing activation of microglia has also been reported post-mortem in patients with TBI [31]. Activated microglia promote inflammation by releasing cytokines and chemokines. Proinflammatory cytokines are known to cause caspase-mediated proteolysis and microglia recruitment [16].
**Toxic proteinopathies and neurodegeneration**

Dysfunction of axonal transport, caused by microtubule disruption, results in the accumulation of amyloid-β precursor protein (APP) [24]. This APP is cleaved into amyloid-β peptides, which aggregate into amyloid plaques, the pathological hallmark finding in Alzheimer’s disease (AD). Tau, a microtubule-stabilizing protein, has been shown to accumulate perivascularly during the disruption of microtubules [32], suggesting that TBI may be associated with neurodegenerative diseases such as AD [16].

The pathophysiological changes in the axons as described above may persist for years after the injury. It has been reported that the neurofibrillary tangles seen in AD are also seen in the brains of boxers and football players who have had repeated concussions [33]. Recent studies have shown that cis-phosphorylated tau protein levels increase throughout the mouse brain after moderate to severe TBI or mTBI. Monoclonal antibodies to cis-phosphorylated tau protein appeared to improve the pathophysiology and behavioral outcome, thus displaying therapeutic potential [34].

**UNRESOLVED CHALLENGES OF MTBI: THE NEED FOR BIOMARKERS**

**Diagnosis**

As mentioned earlier, patients with mTBI do not have any abnormality on standard neuroimaging; hence, the diagnosis is based mainly on the patient’s symptoms and signs and the testimony of witnesses. This reiterates the essential limitation that it is very difficult to diagnose mTBI in patients who present to the emergency department without any objective evidence, such as recorded image data, and in those with confusion or disorientation without any trace of head trauma. The problem becomes more complicated, especially when issues of litigation or secondary gain are involved.

In addition, the diagnostic criteria, including imaging techniques, need more clarification. The criterion of “no abnormality on CT or MR imaging” recommended by the US DV A/DoD guidelines is too ambiguous to be applied in clinical practice because MRI sequences are very diverse and not specified. Microbleeding, which is not clearly detected with traditional methods, may be detected by using modern sequences such as susceptibility-weighted images. It is necessary to modify the diagnostic criteria in light of recent advances in modern medicine with new equipment and imaging techniques being developed rapidly.

There are several other problems associated with the ACRM diagnostic criteria for mTBI. The upper limits of the duration of loss of consciousness or memory loss after trauma are specified as 30 minutes and 24 hours, respectively, with no mention of the lower limits. Consequently, patients who had loss of consciousness for 20 minutes and 10 seconds are both classified into one category. There is also a claim that patients with GCS scores of 13 have a different clinical course from those with scores of 14 and 15 [35]. Further studies are needed to overcome these limitations. Thus, it is difficult to diagnose mTBI in a symptomatic patient in the absence of witnesses, signs of head trauma, and abnormalities on neuroimaging. Despite these difficulties, efforts have been made to accurately diagnose mTBI. Magnetoencephalography (MEG) and electroencephalography (EEG) have been explored as part of such efforts. In a recently published case-control study, it has been found that the control and mTBI groups could be distinguished with 100% accuracy by using MEG [36]; therefore, the application of these
modalities is being actively researched. Additionally, EEG is also being studied as a diagnostic tool for mTBI. Recent studies have shown that the EEG slow wave quantity is a sensitive indicator in the diagnosis and prognosis of mTBI [37]. Recently, blood-based biomarkers that can be measured easily and quantitatively have been recognized as useful in the diagnosis of mTBI. Recent studies have shown glial fibrillary acidic protein (GFAP) and ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) as emerging biomarker candidates for diagnosing TBI [38].

**Prognosis**

A typical mTBI is known to resolve within 24 hours of visiting the emergency department, and symptoms such as headaches, dizziness, loss of concentration, and body and cognitive symptoms disappear within 12 weeks. However, studies have shown that 50% of patients remain symptomatic even after 3 months [39]. In addition, approximately 15% of patients continue to have neurological disorders or symptoms even after 1 year, which is sometimes referred to as post-concussion syndrome (PCS) [40]. Although various studies have been performed to detect PCS subgroups early, there is no reliable clinical model predicting progression to PCS [41]. In addition, a prognostic model with clinical variables for moderate to severe brain injury has been proposed; however, its prediction accuracy is low [42]. Therefore, if a body fluid-based biomarker is developed to predict the prognosis of mTBI, it is expected to help in clinical decision-making. In addition, there are a few predictive models currently available for long-term complications such as chronic traumatic encephalopathy (CTE); however, in the future, biomarkers in blood are expected to be used in making such predictive models.

**BODY FLUID CANDIDATES FOR BIOMARKER RESEARCH IN MTBI**

**Cerebrospinal fluid (CSF)**

Theoretically, CSF is the closest body fluid to the target organ, the brain, and is most likely to reflect the pathophysiology of TBI [14]. However, in practice, there are various associated problems. When the blood-brain barrier (BBB) is damaged along with brain damage, various substances are introduced into the CSF from the blood as well as the brain. Comparing this with signal processing, the substance introduced from the brain to the CSF would be a signal, and the substance introduced from the blood to the CSF would be noise. Hence, the noise in CSF can be increased after TBI.

Another limitation is that CSF is difficult to obtain. In patients with moderate to severe TBI, who are hospitalized and undergo extraventricular drainage (EVD), it is relatively easier to obtain CSF; however, obtaining CSF from mTBI patients is impractical. Patients with mTBI are more likely to refuse to undergo the relatively more invasive lumbar puncture, which can also aggravate headache. In addition, as there are differences in the components of CSF obtained through lumbar puncture and EVD, the interpretation of test results and their application to clinical decision making can be complex.

**Blood**

As blood is the most common body fluid specimen used for various tests in hospitals, it is easy to obtain patient’s consent. Compared with CSF, it is one step more distal to the target organ; however, it has the advantage that the measurement results do not change significantly even with BBB compromise. The fact that reference values for various blood markers are well known is also an advantage [10].
A disadvantage of using blood specimens is that biomarkers released from the brain into the bloodstream are likely to have low levels in blood. The levels of most candidate protein marker levels are in the picogram range ($10^{-12}$ g/mL); hence, the measurement itself is technically challenging. Moreover, to date, the time to detection in blood after the injury is different for each biomarker. For example, GFAP levels in the plasma were reported to increase significantly 8 hours after the injury, whereas markers such as UCH-L1 increase significantly at baseline and then return to normal levels after 12 hours [38]. When the biomarkers are not specific to the brain, blood concentration is vulnerable to “noise” from other organs. For example, s100B levels increase in blood after a brain injury; however, as it is also produced in tissues other than the brain, its blood levels increase even after trauma to other parts of the body without any brain damage [43].

**Urine**

Urine is a body fluid that can ideally be used when the target organ is the kidney. In TBI, as the target organ is the brain, the concentrations of protein markers in urine are likely to show smaller changes than in blood. Changes in the femtogram range ($10^{-15}$ g/mL) are very difficult to measure with traditional immunological methods. In addition, in the research stage, it is necessary to collect a 24-hour urine sample instead of a spot urine sample at a specific time point, and the hassle of obtaining 24-hour urine samples is also a big obstacle.

### BLOOD-BASED BIOMARKERS UNDER RESEARCH

The advantages and disadvantages of individual blood-based biomarkers were summarized (Table 2). Details of the blood-based biomarkers are shown below.

**s100B**

s100B is a calcium-binding protein that exists in astrocytes and Schwann cells. It is known to affect intracellular calcium homeostasis, and is responsible for intracellular signal transduction [44]. Blood and CSF concentrations of s100B increase after trauma,

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<th>Table 2. Potential blood-based biomarkers</th>
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<td><strong>Biomarker</strong></td>
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<tr>
<td>s100B</td>
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<td>UCH-L1</td>
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<td>miRNA</td>
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TBI, traumatic brain injury; CT, computed tomography; mTBI, mild TBI; UCH-L1, ubiquitin carboxy-terminal hydrolase-L1; GCS, Glasgow coma scale; GFAP, glial fibrillary acidic protein; SBDPs, spectrin breakdown products; NSE, neuron-specific enolase; miRNA, micro RNA.
and unlike neuron-specific enolase (NSE), its concentration does not change much in hemolyzed specimens. Therefore, it has been highlighted as a biomarker of brain damage and many studies have been conducted to date [45]. Unfortunately, s100B is also produced by oligodendrocytes, neural progenitor cells, adipocytes, cartilage cells, and tumor tissues such as melanoma, and its levels may also increase after BBB dysfunction [46-49]. Therefore, its concentration increases even in polytrauma without brain damage and may be increased by the exacerbation of systemic conditions such as hemorrhagic shock. Furthermore, it is known that the darker the skin color, the higher the levels of s100B [50]. Therefore, skin color must also be considered when interpreting s100B levels. As s100B is excreted 100% through the kidneys, care must be taken while interpreting s100B levels in patients with renal insufficiency. However, it is known that there are no significant differences in the blood concentrations of s100B in patients with mild to moderate renal dysfunction [51,52].

Several studies have shown the biological half-life of s100B to be 25 minutes to 2 hours, and it is difficult to specify a detection time window due to the relatively short half-life. However, based on the recently published dynamic model of s100B concentration, it was found that the increase in s100B concentration due to brain damage could be specifically confirmed 12–48 hours after the TBI [53]. In addition, the secondary increase of s100B, confirmed via serial sampling, has been shown to reflect secondary brain damage [54-56], increasing the expectations of the clinical use of s100B.

s100B showed a significant difference in concentration according to the degree of brain damage (mild, moderate, and severe TBI) [57,58], and 2 reviews showed a correlation with the GCS scores at admission [59,60]. In addition, a newer version of the Scandinavian CT guidelines published in 2013 may help determine whether brain CT scans are needed for patients with mTBI visiting the emergency department. If the s100B concentration is < 0.10 µg/L, measured within 6 hours from the time of injury, brain CT may not be needed [61]. On the contrary, the predictive power was not sufficiently high for PCS in children [62].

**UCH-L1**

UCH-L1 is a cysteine protease present in neurons and accounts for approximately 2% of the total soluble protein in the brain [63]. Recent studies have shown that UCH-L1 levels rapidly increase in the plasma after brain injury and rapidly decrease subsequently within 36 hours [38]. In a recent study involving mild and moderate TBI, UCH-L1 levels were highly correlated with the GCS scores and the presence of lesions on brain CT [64]. In another prospective study, the UCH-L1 concentration in blood was found to be superior to that of GFAP or s100B in predicting the presence of CT lesions within 6 hours after mild and moderate brain injury [65]. However, a recently published prospective cohort study confirmed that the accuracy of UCH-L1 was lower than that of GFAP in determining the presence of brain damage, presence of lesions on CT, and necessity of neurosurgical intervention [38].

**Spectrin breakdown products (SBDPs)**

Spectrin is a major component of the cytoskeleton, broken down by calpain after brain damage, resulting in 2 αII-spectrin fragments: 150 kDa (SBDP150) and 145 kDa (SBDP145). The αII-spectrin fragments are present in the axons and pre-synaptic terminals of neurons, and caspase-3 again degrades it into 120 kDa (SBDP120) [66]. Although SBDPs reflect the necrosis and apoptosis processes in the brain, they are not specific for brain damage. They also occur in cerebral ischemia, neurodegenerative disease (i.e., AD), and normal aging [67,68]. Increased concentrations of SBDPs occurred within 15 minutes of brain damage and
were found to increase significantly from 3 to 24 hours after brain injury [69,70]. The SBDP concentrations in the CSF are associated with mortality after brain injury [67]. Although techniques to reduce spectrin breakdown by using calpain inhibitors have been introduced, their therapeutic effect on axon survival is yet to be determined in brain injury [71,72].

**NSE**
An enzyme involved in glycolysis, NSE is a marker that reflects apoptosis and is present in the neuron cell body [73]. Unfortunately, hemolyzed samples show false positives [74]. Recent meta-analyses have shown that NSE can be used as an indicator of functional levels and mortality rates after TBI [75].

**GFAP**
GFAP is a protein found only in the central nervous system and constitutes the cytoskeleton of astrocytes. It is known that when astrocytes are damaged, the production of GFAP is greatly increased [76]. It is a marker specific to brain damage because it does not increase after trauma to other parts of the body; however, after TBI, it takes 8 hours for its blood levels to increase [38]. The GFAP blood concentrations in patients with mTBI in the emergency department is better than the s100B blood concentrations in predicting the need for brain CT. Especially, in cases of extracranial injuries, GFAP blood concentrations were also found to have a higher specificity for detecting lesions that could be confirmed with brain CT [77]. In addition, in 2 reviews, the concentrations of GFAP correlated with the initial GCS scores, as with s100B [59,60]. However, the outcome of patients at 6 months after TBI could not be predicted precisely based on the GFAP blood concentrations [45].

**Micro RNA (miRNA)**
miRNA binds to the 3’ untranslated region of messenger RNA (mRNA) and causes degradation of mRNA or inhibits mRNA translation. Even a single miRNA is known to regulate the entire mRNA network [78], and is expected to be used in therapeutics by blocking the pathophysiological cascade after brain injury. In particular, miRNAs are abundantly expressed in the brain tissue, and some are known to have brain-specific functions [79-81]. After brain injury, miRNA expression levels are known to vary significantly [82,83], with the downregulation of miR-23a and miR-27a being confirmed within 4 hours of the brain injury. These changes are believed to be related to the increased expression of proapoptotic Bcl-2 family members (NoxA, Puma, and Bax) [83].

**Other biomarkers**
In addition, several studies have shown that other biomarker candidates, including inflammatory cytokines, amyloid-related markers, phosphorylated tau, TAR DNA-binding protein 43, hormones (steroid and pituitary hormones), neurofilament light chain, and myelin basic protein have the potential for diagnosing mTBI.

**PROSPECTS AND FUTURE CHALLENGES**
Recently, a quantum jump in the technology of protein biomarker development has occurred, which involved the mass spectrometry (MS)-based proteomics workflow based on high-throughput proteomics by using high-performance MS including Orbitrap® liquid chromatography-tandem mass spectrometry (LC-MS/MS; Thermo Fisher Scientific, Waltham, MA, USA) and triple quadrupole LC-MS/MS. Those high-performance technologies
generated huge amount of proteome information. Subsequently, a bioinformatics algorithm was developed for processing large data, which can expedite both the identification and quantitation of proteome data sets. In addition, the tools of systems biology guided the functional annotation among all bits and pieces of proteome data.

However, the importance of clinical knowledge and experience is more emphasized than ever even with the advances in technology because well-designed biorepository and comprehensive functional evaluation is the pivotal requirement for biomarker discovery and validation.

REFERENCES


