

MicroRNA-185 regulates spinal cord injuries induced by thoracolumbar spine compression fractures by targeting transforming growth factor- β 1

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Abstract. The aims of the present study were to examine the expression of transforming growth factor (TGF)- β 1 and microRNA (miR)-185 in the bone tissue, blood and cerebrospinal fluid of patients with spinal cord injuries and to evaluate the regulation of spinal cord injuries by miR-185. A total of 44 patients with spinal cord injuries induced by thoracolumbar spine compression fractures, who were hospitalized at Luoyang Orthopedic-Traumatological Hospital between June 2012 and February 2015 were enrolled in the present study. Among the patients enrolled, 18 underwent surgery between 1 and 7 days following fracture, and 26 patients underwent surgery between 8 and 14 days following fracture. Bone tissue, peripheral blood and cerebrospinal fluid were subsequently harvested from patients for analysis. Reverse transcription-quantitative polymerase chain reaction was performed to determine the expression of miR-185 and TGF- β 1 mRNA. Western blotting was performed to evaluate TGF- β 1 protein expression in bone tissue and ELISA was employed to quantify TGF- β 1 protein expression in the blood and cerebrospinal fluid. TGF- β 1 mRNA and protein levels in bone tissue, blood and cerebrospinal fluid from patients who underwent surgery 8-14 days post-fracture were significantly higher than those who underwent surgery 1-7 days post-fracture ($P < 0.05$). By contrast, miR-185 levels were significantly lower in bone tissue, blood and cerebrospinal fluid from patients who underwent surgery 8-14 days post-fracture compared with those who underwent surgery 1-7 days post-fracture ($P < 0.05$). The results of the present study demonstrate that the upregulation of TGF- β 1 in the bone tissue, blood and cerebrospinal fluid of patients with spinal cord injuries induced by thoracolumbar spine compression fractures is correlated with the downregulation of miR-185. Furthermore, miR-185 may target TGF- β 1, affecting

its transcription and translation, indicating that it serves an important role in spinal cord injuries induced by thoracolumbar spine compression fractures.

Introduction

The spine is perhaps the most important component of the human skeletal system, as weight, impact or pressure in any area of the body is applied to it (1). Spinal cord injuries most frequently occur in thoracic vertebra 12 to lumbar vertebra 1 of the spine in young adults, accounting for 4.3% of all body fractures (1). Thoracolumbar spine compression fractures account for >90% of all spinal lord fractures and are one of the primary causes of long-term chronic lumbar spinal pain, which severely impacts the quality of life of those suffering from it (2). Furthermore, traumatic spinal cord fracture and dislocation may be fatal (3), with the majority of patients experiencing sensory disturbance of limbs and incontinence (4). Symptoms of paralysis following spinal cord injuries are difficult to recover from and require adequate and appropriate nursing, posing a challenge for clinical treatment (5).

In spinal cord injuries induced by compression fracture, changes in the expression of microRNAs (miR) and mRNAs have been detected; therefore, they may be beneficial in the clinical diagnosis and treatment of spinal cord injuries (6,7). Transforming growth factor (TGF)- β 1 is a member of the TGF superfamily, which is generated by various types of cells. In the bones, TGF- β 1 is produced by osteocytes, osteoblasts, osteoclasts and chondrocytes. TGF- β 1 produced by osteoblasts is immediately bound to the bone matrix (8). It has been demonstrated that TGF- β 1, which is a potent chemokine, stimulates the synthesis of collagen. TGF- β 1 increases the growth of extracellular bone matrix and has important regulatory effects on the formation of bones and cartilage (9). During early repair of spinal cord injuries, endogenous TGF- β 1 expression is rapidly upregulated in the spinal cord, exerting its effect by activating glial cells and phagocytes. As a result, connective tissues are formed, angiogenesis is promoted, extracellular matrix is deposited and collagen is synthesized (10-12). These procedures serve important roles in the repair of nervous tissue lesions. However, the effect of TGF- β 1 in spinal cord injuries induced by thoracolumbar spine compression fractures remains unclear. In the present study, the expression of TGF- β 1 and miR-185, and the regulation of TGF- β 1 by

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miR-185 were evaluated in patients with spinal cord injuries induced by thoracolumbar spine compression fractures.

Materials and methods

Patients. A total of 44 patients with spinal cord injuries induced by thoracolumbar spine compression fractures, hospitalized at Luoyang Orthopedic-Traumatological Hospital between June 2012 and February 2015, were enrolled in the present study. Among them, 18 patients underwent surgery between 1 and 7 days post-fracture (Group A), and 26 underwent surgery between 8 and 14 days post-fracture (Group B). These patients had no prior history of spinal cord injury and had no history of receiving hormonal treatment, traditional Chinese medicine or radiotherapy. Among the 44 patients, 20 had thoracic spine compression fractures, 18 had lumbar spine compression fractures and 6 had thoracolumbar spine compression fractures. Regarding the reasons for injury, high falling caused eight cases of thoracic spine compression fractures, nine cases of lumbar spine compression fractures and three cases of thoracolumbar spine compression fractures; traffic accidents caused ten cases of thoracic compression fractures, eight cases of lumbar compression fractures and one case of thoracolumbar spine compression fracture; extreme blunt trauma caused two cases of thoracic spine compression fractures, one case of lumbar spine compression fracture and one case of thoracolumbar spine compression fracture. Three types of sample were harvested from patients: i) Bone tissue at the fracture was harvested during surgery and stored in liquid nitrogen at -80°C ; ii) fasting peripheral blood was harvested on the morning of the day of surgery and subsequently stored in ethylene diamine tetraacetic acid tubes at -20°C ; iii) a total of 2 ml cerebrospinal fluid was harvested during surgery, followed by centrifugation ($1,500 \times g$) at -4°C for 10 min, and was stored at -80°C . All procedures were approved by the Ethics Committee of Luoyang Orthopedic-Traumatological Hospital. Written informed consent was obtained from all patients or their families.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (cat. no. 10606ES60; Yi Sheng Biotechnology Co., Ltd., Shanghai, China) was used to extract total RNA following the manufacturer's protocol. Ultraviolet spectrophotometry (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) was used to determine the purity of RNA, by measuring A260/A280. cDNA was subsequently obtained via reverse transcription using the TIANScript II RT kit (cat. no. KR107; Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. RT-qPCR was performed using iQ5 optical system software (version 2.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA) with SuperReal PreMix (SYBRGreen; cat. no. FP204; Tiangen Biotech, Co., Ltd.) according to the manufacturer's protocol. The primer sequences used were as follows: TGF- β 1, forward 5'-GGACACCACTATTGCTTCAG-3' and reverse 5'-TCCAGACTCCAAATGTAG-3'; β -actin forward 5'-TTC CAGCCTTCCTCCTGG-3' and reverse 5'-TTGCGCTCA GGAGGAGGAAT-3'. PCR amplification conditions were as follows: Initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for

55 sec and elongation at 72°C for 1 min; final extension at 72°C for 10 min. The $2^{-\Delta\Delta\text{Cq}}$ method (13) was used to calculate TGF- β 1 levels, and β -actin was used as a reference gene. Using online prediction websites, including miRanda (<http://www.microrna.org/microrna/home.do>), TargetScan (<http://www.targetscan.org>), PicTar (<http://pictar.mdc-berlin.de/>) and BibiServ (<http://bibiserv.techfak.uni-bielefeld.de/>), miR-185 was predicted to regulate TGF- β 1. The forward primers for miR-185 and U6 small nuclear RNA (internal control) were 5'-TGGAGAGAAAGGCAGTTCCTGA-3' and 5'-GCTTCGGCAGCACATATACTAAAAT-3', respectively. The common downstream primer for miR-185 and U6 was 5'-CGCTTCACGAATTTGCGTGTCTAT-3'. PCR amplification conditions were as follows: Initial denaturation at 95°C for 3 min; 40 cycles of denaturation at 95°C for 12 sec, annealing at 62°C for 35 sec and elongation at $62-95^{\circ}\text{C}$ for 15 sec. The miRcute miRNA qPCR detection kit (SYBR Green) was used to perform qPCR (cat. no. FP401; Tiangen Biotech Co., Ltd.). The $2^{-\Delta\Delta\text{Cq}}$ method (13) was used to calculate miR-185/U6 levels.

Western blotting. Proteins were extracted and a bicinchoninic acid protein concentration determination kit [cat. no. RTP7102; Real-Times (Beijing) Biotechnology Co., Ltd., Beijing, China] was used to determine protein concentration. Protein samples ($20 \mu\text{g}$ per lane) were separated by 10% SDS-PAGE and resolved proteins were subsequently transferred to polyvinylidene difluoride membranes on ice (100 V , 2 h) and blocked for 1 h with 5% skimmed milk at room temperature. Membranes were subsequently incubated at 4°C overnight with polyclonal rabbit anti-human TGF- β 1 primary antibody (1:500; cat. no. ab92486; Abcam, Cambridge, MA, USA) and rabbit anti-human β -actin primary antibody (1:5,000; cat. no. ab129348; Abcam). Following extensive washing, the membranes were incubated with polyclonal goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:3,000; cat. no. ab6721; Abcam) for 1 h at room temperature. Membranes were developed using an enhanced chemiluminescence detection kit (cat. no. FDO0142; Oddfoni Biological Technology Co., Ltd., Nanjing, China) for imaging. To acquire and analyze imaging signals, Image lab software (version 3.0; Bio-Rad) was used. The content of target protein was expressed as a relative value against β -actin.

ELISA. Blood and cerebrospinal fluid underwent determination of TGF- β 1 concentration by ELISA according to the manufacturer's protocol (TGF- β 1 ELISA kit; cat. no. ab100674; Abcam). Briefly, $50 \mu\text{l}$ control and $10 \mu\text{l}$ tissue samples were seeded onto wells in an assay plate, followed by $40 \mu\text{l}$ sample dilution reagent. Horseradish peroxidase-labeled antibody ($100 \mu\text{l}$) was subsequently added to all wells except for the blank, followed by incubation at 37°C for 1 h prior to five plate washes. Substrates ($50 \mu\text{l}$) were added prior to incubation at 37°C for 15 min. Finally, stop solution ($50 \mu\text{l}$) was added to each well and the determination of optical density at 450 nm was performed within 15 min using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical analyses. Results were analyzed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). The data were presented as mean \pm standard deviation. Multi-group measurements

were subjected to one-way analysis of variance. In cases of homogeneity of variance, least significant difference and Student-Newman-Keuls methods were used; in cases of heterogeneity of variance, Dunnett's T3 or Tamhane's T2 method was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of TGF- β 1 mRNA in patients with spinal cord injuries induced by thoracolumbar spine compression fractures increases with time following injury. RT-qPCR was performed in order to determine levels of TGF- β 1 mRNA. The data indicated that TGF- β 1 mRNA levels in the bone tissues, blood and cerebrospinal fluid of Group B were significantly higher than those in Group A ($P < 0.05$; Fig. 1). This suggests that the expression of TGF- β 1 mRNA in patients with spinal cord injuries induced by thoracolumbar spine compression fractures increases with time following injury.

Expression of TGF- β 1 protein in patients with spinal cord injuries induced by thoracolumbar spine compression fractures is increased with time following injury. To measure TGF- β 1 protein expression, western blotting and ELISA were used. Results from western blotting indicated that TGF- β 1 protein expression was significantly higher in the bone tissue of Group B than those in Group A ($P < 0.05$; Fig. 2A), which is consistent with the trend of TGF- β 1 mRNA expression in bone tissues. ELISA indicated that TGF- β 1 protein expression levels were significantly higher in the blood and cerebrospinal fluid of Group B than those in Group A ($P < 0.05$; Fig. 2B and C), which is consistent with the trends of TGF- β 1 mRNA expression in blood and cerebrospinal fluid. These results indicate that the expression of TGF- β 1 protein in patients with spinal cord injuries induced by thoracolumbar spine compression fractures is increased with time following injury.

Expression of miR-185 in patients with spinal cord injuries induced by thoracolumbar spine compression fractures is reduced with time following injury. To measure miR-185 expression, RT-qPCR was performed. The data indicated that the miR-185 levels in bone tissues, blood and cerebrospinal fluid of Group B were significantly lower than those in Group A ($P < 0.01$; Fig. 3). These results suggest that the expression of miR-185 in patients with spinal cord injuries induced by thoracolumbar spine compression fractures decreases with time following injury.

Discussion

Thoracic spine compression fractures typically induce spinal cord injuries (14). Deformation of the spinal canal, which is where the spinal cord is located, induced by thoracolumbar spine compression fractures may directly or indirectly damage spinal nerves. At present, there is no effective method to directly repair damaged spinal nerves (15). Notably, intervening in cellular and molecular mechanisms following spinal cord injury may inhibit secondary injuries to the spinal cord and serve a role in its repair.

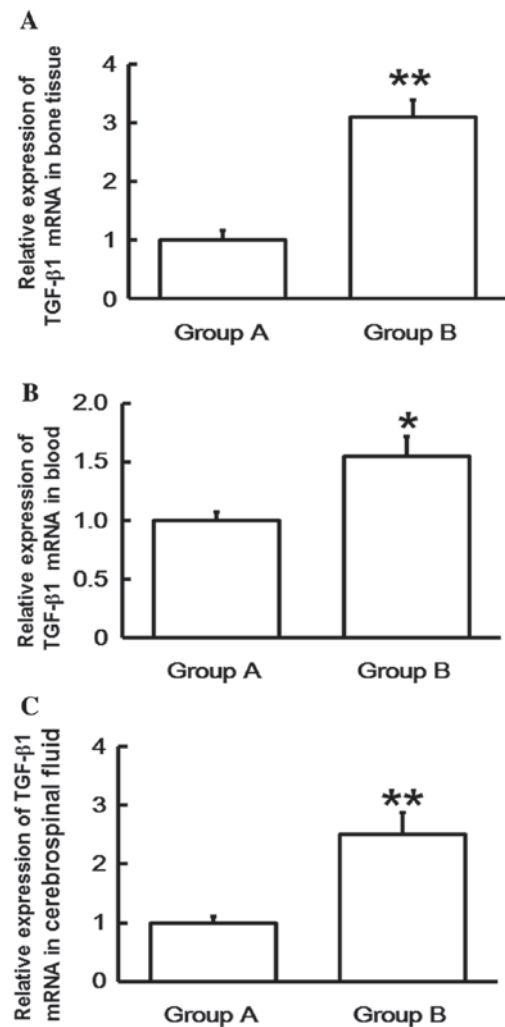


Figure 1. Expression of TGF- β 1 mRNA in (A) bone tissue (B) blood and (C) cerebrospinal fluid. Reverse transcription-quantitative polymerase chain reaction was performed to measure the levels of mRNA. Group A underwent surgery 1-7 days following fracture and group B underwent surgery 8-14 days following fracture. * $P < 0.05$ compared with group A; ** $P < 0.01$ compared with group A. TGF, transforming growth factor.

TGF- β 1, an important coupling agent in the process of bone reconstruction, has an important regulatory effect on bone reconstruction (16). In the present study, TGF- β 1 mRNA and protein levels were significantly higher in the bone tissues of patients who underwent surgery 8-14 days following fracture compared with those that underwent surgery 1-7 days following fracture. In the bone tissue, osteoblasts are one of the most sensitive cell lines to the mitogenetic effect of TGF- β 1 (17). Pfeilschifter *et al* (18) showed that TGF- β is positively correlated with human bone remodeling and formation. Additionally, Joyce *et al* (19) demonstrated that subperiosteal injection of various concentrations of TGF- β 1 induces osteogenesis and cartilage formation. Furthermore, Centrella and McCarthy (20) suggested that osteogenic or platelet-derived TGF- β 1 is able to bidirectionally stimulate DNA synthesis in the osteoblasts of fetal mouse skulls and fetal bovine bones, and the synthetic rate of DNA increases, reaches its peak and then decreases as TGF- β 1 levels steadily increase. The results of the present study are similar to the aforementioned observations (18-20), therefore, it has been demonstrated that

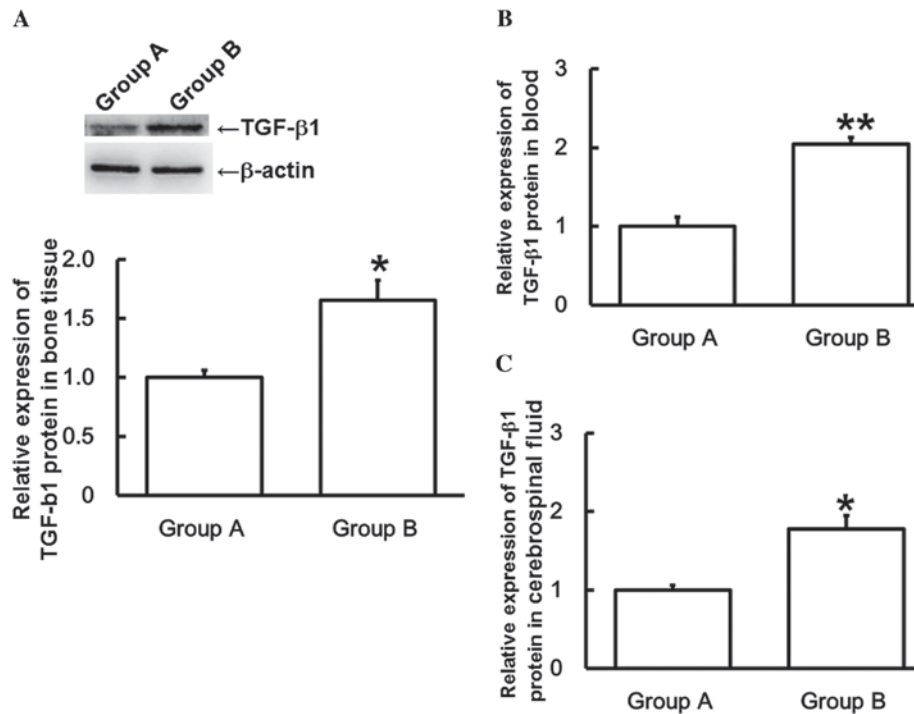


Figure 2. Expression of TGF- β 1 protein in (A) bone tissue, (B) blood and (C) cerebrospinal fluid. Western blotting was performed to determine TGF- β 1 protein expression in bone tissue and ELISA was used to measure TGF- β 1 protein expression in blood and cerebrospinal fluid. Group A underwent surgery 1-7 days following fracture and group B underwent surgery 8-14 days following fracture. * $P < 0.05$ compared with group A; ** $P < 0.01$ compared with group A. TGF, transforming growth factor.

upregulation of TGF- β 1 serves important roles in skeletal repair and regeneration.

Similar to the results from bone tissues, TGF- β 1 mRNA and protein levels in the blood or cerebrospinal fluid of patients who underwent surgery 8-14 days following fracture were significantly higher than those who underwent surgery 1-7 days following fracture. A previous study has reported that TGF- β 1 expression in motor neurons of the spinal cord is associated with the regulation of secondary injuries in the spinal cord (21). Tyor *et al* (22) identified that inhibition of TGF- β 1 following spinal cord injury may reduce the number of secondary injuries and the accumulation of monocyte-macrophages in injury regions. Intravenous injection of TGF- β 1 in rats following spinal cord injury reduces the injury area by 50% 48 h post-injection, compared with a control group (23). Romão *et al* (24) also observed that TGF- β 1 mRNA expression increases with time following spinal cord injury, reaching its peak on day 7. Additionally, Gudi *et al* (25) determined that TGF- β 1 is important in the regulation of neuronal survival and that TGF- β 1 selectively upregulates the expression of endogenous neurotrophic factors with synergistic effects. Furthermore, an association between TGF- β 1 and various central nervous system diseases has been identified. In ischemic brain damage, TGF- β 1 exerts its neuroprotective effects by reducing the concentration of calcium ions, activating the endothelial cells in ischemic areas and promoting the proliferation of blood capillaries (26). These results suggest that injuries in the spinal cord may stimulate the upregulation of TGF- β 1 in the body to try to activate the repair and regeneration of the spinal cord, and to reduce inflammation.

miRNAs may interfere with mRNAs and affect their translation (27). Regulation by miRNA increases or decreases mRNA expression to mediate the activities of coding genes of proteins and serves an important role in the occurrence and development of tumors (28,29). In the present study, bioinformatics was used to predict the upstream genes that regulate TGF- β 1 and it was determined that miR-185 may be closely associated with TGF- β 1. A recent study has demonstrated that miR-185 upregulates the TGF- β 1 signaling pathway in chronic benzene poisoning (30). In addition, Kim *et al* (31) reported that miR-185 inhibits cardiac hypertrophy via multiple signaling pathways. Ma *et al* (32) demonstrated that miR-185 inhibits proliferation and induces the apoptosis of clear cell renal cell carcinoma cells. Furthermore, Bao *et al* (33) reported that miR-185 is able to target suppressors of cytokine signaling 3 and thus inhibit the functional disorder of β cells induced by diabetes. Fu *et al* (34) have recently indicated that miR-185 targets c-met and inhibits human breast cancer cell proliferation and metastasis, whereas Wang *et al* (35) have reported that miR-185 has similar effects in breast cancer cells and targets the vascular endothelial growth factor A gene.

In conclusion, the present study demonstrated that miR-185 may target TGF- β 1 to affect its transcription and translation and thus serve an important role in spinal cord injury induced by thoracolumbar spine compression fracture. Bone tissues, blood and cerebrospinal fluid were harvested as research samples. The fact that miR-185 and TGF- β 1 expression was detected in the blood circulation demonstrates that miR-185 may be useful as a diagnostics tool, while the expression of miR-185 and TGF- β 1 in bone tissues and cerebrospinal fluid represents the physiological status of the respective tissues (36). However, further studies are

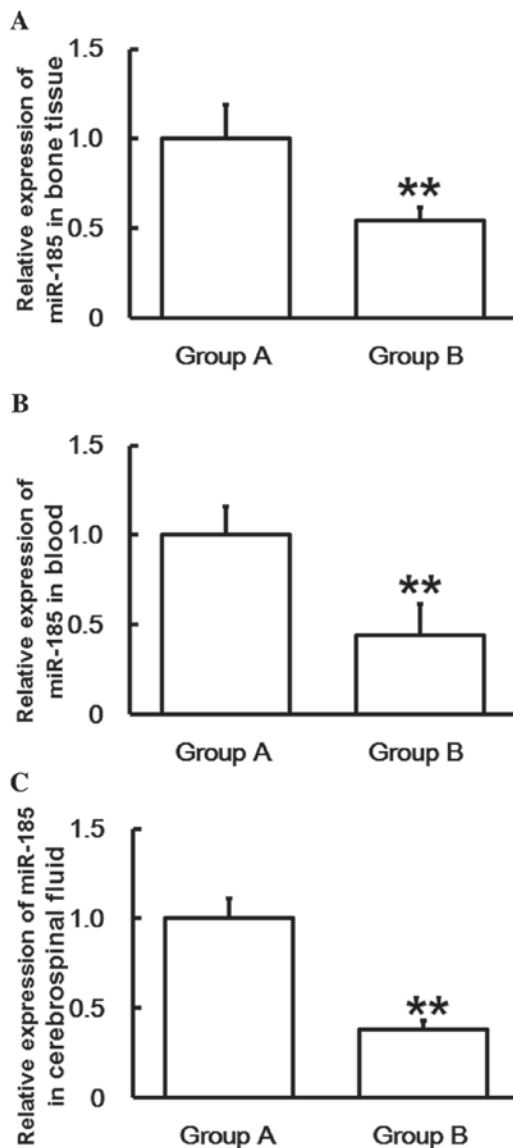


Figure 3. Expression of miR-185 in (A) bone tissue, (B) blood and (C) cerebrospinal fluid. Reverse transcription-quantitative polymerase chain reaction was performed to measure mRNA levels. Group A underwent surgery 1-7 days following fracture and group B underwent surgery 8-14 days following fracture. **P<0.01 compared with group A. miR, microRNA.

required to elucidate direct evidence regarding the association between TGF- β 1 regulation by miR-185 and spinal cord injuries induced by thoracolumbar spine compression fractures.

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