ORIGINAL ARTICLE

Identification of RNA-binding Proteins in Spinal Cord Injury: An In-silico Approach

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ABSTRACT

Introduction: Gene expression is regulated by trans-acting factors such as microRNA, and RNA-binding proteins (RBPs). Dysregulation of RNA-binding proteins (RBPs) are found in neurological diseases. However, the role RBPs in spinal cord injury (SCI) have not been identified. The objective of this study was to identify RBPs by re-analyzing RNA-sequencing data from SCI mice model using the latest version of Tuxedo pipeline. **Methods:** Reads from transcriptomic sequence of acute, subacute, and control mice models, from the Sequence Read Archive (SRA) website, were uploaded to a scientific workflow system called usegalaxy.org. The reads were assessed for their quality using FastQC, before they were mapped to the mouse mm10 reference genome using HISAT2. The fragments were then aligned to full-length transcripts using Stringtie, followed by DESeq2 to find differentially expressed genes (false discovery rate of 0.05 and fold change of -1< x >1). Finally, to find functional annotations, the Protein Analysis through Evolutionary Relationship (PANTHER) and g:Profiler were used. **Results:** There were 24 RBP-coding genes identified in the acute injury, and 27 in the subacute injury. Four RBPs that were commonly expressed at high levels in both acute and subacute injury; *Hnrnpm*, *Ptbp3*, *Rbfox3* and *Znf385a*. These proteins regulate alternative splicing, and RNA transport. Other RBP-coding genes with a role in inflammatory response and apoptosis were also discovered. **Conclusion:** Novel RBP-coding genes differentially expressed in SCI were discovered, suggesting their role in the pathophysiology of SCI. These findings contribute to a better understanding of the regulatory mechanisms employed by RBPs in SCI.
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Keywords: New Tuxedo pipeline; RNA binding proteins; RNA sequencing; Spinal cord injury; Transcriptomic analysis

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INTRODUCTION

Spinal cord injury (SCI) is caused by trauma to the spinal cord, altering normal physiological functions, respiration, heart rate, and blood pressure (1). SCI inflict temporary or permanent change in the normal functions of the cord, which may lead to permanent neurological deficits and disability. It is estimated that up to 500,000 people suffer from SCI annually (1). In Malaysia, approximately 292 patients suffering from SCI were admitted to Hospital Kuala Lumpur between 2005 and 2009 (2,3). Majority of SCI patients in Malaysia demonstrated paraplegia, i.e., paralysis of the lower limbs, as their clinical presentation, whilst tetraplegia, i.e., paralysis of the upper and lower limbs, is more common in the United States (4). Common causes of SCI are due to traumatic or non-traumatic injury. Common leading factors of traumatic SCI in Malaysia include motor vehicle accident, falling, and violence-induced (2). There is currently no effective treatment to restore complete function in patients with SCI. One of the contributing factors to lack of effective treatment is due to poor understanding of the cellular mechanisms pertaining injury to the spinal cord.

The pathophysiology of SCI is divided into two: primary and secondary injury. Primary injury occurs when the spinal cord is mechanically compressed, contused, or stretched, as a result of traumatic event (5). Primary mechanical injury most commonly caused by the loss of the spinal column's biomechanical integrity, which results in compressive stresses on the

spinal cord, causing neuronal axons, blood vessels, and cell membranes to be disrupted (6). The site of tissue damage will spread to the surrounding tissues after a few days, which elicit the secondary injury cascades. The area of trauma enlarges significantly during the secondary injury, which consequently initiate glutamate excitotoxicity, cell death, and inflammatory processes (7,8).

The cellular changes to the SCI pathophysiology are due to trans-acting factors, such as microRNA, long non-coding RNA, and RNA Binding Proteins (RBPs), which strictly regulate the gene expression of RNA transcripts. RBPs are proteins that bind to double- and single-stranded RNA for a number of important reasons; i.e., RNA processing, mRNA stability, and mRNA transport (9). In humans, over 2000 RBPs have been identified that interact with RNA transcripts via specific binding domains such as RNA recognition motif (RRM) and Z-fingers (10). The potential application of RBPs as either a therapeutic target or a diagnostic marker is a fast-growing research topic, and the involvement of aberrantly expressed RBPs in SCI is currently unclear. Identification of these RBPs will hopefully elucidate the mechanisms of SCI further, and could be potential targets for therapeutics of SCI.

MATERIALS AND METHODS

Raw Data Processing and Quality Control

In-silico analysis was performed to identify RNA-binding proteins (RBPs) expressed in injured spinal cord. Mouse RNA-Seq data were downloaded from the Short Read Archive (SRA) database for acute injury (SRR789193, SRR789194, and SRR789195), subacute injury (SRR789196, SRR789198, and SRR922121), and sham control (SRR789190, and SRR789191), as published by (7). The FASTQ raw data were uploaded to the Galaxy server, usegalaxy.org (v21.05) (11). Raw reads were assessed for their quality using FastQC (v0.11.8) (https:// www.bioinformatics.babraham.ac.uk/projects/fastqc/) (12).

Sequence Mapping and Assembly

The 100bp reads were mapped to the Mus musculus (mm10) reference genome using splice-aware HISAT2 (v2.1.0) using default parameters (13). A reference annotation of Mus musculus was retrieved from GENCODE on August 2022 (https://www.gencodegenes.org). Mapped reads were assembled into transcripts by Stringtie (v2.2.1) using default parameters (14). Next, StringTie merge was performed to create a non-redundant transcript which further lead to a uniform set of transcripts that consistent across all samples by combining all gene structures discovered in the samples (14).

Read Count

FeatureCount (v2.0.1) was used to count mapped reads according to the genomic landscape, i.e. exon, introns or location on chromosomes (15). The data counts generated by this tool were used to identify differentially expressed genes.

Differential Expression

The DESeq2 (v1.32.0) was used to identify differentially expressed genes (16). It uses Benjamini-Hochberg (BH) method to correct for multiple testing. Differential expressed genes were identified using the following parameter; fold change -1<x>1, and adjusted p-value ≤0.05.

Functional Analysis

Functional analysis of differentially expressed genes was performed using Protein Analysis through Evolutionary Relationship (PANTHER) software (v17.0), and g:Profiler (https: //biit.cs.ut.ee/gprofiler/) (17,18). Both tools were used to identify Gene Ontology (GO)_Biological-process (BP) and (GO)_Molecular functions (MF) enriched genes. Differentially expressed genes encoding for acute and subacute injuries were plotted on Venn diagrams using Biovenn (19).

RESULTS

Quality of Raw Data

Raw reads were analysed for their quality using FastQC software (v0.11.8) (https:// www.bioinformatics.babraham.ac.uk/projects/fastqc/). Phred Score was generated by the FastQC to determine the quality score of each sequencing reaction. On average, the Phred score for all samples are 37 (data not shown), which indicate that the base call accuracy is 99.98004738% accurate. Therefore, the reads from all samples were of excellent quality and does not require trimming.

Accuracy of Mapping/Alignment

Reads were mapped against the mouse reference genome, mm10/GRCm38 (The GENCODE consortium) using HISAT2 software tool (v2.1.0) (13). Result shows that the percentage of alignment for all samples were ranged between 93.41 – 96.93% (Table I), indicating that most of the reads were aligned to the reference genome.

Differentially Expressed Genes

DESeq2 was used to identify differentially expressed genes between acute or subacute injury with control samples, with the following setting: adjusted p value ≤0.05, and fold change -1< x >1. In total, there were 1068 genes differentially expressed in acute injury (upregulated genes = 673, downregulated genes = 395), and 3189 genes in subacute (upregulated genes = 1043,

Table I : Percentage of aligned reads against mouse reference genome (mm10)

Condition	Run Number	Total Number of Reads	Aligned (%)
Sham	SRR789190	53782816	95.88
	SRR789191	61616902	96.50
Acute	SRR789193	60361186	93.99
	SRR789194	56543424	96.29
	SRR789195	58249284	96.01
Subacute	SRR789196	65973562	96.93
	SRR789198	62595276	93.41
	SRR922121	82099258	93.44

Table II : Number of differentially expressed genes in acute and subacute SCI

Injury	Upregulated	Downregulated	Total
Acute	673	395	1068
Subacute	1043	2146	3189

Note: SCI: Spinal Cord Injury.

Table III : Number of differentially expressed RBP-coding genes in acute and subacute SCI

Injury	Upregulated	Downregulated	Total
Acute	5	19	24
Subacute	10	16	26

Note: SCI: Spinal Cord Injury.

Table IV : List of Differentially Expressed Genes Encoding For RNA-Binding Proteins. Acute injury: Upregulated genes (A), Downregulated genes B), Subacute injury: Upregulated genes (C), Downregulated genes (D).

A)

No	Genes	Protein function
1	Heterogeneous nuclear ribonucleoprotein M (HnRNPM)	Pre-mRNA binding protein in vivo, binds avidly to poly(G) and poly(U) RNA homopolymers in vitro. Involved in splicing. Acts as a receptor for carcinoembryonic antigen in Kupffer cells, may initiate a series of signalling events leading to tyrosine phosphorylation of proteins and induction of IL-1 alpha, IL-6, IL-10 and tumor necrosis factor alpha cytokines (By similarity).
2	Polypyrimidine tract-binding protein 3 (Ptbp3)	RNA-binding protein that mediates pre-mRNA alternative splicing regulation. Plays a role in the regulation of cell proliferation, differentiation and migration. Positive regulator of EPO-dependent erythropoiesis. Participates in cell differentiation regulation by repressing tissue-specific exons. Promotes Fas exon 6 skipping. Binds RNA, preferentially to both poly(G) and poly(U) (By similarity).
3	RNA binding protein fox-1 homolog 3 (Rbfox3)	Pre-mRNA alternative splicing regulator. Regulates alternative splicing of RBFOX2 to enhance the production of mRNA species that are targeted for nonsense-mediated decay (NMD).
4	Pre-mRNA-splicing factor RBM22 (Rbm22)	Involved in the first step of pre-mRNA splicing. Binds directly to the internal stem-loop (ISL) domain of the U6 snRNA and to the pre-mRNA intron near the 5' splice site during the activation and catalytic phases of the spliceosome cycle. Involved in both translocations of the nuclear SLU7 to the cytoplasm and the cytosolic calcium-binding protein PDCD6 to the nucleus upon cellular stress responses (By similarity); Belongs to the SLT11 family.
5	Zinc finger protein 385a (Znf385a)	RNA-binding protein that affects the localization and the translation of a subset of mRNA. May play a role in adipogenesis through binding to the 3'-UTR of CEBPA mRNA and regulation of its translation. Targets ITPR1 mRNA to dendrites in Purkinje cells, and may regulate its activity-dependent translation. With ELAVL1, binds the 3'-UTR of p53/TP53 mRNAs to control their nuclear export induced by CDKN2A. Hence, may regulate p53/TP53 expression and mediate in part the CDKN2A anti- proliferative activity. May also bind CCNB1 mRNA. Alternatively, may also regulate apoptosis and cellular response to DNA damage stimulus.

B)

No	Genes	Protein function
1	Calcium-regulated heat stable protein 1 (Carhsp1)	Binds mRNA and regulates the stability of target mRNA.
2	Cytoplasmic polyadenylation element-binding protein 3 (Cpeb3)	Sequence-specific RNA-binding protein which acts as a translational repressor in the basal unstimulated state but, following neuronal stimulation, acts as a translational activator. In contrast to CPEB1, does not bind to the cytoplasmic polyadenylation element (CPE), a uridine-rich sequence element within the mRNA 3'-UTR, but binds to a U- rich loop within a stem-loop structure. Required for the consolidation and maintenance of hippocampal-based long term memory. In the basal state, binds to the mRNA 3'-UTR of the glutamate receptor. Increased Cpeb3, increased apoptosis.
3	Elav like protein 2 (Elavl2)	Seems to recognize a GAAA motif. Can bind to its own 3'-UTR, the FOS 3'-UTR and the ID 3'-UTR. Important for neuronal function and clinically relevant to autism spectrum disorder.
4	ELAV-like protein 4 (Elavl4)	May play a role in neuron-specific RNA processing. Protects CDKN1A mRNA from decay by binding to its 3'-UTR. Binds to AU-rich sequences (AREs) of target mRNAs, including VEGF and FOS mRNA (By similarity).
5	Epithelial splicing regulatory protein 1 (Esrp1)	mRNA splicing factor that regulates the formation of epithelial cell-specific isoforms. Specifically regulates the expression of FGFR2-IIIb, an epithelial cell-specific isoform of FGFR2. Also regulates the splicing of CD44, CTNND1, ENAH, 3 transcripts that undergo changes in splicing during the epithelial-to-mesenchymal transition (EMT). Acts by directly binding specific sequences in mRNAs. Binds the GU-rich sequence motifs in the ISE/ISS-3, a cis-element regulatory region present in the mRNA of FGFR2 (By similarity); Belongs to the ESRP family.
6	Fragile X mental retardation syndrome-related protein 1 (Fxr1)	RNA-binding protein required for embryonic and postnatal development of muscle tissue. May regulate intracellular transport and local translation of certain mRNAs.
7	Fragile x mental retardation, autosomal homolog 2 (Fxr2)	RNA-binding protein; Regulates adult hippocampal neurogenesis.
8	Leucine-rich PPR motif-containing protein (Lrppre)	May play a role in RNA metabolism in both nuclei and mitochondria. In the nucleus binds to HNRPA1-associated poly(A) mRNAs and is part of nmRNP complexes at late stages of mRNA maturation which are possibly associated with nuclear mRNA export. May bind mature mRNA in the nucleus outer membrane. In mitochondria binds to poly(A) mRNA. Plays a role in translation or stability of mitochondrially encoded cytochrome c oxidase (COX) subunits. May be involved in transcription regulation.
9	Putative RNA-binding protein Luc7-like 1 (Luc7l)	May bind to RNA via its Arg/Ser-rich domain. Might be involved in splicing.
10	RNA-binding protein Nova-1 (Nova1)	Functions to regulate alternative splicing in neurons by binding pre-mRNA in a sequence-specific manner to activate exon inclusion. Nova1 KD increases apoptosis under basal condition.
11	Poly(a) binding protein, cytoplasmic 1-like (Pabpc1l)	Binds the poly(A) tail of mRNA. Predicted to act upstream of or within several processes, including mRNA polyadenylation. Predicted to be part of ribonucleoprotein complex. Predicted to be active in cytoplasmic stress granule; cytosol; and nucleus. Orthologous to human PABPC1L (poly(A) binding protein cytoplasmic 1 like); Participates in mRNA decay pathway; RNA degradation pathway; RNA transport pathway.
12	Polyadenylate-binding protein (Pabpc4)	Binds the poly(A) tail of mRNA. t is suggested that PABPC4 might be necessary for regulation of stability of labile mRNA species in activated T cells. PABPC4 may also be involved in the regulation of protein translation in platelets and megakaryocytes or may participate in the binding or stabilization of polyadenylates in platelet dense granules.
13	Embryonic polyadenylate-binding protein 2 (Pabpn1l)	Binds the poly(A) tail of mRNA.
14	RNA-binding protein PNO1 (Pno1)	Positively regulates dimethylation of two adjacent adenosines in the loop of a conserved hairpin near the 3'-end of 18S rRNA.
15	RNA binding protein fox-1 homolog 1 (Rbfox1)	RNA-binding protein that regulates alternative splicing events by binding to 5'-UGCAUGU-3' elements. Prevents binding of U2AF2 to the 3'-splice site. Regulates alternative splicing of tissue-specific exons and of differentially spliced exons during erythropoiesis. It is also important in alternative splicing of genes critical for neuronal development.
16	RNA-binding protein 15 (Rbm15)	RNA-binding protein that acts as a key regulator of N6- methyladenosine (m6A) methylation of RNAs, thereby regulating different processes, such as hematopoietic cell homeostasis, alternative splicing of mRNAs and X chromosome inactivation mediated by Xist RNA.
17	RNA-binding protein 3 (Rbm3)	Cold-inducible mRNA binding protein that enhances global protein synthesis at both physiological and mild hypothermic temperatures. Reduces the relative abundance of microRNAs, when overexpressed. Enhances phosphorylation of translation initiation factors and active polysome formation.
18	RNA-binding protein 41 (Rbm41)	May bind RNA. Predicted to be involved in mRNA splicing via spliceosome.
19	Zinc finger Ran-binding domain-containing protein 2 (Zranb2)	Splice factor required for alternative splicing of TRA2B/SFRS10 transcripts. May interfere with constitutive 5'- splice site selection (By similarity).

downregulated genes = 2146) (Table II). There were genes that were expressed in both acute and subacute injury; upregulated genes: 1162, and downregulated genes: 285 (Fig. 1A and B). Expression of identical genes in both acute and subacute injury suggest that they are involved in the molecular pathology of SCI. Functional analysis showed that the differentially expressed genes, that were highly expressed in both SCI conditions, have ribonucleotide binding function, and were associated with biological, and cellular processes; characteristics associated with RBPs (Fig. 2). In contrast, there were more genes that were found to be downregulated in SCI, which include genes that encode for proteins with the following functions: ion channel activity, and molecular binding functions, which includes ribonucleotide binding; a common ability of RBPs.

Expression of Genes Encoding for RNA-Binding Proteins (RBPs) in Acute and Subacute SCI

To identify differentially expressed genes encoding for RBPs in acute and subacute SCI, gene ontology (GO) analysis was performed using PANTHER software. Results revealed a total of 24 RBP-coding genes were differentially expressed in acute condition, and 26 in subacute injury (Table III). We further identified that in acute injury, the expression of five genes encoding for RBPs were upregulated, and 19 were downregulated. In contrast, subacute injury revealed 10 upregulated-, and 18 downregulated- RBP-

C)

No	Genes	Protein function
1	Heterogeneous nuclear ribonucleoprotein M (Hnrnpm)	Pre-mRNA binding protein in vivo, binds avidly to poly(G) and poly(U) RNA homopolymers in vitro. Involved in splicing. Acts as a receptor for carcinoembryonic antigen in Kupffer cells, may initiate a series of signalling events leading to tyrosine phosphorylation of proteins and induction of IL-1 alpha, IL-6, IL-10 and tumor necrosis factor alpha cytokines (By similarity).
2	Insulin-like growth factor 2 mRNA-binding protein 3 (Igf2bp3)	RNA-binding factor that may recruit target transcripts to cytoplasmic protein-RNA complexes (mRNPs). This transcript 'caging' into mRNPs allows mRNA transport and transient storage. It also modulates the rate and location at which target transcripts encounter the translational apparatus and shields them from endonuclease attacks or microRNA-mediated degradation. Binds to the 3'-UTR of CD44 mRNA and stabilizes it, hence promotes cell adhesion and invadopodia formation (By similarity).
3	Far upstream element-binding protein 2 (Khsrp)	Binds to the dendritic targeting element and may play a role in mRNA trafficking. Part of a ternary complex that binds to the downstream control sequence (DCS) of the pre-mRNA. Mediates exon inclusion in transcripts that are subject to tissue-specific alternative splicing. May activate gene expression. Also involved in degradation of inherently unstable mRNAs that contain AU-rich elements (AREs) in their 3'-UTR, possibly by recruiting degradation machinery.
4	Poly(a) binding protein, cytoplasmic 6 (Pabpc6)	Binds the poly(A) tail of mRNA. Predicted to be active in cytoplasmic stress granule; cytosol; and nucleus.
5	Polypyrimidine tract-binding protein 3 (Ptbp3)	RNA-binding protein that mediates pre-mRNA alternative splicing regulation. Plays a role in the regulation of cell proliferation, differentiation and migration.
6	RNA binding protein fox-1 homolog 3; (Rbfox3)	Pre-mRNA alternative splicing regulator. Regulates alternative splicing of RBFOX2 to enhance the production of mRNA species that are targeted for nonsense-mediated decay (NMD).
7	RNA binding motif protein, X-linked-like-1 (Rbmxl1)	RNA-binding protein which may be involved in pre-mRNA splicing.
8	RNA-binding protein with multiple splicing 2 (Rbpms2)	Contributes to the regulation of smooth muscle cell differentiation and proliferation in the gastrointestinal system. Binds NOG mRNA. Mediates an increase of NOG mRNA levels, and thereby contributes to the negative regulation of the BMP signalling pathway. This promotes reversible dedifferentiation of smooth muscle cells and promotes smooth muscle cell proliferation.
9	Survival motor neuron protein (Smn1)	The SMN complex plays a catalyst role in the assembly of small nuclear ribonucleoproteins (snRNPs), the building blocks of the spliceosome. Thereby, plays an important role in the splicing of cellular pre-mRNAs. Associated with cell viability (decreased Smn1, decreased viability). Loss of SMN1 leads to motor neuron cell death through apoptosis.
10	Zinc finger protein 385a (Znf385a)	RNA-binding protein that affects the localization and the translation of a subset of mRNA. May play a role in adipogenesis through binding to the 3'-UTR of CEBPA mRNA and regulation of its translation. Targets ITPR1 mRNA to dendrites in Purkinje cells, and may regulate its activity-dependent translation. With ELAVL1, binds the 3'-UTR of p53/TP53 mRNAs to control their nuclear export induced by CDKN2A. Hence, may regulate p53/TP53 expression and mediate in part the CDKN2A anti- proliferative activity. May also bind CCNB1 mRNA. Alternatively, may also regulate apoptosis and cellular response to DNA damage stimulus.

D)

No	Genes	Protein function
1	Calcium-regulated heat stable protein 1 (Carhsp1)	Binds mRNA and regulates the stability of target mRNA.
2	Cytoplasmic polyadenylation element-binding protein 3 (Cpeb3)	Sequence-specific RNA-binding protein which acts as a translational repressor in the basal unstimulated state but, following neuronal stimulation, acts as a translational activator. In contrast to CPEB1, does not bind to the cytoplasmic polyadenylation element (CPE), a uridine- rich sequence element within the mRNA 3'-UTR, but binds to a U- rich loop within a stem-loop structure. Required for the consolidation and maintenance of hippocampal-based long term memory. In the basal state, binds to the mRNA 3'-UTR of the glutamate receptor. Increased Cpeb3, increased apoptosis.
3	Elav like protein 2 (Elavl2)	Seems to recognize a GAAA motif. Can bind to its own 3'-UTR, the FOS 3'-UTR and the ID 3'-UTR. Important for neuronal function and clinically relevant to autism spectrum disorder.
4	ELAV-like protein 4 (Elavl4)	May play a role in neuron-specific RNA processing. Protects CDKN1A mRNA from decay by binding to its 3'-UTR. Binds to AU-rich sequences (AREs) of target mRNAs, including VEGF and FOS mRNA (By similarity).
5	Fragile X mental retardation syndrome-related protein 1 (Fxr1)	RNA-binding protein required for embryonic and postnatal development of muscle tissue. May regulate intracellular transport and local translation of certain mRNAs.
6	Fragile x mental retardation, autosomal homolog 2 (Fxr2)	RNA-binding protein; Regulates adult hippocampal neurogenesis.
7	Leucine-rich PPR motif-containing protein (Lrpprc)	May play a role in RNA metabolism in both nuclei and mitochondria. In the nucleus binds to HNRPA1-associated poly(A) mRNAs and is part of mRNP complexes at late stages of mRNA maturation which are possibly associated with nuclear mRNA export. May bind mature mRNA in the nucleus outer membrane. In mitochondria binds to poly(A) mRNA. Plays a role in translation or stability of mitochondrially encoded cytochrome c oxidase (COX) subunits. May be involved in transcription regulation.
8	Putative RNA-binding protein Luc7-like 1 (Luc7l)	May bind to RNA via its Arg/Ser-rich domain. Might be involved in splicing.
9	RNA-binding protein Nova-1 (Nova1)	Functions to regulate alternative splicing in neurons by binding pre-mRNA in a sequence-specific manner to activate exon inclusion. Nova1 KD increases apoptosis under basal condition.
10	Poly(a) binding protein, cytoplasmic 1-like (Pabpc1l)	Binds the poly(A) tail of mRNA. Predicted to act upstream of or within several processes, including epigenetic maintenance of chromatin in transcription-competent conformation; mRNA polyadenylation; and oocyte maturation. Predicted to be part of ribonucleoprotein complex. Predicted to be active in cytoplasmic stress granule; cytosol; and nucleus. Orthologous to human PABPC1L (poly(A) binding protein cytoplasmic 1 like); Participates in mRNA decay pathway; RNA degradation pathway; RNA transport pathway.

11	Polyadenylate-binding protein (Pabpc4)	Binds the poly(A) tail of mRNA. t is suggested that PABPC4 might be necessary for regulation of stability of labile mRNA species in activated T cells. PABPC4 was also identified as an antigen, APP1 (activated-platelet protein-1), expressed on thrombin-activated rabbit platelets. PABPC4 may also be involved in the regulation of protein translation in platelets and megakaryocytes or may participate in the binding or stabilization of polyadenylates in platelet dense granules.
12	Embryonic polyadenylate-binding protein 2 (Pabpn1l)	Binds the poly(A) tail of mRNA.
13	RNA-binding protein PNO1 (Pno1)	Positively regulates dimethylation of two adjacent adenosines in the loop of a conserved hairpin near the 3'-end of 18S rRNA.
14	RNA binding protein fox-1 homolog 1 (Rbfox1)	RNA-binding protein that regulates alternative splicing events by binding to 5'-UGCAUGU-3' elements. Prevents binding of U2AF2 to the 3'-splice site. Regulates alternative splicing of tissue-specific exons and of differentially spliced exons during erythropoiesis. It is also important in alternative splicing of genes critical for neuronal development.
15	RNA binding protein 15 (Rbm15)	RNA-binding protein that acts as a key regulator of N6- methyladenosine (m6A) methylation of RNAs, thereby regulating different processes, such as hematopoietic cell homeostasis, alternative splicing of mRNAs and X chromosome inactivation mediated by Xist RNA.
16	RNA-binding protein 20 (Rbm20)	RNA-binding protein that acts as a regulator of mRNA splicing of a subset of genes involved in cardiac development. Regulates splicing of TTN (Titin).
17	RNA-binding protein 3 (Rbm3)	Cold-inducible mRNA binding protein that enhances global protein synthesis at both physiological and mild hypothermic temperatures. Reduces the relative abundance of microRNAs, when overexpressed. Enhances phosphorylation of translation initiation factors and active polysome formation.
18	RNA-binding protein 41 (Rbm41)	May bind RNA. Predicted to be involved in mRNA splicing via spliceosome.

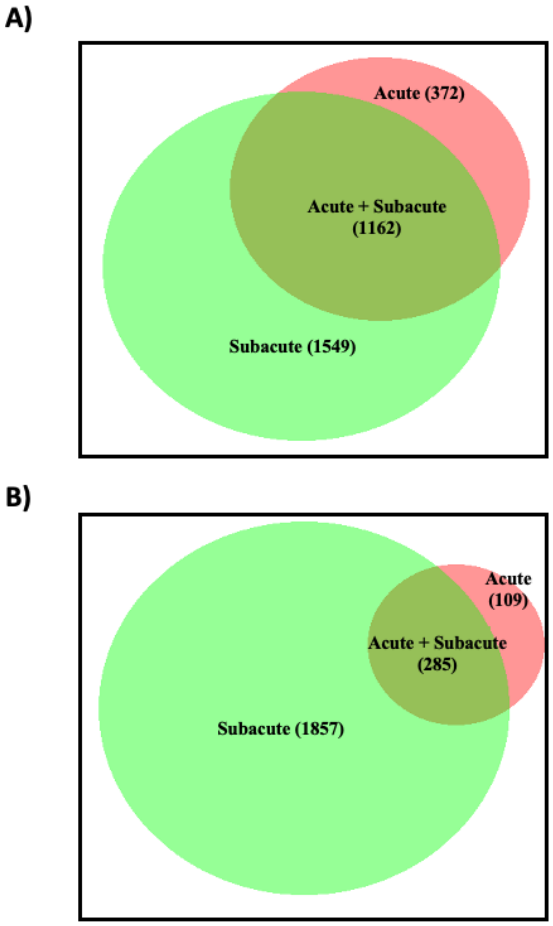
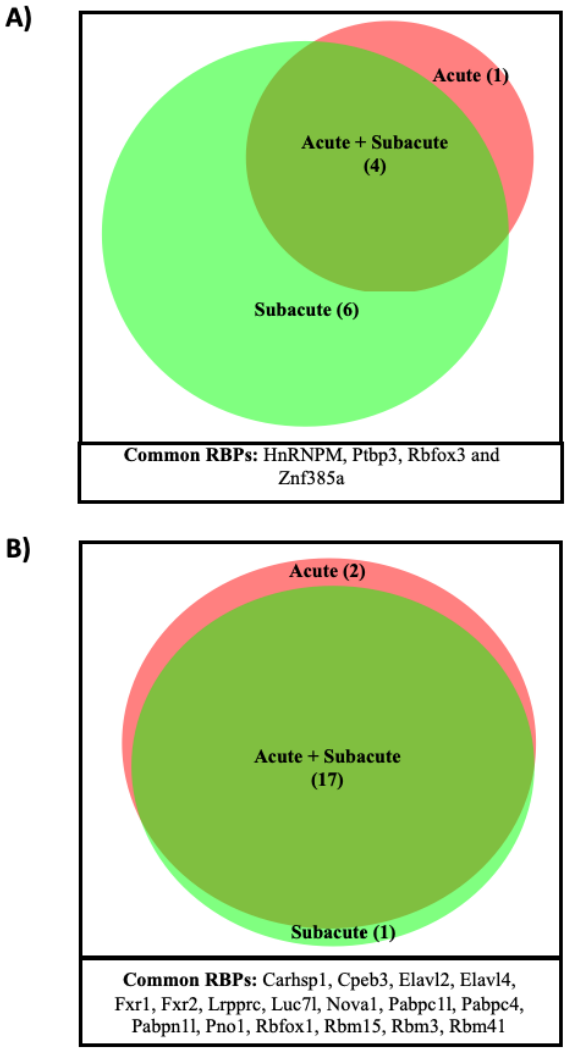


Figure 1 : Differentially expressed genes in acute and subacute spinal cord injury. A) Number of differentially expressed genes (upregulated and downregulated). Comparison of differentially expressed upregulated (B) and downregulated (C) genes between acute, subacute and acute and subacute. Fold change -1<x >1. p-adjusted value ≤ 0.05.

Figure 2 : Identification of Gene Ontology Biological Process (BP) and Molecular Functions (MF) using g:Profiler. Acute SCI: Upregulated genes (A), Downregulated genes (B). Subacute SCI: Upregulated genes (C), Downregulated genes (D). p-adjusted value ≤ 0.05.

coding genes. Four of the five RBP-coding genes that were highly expressed in acute SCI are associated with mRNA splicing. i.e., Heterogeneous nuclear ribonucleoprotein M (*Hnrnpm*), Polypyrimidine tract-binding protein 3 (*Ptbp3*), RNA binding protein fox-1 homolog 3 (*Rbfox3*), and Pre-mRNA-splicing factor RBM22 (*Rbm22*) (Table IV A). The gene encoding for Zinc finger protein 385a (*Znf385a*) was also found to be highly expressed in acute injury. The protein is implicated in the localization and translation of mRNA in the cell. In contrast, there were more RBP-coding genes (n=17) with downregulated expression following acute injury. The genes-coding



proteins are implicated in the following cellular processes: splicing (n=7), translation (n=4), mRNA stability (n=2), mRNA transport (n=2), mRNA decay (n=1), neurogenesis (n=1), and unknown post-transcription function (n=2). Acute injury triggered downregulation of 19 RBP-coding genes (Table IV B). These RBPs have been implicated in the following functions: splicing (n=7), translation (n=4), mRNA stability (n=3), mRNA decay (n=2), transport (n=3), polyadenylation (n=1), neuronal functions (n=1), neurogenesis (n=1), and unknown post-transcription function (n=2).

In subacute SCI, there were 10 RBP-coding genes with elevated expression, that are associated with the following functions: splicing (n=6), mRNA transport (n=1), mRNA degradation (n=1), mRNA localization (n=1), translation (n=1) and unknown post-transcription function (n=1) (Table IV C). Subacute injury resulted in downregulation of RBP-coding genes involved in splicing (n=6), translation (n=4), mRNA stability (n=2), mRNA decay (n=2), polyadenylation (n=1), neurogenesis (n=1), and unknown post-transcription function (n=3) (Table IV D).

Identification of Common RBP-Coding Genes Expression in Acute and Subacute SCI

To identify common RBP-coding genes expressed in acute and subacute injury level, differentially expressed genes were plotted for Venn Diagram. Result shows increased expression of genes coding as follows: i) *Hnrnpm* - acute:1.20-fold, subacute:1.77-fold; ii) Ptbp3 - acute: 1.46-fold, subacute: 2.59-fold; iii) *Rbfox3* - acute: 2.29-fold, subacute: 2.88-fold; and iv) *Znf385a* - acute: 2.73-fold, subacute: 2.72-fold in acute and subacute injury (Fig. 3A). The list of RBP-coding genes that were downregulated in acute and subacute injury were mostly identical, with the exception of three RBPs (Fig. 3B). Loss of function of these common RBP-coding genes suggest that their expression are not essential in cellular response to injury of the spinal cord.

DISCUSSION

Spinal cord injury (SCI) is a serious, incurable condition that has a negative impact on both one's quality of life and society. SCI clinical features are classified into acute, subacute, and chronic phases based on pathophysiological events. The spinal cord injury primarily interferes with motor function defects, which significantly limit normal daily activities, resulting in severe psychological trauma. There are between 250 000 to 500 000 people suffered from SCI annually (1). RBPs are a class of proteins that interacts with RNA, DNA, and other proteins. There are approximately 1542 RBPs in human proteome (20). As RBPs are known to be key regulators in post-transcriptional processes, any alteration to RBP expression and interaction may affect alternative splicing, mRNA transport, mRNA stability and many more. RBP dysregulation is a common hallmark of neurological disorders, such as amyotrophic lateral sclerosis (ALS), frontotemporal lobar dementia (FTLD) (21,22), and mental retardation (23), as well as traumatic brain injury (24). Currently, there is lack of information about the role of RBPs in pathophysiology of SCI.

RBPs in Inflammatory Response of SCI

This study analysed RNA-Seq data from (9) using adapted "New Tuxedo" pipeline method (14). In the original paper, spinal cord tissues of injured mice were isolated two days (acute) and seven days (subacute) post injury before the samples were sequenced to elucidate the global changes of transcriptomic expression following SCI in mice (7). Our transcriptomic analysis revealed four genes-encoding RBPs that were highly expressed in both acute and subacute SCI, which are *Hnrnpm, Ptbp3, Rbfox3* and *Znf385a*. Majority of these RBP-coding genes; i.e., Hnrnpm, Ptbp3 and Rbfox3, have a role in regulating alternative splicing activity. One of known functions

of Hnrnpm protein is that it regulates the inflammatory response via IL-1α, IL-6, IL-10 and tumor necrosis factor -α (25,26). Previous study demonstrated that Hnrnpm repressed the expression of immune genes, which include IL-6, via splicing mechanism (27). Inflammatory response is activated during acute SCI as the injury area is infiltrated by neutrophils following trauma to the spinal cord. Therefore, it is possible that Hnrnpm attempts to limit inflammatory response via repressing the expression of IL-6. The role of Hnrnpm in regulating IL-1α, IL-10 and tumor necrosis factor -α are still unclear.

RBPs in Cell Death of SCI

There are two types of cell death, programmed or non-programmed cell death. Programmed Cell Death (PCD) is activated through intracellular signalling pathway while non-PCD is through unexpected cell injury (28). PCD is further classified into apoptosis and non-apoptotic cell death, while necrosis is a non-PCD. Necrosis occurs due to injury caused by chemical agents, trauma, or pathogens. In response, it triggers inflammatory responses (29). Functional analysis using g:Profiler software has identified an enrichment of upregulated genes involved in cell death related activities in acute and subacute SCI; i.e., regulation of cell death, programmed cell death, and apoptosis process. None of the enriched genes involved in cell death were found to be downregulated. Cell death is one of the pathophysiological changes that occurs during secondary SCI. The causes of neuronal and glial cell death include excessive activation of glutamate receptors, which leads to excitotoxicity in cells (30). Therefore, it is expected that there are enrichment of upregulated genes involved in cell death in both acute and subacute injury.

The list of enriched genes were further dissected to identify RBPs that are implicated in cell death. Functional analysis using PANTHER software has identified four RBP-coding genes, which are Cytoplasmic polyadenylation element-binding protein 3 (Cpeb3), Zinc finger protein 385a (Znf385a), Survival motor neuron protein (Smn1) and RNA-binding protein Nova-1 (Nova1); all of these RBPs are involved in modulating apoptosis. Interestingly, Cpeb3 and Znf385a proteins were differentially expressed in both acute and subacute SCI.

We discovered that the expression of Znf385a has increased following acute (2.73-fold) SCI, compared to control, and subacute injury (2.72-fold), suggesting that the protein might be important in the pathophysiology of the injury, regardless of the injury level. Znf385a is implicated in mRNA localisation and translation by binding to the 3'-UTR of target mRNA. Znf385a binds to p53, which results in apoptosis (31). Therefore, we postulate that upregulation of Znf385a expression increases cell death by apoptosis, a common hallmark of the tissue degeneration in SCI.

In contrast, our findings show that the expression for Cpeb3 were reduced following acute (-1.23-fold) and subacute (-1.84-fold) injury. Cpeb3 protein play an important role as translational regulator in neurons for mRNA transcripts such as glutamate receptors, GluR2 (32). In a study using human samples, CPEB3 knockdown reduced apoptosis via p53 pathway, suggesting that the RBP is important for apoptosis (33). It is unclear as to why *Cpeb3* expression is downregulated during acute and subacute injury as the gene-coding protein activates apoptosis. Further investigation needs to be performed to address this finding.

Smn1 plays an important role in the pre-mRNA splicing (34). Loss of SMN1 resulted in spinal muscular atrophy (35). Previous study shows that Smn1 knockdown increased caspase-3 expression, but did not improve cell viability, thus suggesting that Smn1 confers protection to neurons against injury (35, 36). In this study, the expression of Smn1 was increased in subacute SCI (3.43-fold), which indicate that the protein-coding gene promote protection to neurons during in the excitotoxic cellular environment caused by the injury; i.e., glutamate excitotoxicity and formation of free radicals.

Nova1, a neuron-specific protein, is one of the key players in alternative splicing. It is vital for neuronal viability as Nova1 knockout mice died due to apoptosis in spinal and brainstem neurons (37). Our transcriptomic analysis show that Nova1 expression is downregulated in subacute SCI in-vivo model (-1.21-fold). This correlates with the previous studies on the pathophysiology of SCI, which suggest that apoptosis occurs in the subacute level (38,39). It is possible that downregulation of Nova1 expression further exacerbate apoptotic cell death in the injured spinal cord.

Using RBPs as novel therapeutic strategy

SCI causes irreversible damage to the spinal cord tissue, which could ultimately cause paralysis. Despite the high prevalence, there is lack of effective treatment for SCI (1). There are a number of pharmacological treatments, such as methylprednisolone and pregabalin, however, they failed to restore the normal physiological function of SCI patients (40,41). One of the factors contributing to the lack of success at identifying effective treatment is due to the complexity of the cellular mechanisms within the SCI, i.e. primary and secondary injury. The primary injury is caused by initial impact of trauma to the spinal cord, which leads to cell death and loss of tissue at the local

injury site. Ongoing injury leads to the secondary injury which is caused by plethora of changes at biochemical and cellular processes, such as activation of inflammation (42) and oxidative stress (43), thus propagating more cells to die which leads to degeneration of spinal cord tissues. Many of the current research into SCI are focusing on treatment to repair the injured spinal cord, as well treatment that can limit the severity of SCI. Therefore, it is important to identify novel therapeutic strategies that can protect cells from dying, thus conserving the normal physiological function of spinal cord.

In this study, we have identified two potential molecular therapeutic strategies to enhance cell survival. The first therapeutic strategy is by reducing the inflammatory response, which would limit the impact of injury. We have identified one RBP-coding gene that was highly expressed in both acute and subacute injury, which is Hnrnpm (1.20-fold in acute injury, 1.77-fold in subacute injury). The role Hnrnpm protein is to repress the expression of genes involved in the immune system, suggesting that this RBP is vital in preventing/limiting the inflammatory response following injury (27). Therefore, Hnrnpm might be a feasible pharmacological target since boosting its expression after SCI could decrease inflammation, thus avoiding loss of spinal cord tissue.

The second potential therapeutic strategy is to limit the activation of cell death in order to preserve the spinal cord's physiological function. We have identified a few RBP-coding genes with the role in regulating apoptosis; Znf385a (2.73-fold in acute injury, 2.72-fold in subacute injury), Smn1 (3.43-fold in subacute injury), and Nova1 (-1.21-fold in subacute injury). Znf385a protein has been reported to promote apoptosis by interacting with p53 (31), therefore, decreasing Znf385a expression may boost cell survival. Furthermore, Smn1 and Nova1 are critical for cell viability, because Smn1 regulates the expression of caspase-3, one of the primary regulators of apoptosis (35, 36), while Nova1 downregulates the activation of apoptosis (37). As a result, we suggest that cell viability can be improved/maintained by adding exogenous expression of either Smn1 or Nova1 after SCI,.

Despite the potential of RBPs used as molecular therapeutic strategy for SCI, cellular activities are governed by a complicated network of protein-RNA interactions. To maintain homeostasis, cells frequently adopt compensating mechanisms via positive- or negative-feedback loops. As a result, increasing or decreasing RBP expression may or may not alter cellular function.

CONCLUSION

Using transcriptomic analysis, we have identified RNA-binding proteins (RBPs) in acute and subacute spinal cord injury (SCI). There were 24 RBPs expressed during acute injury, and 27 RBPs in subacute condition. From these numbers, four RBP-coding gene were highly expressed in both acute and subacute injury; Heterogenous nuclear ribonucleoprotein M (Hnrnpm), Polypyrimidine tract-binding protein 3 (Ptb3), RNA-binding protein fox-1 (Rbfox1) and Zinc finger protein 385a (Znf385a). Furthermore, RBP-coding genes implicated in inflammation and cell death were identified following acute and subacute injury, suggesting that they play essential role in pathophysiology of SCI.

There are several limitations to this study. The first limitation is the number of biological replicates for the control (sham) condition. Although two replicates are acceptable for RNA-sequencing study, however, the effect size (also known as fold-change) is directly proportionate on the number of biological replicates (44). By increasing the number of replicates, it would increase the gene count, resulting in better statistical significance for low expressed genes. It is possible that by increasing the number of sham samples, more differentially expressed genes could be identified. The second limitation is that this analysis did not investigate the expression of different transcript isoforms belonging to the same gene. Transcript isoform form by alternative splicing activity, which include or exclude certain exon(s). It is possible that different isoforms of RBPs play an important role in the pathophysiology of SCI.

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