Analysis m6A-related genes of peripheral white blood cell in spinal cord injury as potential targets for prognosis and treatment

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Research Article

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Abstract

Spinal cord injury (SCI) is a destructive neurological and pathological state that causes major motor, sensory and autonomic dysfunctions. N6-methyladenosine (m6A) is a reversible RNA modification and has been implicated in various biological processes. However, there are few studies on m6A in SCI. This project mainly explores the prognostic value of m6A-related genes as potential biomarkers in SCI, in order to establish a set of accurate diagnosis and prognostic prediction model. In this study, we used GSE151371 dataset in GEO database including 38 SCI and 10 healthy samples to screen out 289 SCI related candidate genes and 5 m6A-related candidate genes with high SCI correlation and high differential expression through differentially expressed gene analysis, WGCNA and m6A correlation analysis. These genes are involved in inflammatory response, negative regulation of immune system process and molecular function of carbohydrate binding, iron ion binding, organic acid binding, long-chain fatty acid binding and so on. Furthermore, the key genes were evaluated through a protein-protein interaction (PPI) network combined with a co-expression network. The genes associated with the prognosis of SCI were screened out by MRI related analysis. The comparatively higher expression of PPARG in WBC correlates with worse prognosis in SCI patients, while lower expression of AK5 predicts worse prognosis. Our integrated analysis illustrated the hub genes involved in SCI, shedding light on the development of prognostic markers. Further understanding of the function of the identified SCI hub genes could provide deep insights into the molecular mechanisms of SCI.

Introduction

Spinal cord injury (SCI) is a pathological and neurological condition associated with severe motor, sensory, and autonomic dysfunctions\(^1\). A cascade of damaging processes, including ischemia, oxidative stress, inflammatory events, apoptotic pathways, and locomotor dysfunctions, are included in its pathogenesis, which has acute and chronic stages\(^2\).

Depending on their phenotype, macrophages and microglia can have either positive or negative consequences following spinal cord injury. In addition to playing a crucial part in inflammatory reactions, infections, apoptotic cells, and tissue debris are all recognized, engulfed, and degraded by macrophages and microglia. By eliminating inhibiting myelin components and cellular detritus, they encourage remyelination and axonal regrowth\(^3\). However, the lack of an effective method for cholesterol clearance in the central nervous system leads to an excessive accumulation of myelin-derived cholesterol, which makes it more difficult to recover from spinal cord injury\(^4\). The formation of foamy macrophages is a result of the excessive intracellular presence of lipids and dysregulated intracellular lipid homeostasis. They develop a pro-inflammatory phenotype which can contribute to additional neurologic decline\(^5\).

RNA modifications have recently attracted attention, which are essential for development and regeneration. The m6A modification is the most abundant RNA base methylations. M6A methylation levels are finely regulated by interaction among m6A methyltransferase (writers), demethylase (erasers), and binding proteins (readers)\(^6\). However, the changes in m6A modifications in WBC of SCI have not
been reported yet. We identified the gene and m6a expression changes in WBC which may provide new strategies for SCI.

Systematic analysis of the SCI gene signature to identify novel biomarkers of SCI is necessary. It not only benefits SCI prognosis but also provides novel drug targets for SCI treatment in the future. Gene co-expression network analysis has recently been employed to identify candidate genes associated with SCI. To connectivity among different candidate genes can also be evaluated by using weighted gene co-expression network analysis (WGCNA). Based on a microarray dataset (GSE151371) extracted from the Gene Expression Omnibus (GEO) database, we constructed a gene co-expression network as well as m6A correlation to screen candidate genes involved in SCI.

Materials and methods

2.1 Data collection and processing

First, we searched the GEO database (https://www.ncbi.nlm.nih.gov/geo/) for keywords such as “spinal cord injury”, “white blood cell”, “blood”, etc. Then we eventually included the GSE1513171 datasets into the study, which was used as the experimental dataset. The GSE151371 dataset contains the gene expression profiles of peripheral white blood cell (WBC) and their corresponding clinical data of 38 SCI patients and 10 healthy people. The probes were converted to corresponding gene symbols by referring to the annotation information of the GPL20301 [Illumina HiSeq 4000 (Homo sapiens)] platform.

2.2 Construction of weighted gene co-expression network analysis

In order to explore the modules and genes related to the clinical characteristics of healthy people and SCI patients, the data of GSE151371 were analyzed by using the WGCNA package of R language, and the samples were clustered. In order to ensure the reliability of the results, we analyzed the samples and removed the samples that were not clustered, that is, the outlier samples. In order to ensure that the network conforms to the scale-free network distribution, the “pick Soft Threshold” function in the WGCNA package is used to calculate the correlation coefficient of β value and the mean of gene connectivity, and the appropriate soft threshold is selected to make the network conform to the standard of scale-free network. Then, the modules were clustered with a minimum cluster of 30 genes and a cut height of 0.5. Finally, the gene significance (GS) and module membership (MM) were calculated and correlated with clinical traits. The two modules with the highest correlation with IPF were selected, and the genes in the modules were further analyzed. Genes in the co-expression module have high connectivity and genes in the same module may have similar biological functions.

2.3 DEG analysis

Principal component analysis (PCA) was used to determine the significant difference dimensions with P<0.05. Using R language (R) 4.0.3 limma package to analyze the gene differences between the gene expression matrix of peripheral blood monocytes of healthy people and SCI patients. Set the screening
criteria as $|\log_{2}FC|>2$, $P<0.05$. (Correction method is FDR)\textsuperscript{10}. The up-and down-regulated genes were represented by mapping volcanoes.

2.4 Screening of differentially expressed genes associated highly with idiopathic pulmonary fibrosis

The common genes obtained by WGCNA analysis and DEG analysis were defined as IPF highly correlated differential genes. Use the Venn diagram to show all the differentially expressed genes associated highly with SCI.

2.5 Gene function and pathway enrichment analysis

The online website DAVID (https://david.ncifcrf.gov/) was used to analyze the module function and pathway enrichment of m6A-related candidate genes to further explore the biological functions of these genes\textsuperscript{11}. GO analysis was used to annotate the functions of genes and their products in three aspects, including biological process (BP), molecular function (MF) and cellular component (CC). KEGG database is a collection of information about genes, proteins, chemical components and their interactions, reactions and relationship networks to annotate gene functions and metabolic pathways. We uploaded all identified genes to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database to construct a PPI network\textsuperscript{12}.

2.6 Identification of m6A-related candidate genes

The cor () and cor. test () functions of R language were used to calculate the correlation between the expression levels of 23 m6A regulators (METTL3, METTL14, METTL16, WTAP, VIRMA, ZC3H13, RBM15, RBM15B, YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, HNRNPC, FMR1, LRPPRC, HNRNPA2B1, IGFBP1, IGFBP2, IGFBP3, RBMX, FTO, ALKBH5) and the expression levels of IPF highly correlated differential genes and calculate the p value\textsuperscript{6}. The genes significantly associated with either m6A regulator ($|\text{Pearson R}|>0.7$ and $P<0.05$) was defined as candidate genes related to m6A\textsuperscript{13}.

2.7 Patients and clinical data

We recruited 10 patients in the First Affiliated Hospital of Nanjing Medical University with SCI caused by acute trauma between 2022 and 2023. All the patients were neurologically assessed by a certified Orthopedic Surgeons. Neurological assessment, including sensory, motor and reflex examinations below the injured segment, was graded according to the American Spinal Injury Association (ASIA) Impairment scale\textsuperscript{14}. The inclusion criteria were: (1) trauma-induced acute SCI between C2 and L1; (2) obvious symptoms of paraplegia or quadriplegia, assessed as ASIA grades A–C; (3) according to the Glasgow Coma scale, eye-opening had to be 4 and verbal response 6; (4) age $>18$ years; (5) abnormal spinal cord signal detected by magnetic resonance imaging (MRI); and (6) completed effective and reliable neurological function tests. The exclusion criteria were: (1) ASIA scores D and E; (2) MRI showed no definite changes in spinal cord signaling; (3) combination with severe craniocerebral injury and
intracranial hypertension; and (4) history of tumor or autoimmune disease. All patients provided informed consent and third-party assent was not allowed.

Ten patients undergoing intrathecal anesthesia (with non-neurological and neoplastic diseases) were recruited as controls. The control group was aged >18 years and had no obvious abnormality after neurological evaluation. Nervous system diseases, tumors, rheumatic diseases, and fractures were excluded. All patients provided informed consent and third-party assent was not allowed. The study protocols were approved by ethics committees at the First Affiliated Hospital of Nanjing Medical University (2022-SR-697). The patients' whole blood was collected at the 24-48 hours after spinal cord injury. 2 ml of blood was drawn each time, and the WBC was collected by adding red blood cell lysate after centrifugation at 3000 g for 5 minutes.

2.8 RNA isolation and real-time PCR

For expression analysis, WBC of spinal cord injury patients was isolated by Red Blood Cell Lysis Buffer (BL503A, Biosharp). Total RNA was isolated according to the manufacturer's manual (R6834-02, omega), and the RNA concentration and quality were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). After cDNA synthesis with a PrimeScript RT Master Mix (R222, Vazyme), real-time PCR was performed using SYBR qPCR Master Mix (Q341, Vazyme) on a QuantStudio 7 Flex real-time PCR system (Thermo Fisher Scientific). All the primers used are listed in Table 1. Using the $\Delta \Delta Ct$ method, the expression levels of target genes were calculated (GAPDH as a housekeeping gene, healthy control group as reference samples).

2.9 Magnetic resonance imaging (MRI)

All patients with SCI underwent MRI to determine the extent of the injury. Maximum canal compromise (MCC) and maximum spinal cord compression (MSCC) were used for the measurement of spinal cord compression\textsuperscript{15} (Fig. 2G). Mid sagittal T2-weighted MRI images were used to determine MCC and MSCC. MCC and MSCC were calculated as follows: MCC=\([(D_a+D_b)/2−D_i)/(D_a+D_b)/2\times100;\) MSCC=\([(d_a+d_b)/2−d_i)/(d_a+d_b)/2\times100.\) $D_i$=midsagittal diameter of the spinal canal at the site of injury; $D_a, D_b$=the diameter of the spinal canal above/below the level of spinal injury; $d_i$ =the diameter of the spinal cord at the level of SCI; $d_a, d_b$=the diameter of the spinal cord at a normal segment above/below the level of SCI\textsuperscript{16}. By measuring the vertebral heights at the upper and lower stages of the injured area, the relative length of SCIs was normalized. The genes significantly associated with MCC and MSCC ($|\text{Pearson } R|>0.5$ and $P<0.05$) was defined as candidate genes related to prognosis.

2.10 Statistical analysis

In this study, the R (version 4.2.3) and R Studio software were utilized to carry out the statistical analysis and figure preparation. $P$-values less than 0.05 were defined as statistically significant.

Results
3.1 WGCNA module construction and selection of modules with high correlation with idiopathic pulmonary fibrosis

WGCNA analysis was performed using gene expression matrix. After setting the high degree, 1 outlier samples (GSM4576310, Healthy Control 3) were removed (Figure 2A). Finally, 38 SCI samples and 9 normal samples were analyzed later (Figure 2B). When the scale-free topological fitting index R2 reaches 0.9, the appropriate β value is chosen as 16 (Figure 3A). The dynamic clipping tree algorithm was provided to segment the modules and construct the network diagram. Cluster analysis was performed on the modules and the modules with similarity greater than 0.5 were merged into new modules, in which the minimum module had 30 genes and the clipping height was 0.5 (Figure 3B). On this basis, the WGCNA method based on sequence free network was used to modularize genes, and the topological overlap matrix between all genes was described by heat map, and the relationship between sample features and modules was analyzed. The colors corresponding to the modules are blue, brown, turquoise and yellow. BCL11B, UGT2B28, POLE2 and FCAR were identified as the key genes in each module by choose Top Hub In Each Module function. Among them, the grey module is the gene that cannot be clustered to other modules, so it will not be analyzed in the subsequent analysis (Figure 3C).

Key modules were identified according to the correlation coefficient between module features and traits, in which the yellow module had the highest positive correlation (cor=0.65, p<6.8e-81), and the brown module had the highest negative correlation (cor=0.81, p<1e-200), and finally determined that the yellow module and the brown module were the two modules with the highest degree of SCI correlation. A scatter plot was used to represent the correlation between yellow or brown modules and SCI, and a total of 3311 genes were found (Figure 3D, E).

3.2 The differentially expressed genes between idiopathic pulmonary fibrosis samples and normal samples were screened

Principal component analysis (PCA) showed that the 2 groups were clearly distinguishable from each other (Figure 3F). Using the limma package in R language to screen differentially expressed genes, based on |log2FC|>2 and \(P<0.05\) as the threshold, the differential genes in the SCI patients and healthy population samples in the GSE151371 dataset were screened. A total of 769 differentially expressed genes were found, of which 602 genes were up-regulated and 167 were down-regulated. The results of differentially expressed genes were used to construct a volcano plot, where red represents up-regulated genes, blue represents down-regulated genes, and grey represents genes defined as non-differential (Figure 4A).

3.3 Screening of SCI highly correlated differentially expressed genes

There are 747 genes in yellow and 2564 genes in brown modules obtained by WGCNA analysis were highly correlated with SCI, and the 769 genes obtained by DEG analysis were significantly different. There were 228 differentially expressed genes in the yellow module and 61 differentially expressed genes in the brown module. Therefore, a total of 289 genes were obtained by taking the intersection of the two genes through Venn diagram, and these genes were defined as SCI highly correlated differentially expressed genes (Figure 4B). Among BCL11B, UGT2B28, POLE2 and FCAR, only FCAR was significantly
differentially expressed. Among m6A regulators only IGFBP2 in brown module was significantly
differentially expressed.

3.4 The enrichment analysis of the SCI highly correlated differentially expressed genes.

The results showed that the candidate genes mainly focused on the biological process of regulation of
inflammatory response, negative regulation of immune system process and molecular function of
carbohydrate binding, iron ion binding, organic acid binding, long-chain fatty acid binding and so on
(Figure 4C, D). GSEA show that these genes are mainly concentrated in glycerophospholipid metabolism,
autophagy and pathway of spinal cord injury, adipogenesis and transcription factor regulation in
adipogenesis (Figure 4E). The relationship between lipid metabolism-related genes and m6A regulatory
proteins was further analyzed by protein-protein interaction (PPI) (figure 4F).

3.5 Screening and enrichment analysis of m6A-related candidate genes

Lacking of IGFBP1 expression data, the correlation between the expression levels of 22 m6A regulators
were calculated (Figure 5A). Pearson correlation analysis was used to screen out 5 candidate genes
(IMMP2L, PKIA, AK5, GALK1, WFDC1) related to m6A from SCI highly correlated differentially expressed
genes (|Pearson R|>0.7, P< 0.05) (Figure 5B).

3.6 Validating the correlation between expression level and prognosis of m6A-related candidate genes.

The clinical blood samples were used to validate the genes (IMMP2L, PKIA, AK5, GALK1, WFDC1, PPARG,
FABP2, IGFBP2) expression by rt-qPCR. (Figures 6A). We assessed maximum canal compromise (MCC)
and maximum spinal cord compression (MSCC) by MRI (Fig.6B). In the correlation analysis of with genes
and MRI characteristic, the WBC level of AK5 (cor=-0.656, p=0.0396) significantly correlated with MCC
and PPARG (cor=0.84, p=0.0022) significantly correlated with MSCC (Figures 6C-J). These results
reflected that AK5 and PPARG were related to the degree and prognosis of spinal cord compression.

Discussion

Spinal cord injury is a traumatic disease, and it is difficult to accurately judge the prognosis of the
disease.

In this study, we downloaded GSE151371 dataset from GEO database, which included peripheral white
blood cell gene expression profiles of 38 SCI and 10 healthy samples, and analyzed the obtained data. The
gene expression matrix was used for differential gene analysis, and 602 up-regulated genes and 167
down-regulated genes were screened. The correlation between each module and the trait was obtained by
WGCNA analysis combined with correlation heat map. The yellow and brown modules with the highest
positive and negative correlations were selected, and 289 intersection genes were obtained by
intersection of the DEG and the module genes with the highest correlation in the selected WGCNA. Then, 5
m6A-related candidate genes were screened out from the 289 SCI highly correlated differentially
expressed genes by Pearson correlation analysis, and the enrichment analysis of these genes showed that the above genes were mainly enriched in long-chain fatty acid binding, transcription factor regulation in adipogenesis and other pathways.

Myelin contains extensively a large amounts of lipids (80% of dry weight)\textsuperscript{18}. To prevent peroxidation of myelin-derived lipids at the lesion site, macrophages must be able to take up excessive amounts of myelin debris after SCI\textsuperscript{5}. However, following phagocytosis of myelin debris in SCI, lipids persist in macrophages as a result of dysregulated mechanisms of lipid autophagy or efflux\textsuperscript{19}. The formation of foamy macrophages is the result of the excessive intracellular presence of lipids, which leads to a dysregulated intracellular lipid homeostasis. It is common for these foam cells to be observed a week after SCI.

Macrophages are considered to be among the most important cells involved in the clearance of myelin fragments\textsuperscript{20}. It has been shown that CD68+/CD163- macrophages continue to phagocytose myelin at the lesion site beyond 16 weeks post-injury in a SCI model\textsuperscript{21,22}.

Foam cells are formed due to a dysregulated lipid homeostasis when myelin debris is taken for a long time. This will initiate cytokine release, leading to neuronal apoptosis. By directly or indirectly stimulating lipid efflux transporters ABCA1 and ABCG1, PPARG can prevent foam cell formation and promote remyelination through reversed cholesterol transport into the injured spinal cord.

After SCI, PPARG, ABCA1 and ABCG1 expression is significantly increased in the lesion site and this improved lipid efflux, leading to a reduction in foam cell formation. The high expression of these genes can significantly promote lipid efflux, which has an important protective effect on cells. Importantly, the effect was extrapolated to an acute in vivo SCI model and was associated with an improvement in functional outcomes, emphasizing ABCA1 and ABCG1 as promising targets for SCI treatment.

Additionally, activation of the peroxisome proliferator-activator receptor (PPARG), an inducer of the ABCA1 expression, has been shown to reduce the formation of foam cells through stimulation of intracellular lipid efflux\textsuperscript{23}. In the same way, atorvastatin may reduce injury and improve recovery after SCI by activating PPARG and, consequently, ABCA1\textsuperscript{24}. Thus, the indirect up regulation of ABCA1 via PPARG activation could make PPARG a promising therapeutic target for stimulating lipid efflux of foamy macrophages in SCI\textsuperscript{25,26}.

Targeting the phagocytic process can have a positive impact on SCI recovery through a variety of tactics. However, one must also consider that stimulating phagocytic receptors could eventually turn macrophages into foam cells, which could then negatively impact functional recovery after SCI\textsuperscript{27}. In agreement, the genetic deletion of CD36 led to a reduction in lipid uptake and an increase in functional recovery in SCI mice. Therefore, myelin clearance alone is not always the best approach, as prolonged clearance can lead to foam cells formation and a corresponding harmful, pro-inflammatory cytokine storm. Targeting foam cell formation processes with the stimulation of lipid efflux transporters ABCA1
and ABCG1 directly or indirectly via PPARG is our recommended approach. In particular, stimulating reversed cholesterol transport has been proven to be sufficient in restoring the capacity to remyelinate lesioned tissues⁴.

M6A is the most abundant post-transcriptional modification in mRNA in most eukaryotes²⁸. In addition, studies have reported that m6A is involved in posttranscriptional modification, cell differentiation, cell recoding, cell stress and other processes, and plays an important role in neurosciences²⁹. However, there are few studies on m6A in SCI. Therefore, it is necessary to explore the prognostic value of m6A related genes in SCI and improving the prognosis of patients. The RMVar analysis showed that all five key genes (IMMP2L, PKIA, AK5, GALK1, WFDC1) had m6A sites, which added confidence to our results³⁰.

In order to explore the role of hub gene and m6A-related candidate genes in the prognosis of SCI, we screened out 8 genes associated with patient prognosis by rt-qPCR and MRI correlated analysis, and then screened out 2 key genes (AK5 and PPARG). The comparatively higher expression of PPARG correlates with worse prognosis in SCI patients, while lower expression of AK5 predicts worse prognosis. The above studies indicate that the AK5 are significantly correlated with m6a regulators (LRPPRC and RBMX). This regulation can be direct or indirect, but its specific mechanism is still unknown.

In conclusion, we speculate that the above two genes are closely related to the progression of SCI. However, studies on PPARG and AK5 in SCI are rare.

These results indicated that the key genes screened by bioinformatics methods were highly correlated with the occurrence and development of SCI, and had a significant correlation with the prognosis of SCI patients. Therefore, the above two key genes can provide reference for the prognosis and treatment of SCI.

However, the study also has certain limitations. First, our results are based on data from existing public databases and small clinical samples. Therefore, a largescale, prospective, multicenter study is needed to further validate our results. Secondly, our study population is mainly from China populations. Therefore, our findings may not be optimal for patients from other countries and ethnicities. Finally, the correlation between some key genes and the development and progression of SCI has not been confirmed by biological experiments. In follow-up studies, experimental validation will be performed to reveal the relationship between key genes and SCI. In this way, we can determine their suitability as new prognostic and therapeutic targets to provide a rationale for the clinical prognosis and treatment of SCI.

**Declarations**

* Ethics approval

This study was approved by Ethics Committee of The First Affiliated Hospital of Nanjing Medical University (2022-SR-697).
* Consent to participate

Informed consent was obtained from all individual participants included in the study.

* Consent for publication

The authors affirm that human research participants provided informed consent for publication.

* Availability of data and materials

GSE151371

* Competing interests

The authors declare no competing interests

* Funding

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* Authors' contributions

Mingran Luo performed the data analysis and the manuscript drafting. Qian Wang collected and analyzed clinical data. All authors discussed the results and was involved in revision of the manuscript.

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References


Table

Table1 Primer sequences
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Figure 1

The workflow for prognostic analysis of m6A-related genes as potential biomarkers for spinal cord injury.
Figure 2

WGCNA module construction and selection of modules with high correlation with SCI. (A) Sample clustering diagram (delete 1 outlier samples by setting the height to 100); (B) Sample clustering diagram with clinical features.
Figure 3

(A) Determination of the optimal soft threshold (in the process of module selection, the adjacency matrix is converted into a topology matrix, and the optimal soft threshold $\beta=16$ is determined); (B) Cluster tree of co-expressed gene modules (similar genes are grouped into the same module through dynamic splicing and cluster analysis); (C) The correlation between gene modules and clinical information (The redder the color, the higher the positive correlation; the greener the color, the higher the negative correlation. Numbers...
in the figure are Pearson's correlation coefficient, and corresponding p-values are in parentheses); (D-E) The correlation between Black and Pink modules and IPF is represented by scatter plot. (F) PCA (principal component analysis) of two group (blue are healthy control, red are SCI).
(A) Volcano map of differentially expressed genes (red are up-regulated genes, blue are down-regulated genes, grey are non-differentially expressed genes); (B) A Venn diagram was utilized to screen the hub genes between the DEGs and WGCNA. 289 genes were screened as candidates for further analysis and validation; GO and KEGG enrichment analysis were performed for SCI highly correlated differentially expressed genes. (C) Bubble pattern; (D) Chord diagram; (E) GSEA; (F) Protein-protein interaction.

Figure 5

Screening m6A related candidate genes; (A) the correlation between the expression levels of 21 m6A regulators; (B) Pearson correlation analysis was used to screen out m6A-related candidate genes in SCI;
Figure 6

Screening of key genes associated with SCI diagnosis; (A) Gene expression levels between (spinal cord injury) SCI and healthy control (HC); (B) Representative MRI of SCI patients and measurement of MCC and MSCC (n=10); (C-J) The relationship between genes and MCC/MSCC length. (Red are MCC correlation coefficient, black are MSCC correlation coefficient). *P≤0.05. Pearson's correlation test in C-J. MCC=maximum canal compromise, MSCC=maximum spinal cord compression, Di=midsagittal diameter of the spinal canal at the site of injury, Da, Db=the diameter of the spinal canal above/below the level of spinal injury, di =the diameter of the spinal cord at the level of SCI, da, db=the diameter of the spinal cord at a normal segment above/below the level of SCI.