Alteration of cystatin C in cerebrospinal fluid of patients with sciatica revealed by a proteomical approach

Xudong Liu, *MD, PhD,* ***Bingfang Zeng,*** *MD, PhD,* ***Jianguang Xu,*** *MD, PhD.*

ABSTRACT

Objective: To better understand the pathophysiological mechanisms underlying sciatica induced by lumbar intervertebral disk herniation and to ascertain the protein that presents with the most observable changes in the cerebrospinal fluid (CSF) of patients with sciatica.

Method: We conducted the study in the Key Laboratory of Shanghai 6th People's Hospital, Shanghai Jiaotong University, Shanghai, Peoples Republic of China, during the period June 2004 to March 2005. In 2 separate experiments, we carried out the study involving the CSF of sciatica patients (the case group) and the CSF of otherwise, healthy volunteers (the control group). We utilized a proteomical analysis to compare the samples of 10 patients with sciatica with 10 volunteers in the control group. We individually separated each of the groups' CSF by 2-dimensional gel electrophoresis. We analyzed the harvested gel images with PD Quest 2D-gel software (Bio-Rad) to ascertain the differential proteins between the 2 groups. We based the enzyme linked immuno-absorbent assay (ELISA) experiment, which followed, on the results of the first experiment.

Results: We found 15 of the protein spots in the CSF differed appreciably in varying degrees between the 2 groups, and identification made by LC-MS/MS revealed that the most significant disparity was with cystatin C. The result of ELISA experiment confirmed a considerable decrease in the level of cystatin C ($p<0.01$) in the patients with sciatica.

Conclusion: In the CSF of patients with sciatica, the volume of cystatin C increased markedly indicating that it may play an important role in the pathophysiological processes of sciatica.

Saudi Med J 2005; Vol. 26 (11): 1699-1704

Sciatica is one of the main complaints in patients with lumbar disk herniation; however, we do not yet fully understand its pathophysiological mechanism. Besides, the effect of the compression of the bulging disk directly on the nerve roots, biochemical, autoimmune effects, or both, of the nucleus pulposus on the nerve root may play an important role.[1-4] As the cerebrospinal fluid (CSF) surrounds the spinal nerve roots, after injury to the roots there may be leakage of metabolites into the CSF as a result of the destruction of the blood-nerve barrier. This leads to the changes in the protein components and composition. Through proteomical analysis with high-resolution, 2-dimensional gel electrophoresis (DE) followed by mass spectrometry and database searching, we can analyze, on a global scale, the proteins associated with specific disease.[5-7] We carried out a comparative study between patients with sciatica caused by herniated lumbar disk, and a control group of volunteers to investigate, compare and analyze the proteins taken from the CSF of the participants of the 2 groups.

From the Department of Orthopedic Surgery, Shanghai No.6 People's Hospital, Shanghai JiaoTong University, Yishan Road, Shanghai, *Peoples Republic of China.*

Received 22nd May 2005. Accepted for publication in final form 17th September 2005.

Address correspondence and reprint request to: Dr. Bingfang Zeng, Department of Orthopedic Surgery, Shanghai No.6 People's Hospital, Shanghai JiaoTong University, 600 Yishan Road, Shanghai 200233, *Peoples Republic of China.* Tel. +86 (21) 64369181. Fax. +86 (21) 64701361. E-mail: bingfangzeng@yahoo.com.cn

The goal was to identify the protein most closely related to sciatica resulting from the lumbar intervertebral disk herniation.

Methods. This study was conducted in the Key Laboratory of Shanghai 6th People's Hospital, Shanghai Jiaotong University, Shanghai, Peoples Republic of China, during the period June 2004 to March 2005. In the case group, the CSF samples were taken from patients who suffered from lumbar intervertebral disk herniation who, in the opinion of orthopedic experts, needed diskectomies. Ten patients (5 male and 5 female, aged 43.5 ± 8.2 years) were involved and presented with the condition of sciatica, which had lasted on average 10 (5-23) months. The sciatica, diagnosed by 2 orthopedic surgeons before surgery, and confirmed by intraoperative findings, was believed to have been caused by one S1 spinal nerve root compressed by posterolateral herniated lesions of the intervertebral disks between the levels of L5-S1. All disks were extruded according to the pathologic class of Spengler.[8] A CSF sample was also taken from the control group volunteers. Ten patients (6 male and 4 female, aged 46.4 ± 7.3 years), who were to have the AO unreamed tibial nail removed after fractures in the lower limbs had healed, were involved and presented with no nerve injuries involving the initial fractures and their treatments. They experienced no pain before the removal of their implants, which was carried out 10-31 (mean 16) months after fracture and its internal fixation. The exclusions of this study included spine or brain disorders, such as spinal stenosis and recess stenosis. Some other systemic diseases such as diabetes mellitus, hypertension, and tumor were also excluded. A CSF sample, that became turbid or mixed with blood, was also excluded, so as not to influence the analysis of protein in the CSF. The study was approved by the local Ethical Committee at Shanghai 6th People's Hospital, Shanghai Jiaotong University, Shanghai, China. All sciatica patients and volunteers gave informed consent to participate in the study, which was performed in accordance to the Helsinki Declaration.

Lumbar puncture was performed in the L4-L5 intervertebral space, with the patients lying horizontally on the laterality of the sciatic nerve pain. Each patient had 2 ml of CSF sample obtained and centrifuged at 2000 x g for 10 minutes to eliminate cells and other insoluble materials. They were then stored at –80^{O}C. The solubilization was carried out according to the protocol described by Sanchez and co-workers,[9] with our own modifications. Briefly, 500 µl CSF was mixed with 2000 µl ice-cold acetone and stored overnight at -20^{O}C to remove salt from the sample. Then the mixture was centrifuged at 10000 x g at 4^{O}C for 30 minutes. The pellet was washed twice with alcohol and air-dried. The precipitated proteins were solubilized in a lysis buffer containing 8M urea, 4% CHAPS, 40 mM Tris and 65 mM DTT (Amersham), and the protein concentrations were measured using the Bradford assay. The first dimension of immobilized pH gradients (IPG)-DALT 2-DE was run on an IPGphor isoelectric focusing system (Amersham). We mixed 100 µg of proteins for analytical runs or 400 µg for preparative runs with a rehydration solution containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, 18 mM DTT and a trace of bromophenol blue, to a total volume of 250 µl, and applied to IPG dry strips. After rehydration for 12 hour, isoelectrofocus (IEF) was conducted automatically to a total of 50 kVh for analytical runs, or 70 kVh for preparative runs. Following IEF separation, the gel strips were equilibrated for 2 x 15 minutes in an equilibration buffer containing 50 mM Tris-HCl, pH 8.0, 6 M urea, 30% glycerol, 2% SDS (Bio-Rad), and a trace of bromophenol blue. We added 1% DTT to the first equilibration buffer, and in the second equilibration buffer, DTT was replaced with 2.5% iodoacetamide (Sigma). The equilibrated gel strip was placed on the top of a 12.5% T slab gel and sealed with 0.5% agarose. We performed SDS-PAGE for 15 minutes at a constant current of 15 mA, and then at 30 mA until the Bromophenol Blue reached the bottom of the gels. After 2-DE, the gels were stained with ammoniacal silver nitrate. The silver-stained 2-DE gels were scanned at an optical resolution of 84.7 um/pixel using a GS-710 imaging densitometer (Bio-Rad) in transmissive mode. Spot detection, quantification and matching were performed using PDQuest 7.3.1 software package (Bio-Rad). The mean quantity of each matched protein spot in case group was compared with the mean quantity of the same protein spot in controls. The significance of expression differences of protein spots between case and control groups was estimated by Mann-Whitney U test, $p<0.05$. The Mr and pI of each protein spot were calibrated with protein Markers (HyClone).

The protein spot that was found altering most significantly by the statistical analysis was excised from the gel, destained for 20 minutes in 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1 v/v) (Sigma) and washed in Milli-Q water until the gel became clear. The spot was kept in 0.2 M NH_4HCO_3 for 20 minutes, dried by lyophilization, and digested overnight in 12.5 ng/mL trypsin in 0.1 M NH_4HCO_3. The peptides were extracted 3 times with 50% ACN, 0.1% TFA and dried in vacuo. All electrospray mass spectra were acquired using Finnigen MAT LCQTM ion trap mass spectrometer equipped with an electrospray ionization source. An HP1100 HPLC (Hewlett-Packard) equipped with a C8 column (ABI RP300, 1.0 x 50 mm) was used, at

a flow rate of 0.05 mL/min for LC/MS. Protein identification using MS/MS raw data was performed with the SEQUEST (University of Washington, licensed to Thermo Finnigan) searching program against the The National Center for Biotechnology Information (NCBI) human protein database (version from January 16, 2005, 37,490 protein entries). The species is homosapien.

Another 2 batches of CSF samples were collected, one from 30 patients with sciatica caused by lumbar intervertebral disk herniation and one from 40 healthy volunteers matched with age. The sample collection criterion was the same as above, and all participants gave informed consent prior to enrollment. Diskectomies were performed on the sciatica patients, who participated in a follow-up evaluation 2-3 months after their surgeries. The CSF concentration of cystatin C was measured using an ELISA kit purchased from Biovendor (Brno, Czech Republic). The assay was run according to the manufacturer's directions and data was acquired on an ISA SPEX Fluoromax 2 fluorimeter with MicroMax plate reader. The ELISA data were statistically analyzed using the Statistical Package for the Social Science (SPSS, Chicago, IL, USA). Differences between control group and case groups were assessed by the Mann-Whitney U test. The Wilcoxon signed-rank test was used for comparison of patients before surgery and at the time of follow-up. Statistical significance was defined as $p<0.01$.

Results. In this study, 2-DE was carried out on the CSF samples taken from both the case and control groups. The typical silver-stained 2-DE gel of CSF in the sciatica patients is shown in **Figure 1** compared to the control group. The gels were digitized and analyzed by PD-Quest 7.3.1 software. Totally 493 ± 37 protein spots were detected in the case group and 487 ± 45 in the control group. One gel from the case group was selected as a reference gel, and a matching rate of 94.3% was obtained in the protein gels from the case group and 92.5% from the control groups. Statistical analysis is shown in **Table 1**. The regulation differential in 15 protein spots was found to differ significantly in the gels between the case and control groups. Nine of the case group had expression levels increased, and 6 of the case group were reduced in comparison to the control group. In these differing spots, spot 14 was found to have the most significant change between the case and control groups. Then it was measured with LC-MS/MS. As a result, 9 peptides were obtained (**Table 2**). A database search using MS/MS raw data of peptides derived from Spot 14 by SEQUEST Software revealed that the best match was cystatin C (Homo), which consists of 146 amino acids. The obtained 9 peptides after tryptic digestion covered 74 amino acids in the whole sequence, resulting in the sequence coverage of 50.7%. The calculated mass of cystatin C was 15799.23 ku, and pI was 8.66, corresponding to those of spot 14 displayed by 2-DE.

We measured the CSF concentrations of cystatin C in the healthy volunteers, sciatica patients and patients at follow-up 2-3 months after diskectomy by ELISA (**Figure 2**). For these samples, we measured levels of cystatin C to be 2.26 ± 0.34 μg/ml in the healthy volunteers, 2.93 ± 0.49 μg/ml in sciatic patients, and 2.23 ± 0.27 μg/ml in patients

Table 1 - Protein spots whose expression levels were significantly changed between the cerebrospinal fluid of case and control group.

Protien index (a)	Measured (b)		Normalized volume (c)		*p*-value
	Mr	pI	case	control	
1	19.51	5.37	341.48 ± 150.08	658.03 ± 189.99	0.01993
2	42.63	5.17	273.65 ± 101.7	334.62 ± 55.37	0.03415
3	45.42	5.23	768.29 ± 79.71	242.53 ± 34.61	0.01074
4	57.23	5.29	1165 ± 124.54	856.32 ± 175.68	0.01835
5	68.54	5.21	354.61 ± 82.34	184.46 ± 59.68	0.01219
6	23.67	5.52	426.95 ± 154.36	212.68 ± 121.54	0.01523
7	22.37	5.66	365.12 ± 66.23	551.68 ± 84.46	0.00944
8	20.61	5.72	433.87 ± 186.12	673.55 ± 138.27	0.01078
9	40.37	6.68	926.37 ± 139.46	756.42 ± 97.26	0.02839
10	29.53	7.05	1572.88 ± 241.37	1244.41 ± 149.54	0.03155
11	26.33	7.06	522.94 ± 175.11	769.55 ± 124.33	0.02878
12	26.21	7.66	1362.45 ± 165.35	1733.56 ± 117.71	0.02113
13	15.57	7.21	4377.25 ± 558.22	2747.52 ± 384.29	0.01325
14	15.80	8.66	4119.11 ± 476.33	1787.65 ± 246.66	0.00137
15	15.31	8.73	2573.35 ± 476.24	968.27 ± 213.65	0.00859

(a) Index in the reference gel, (b) Calculated from PD-Quest software, (c) The individual protein spot volumes were normalized as a percentage of the total volume in all of the protein spots present in the gel, and are expressed as ppm.

Table 2 - Peptides of cystatin C detected by mass spectrometry.

Peptide fragment	Amino acid	Masses	Sequest parameters (a) Xcor	Delta	Peptide sequence
T1	73 - 81	1079.13	4.4485	0.4379	ASNDMYHSR
T2	45 - 61	1800	2.5823	0.3073	LVGGPMDASVEEEGVRR
T3	45 - 60	1643.82	3.7778	0.4104	LVGGPMDASVEEEGVR
T4	52 - 62	1225.34	3.1923	0.4521	ALDFAVGEYNK
T5	51 - 62	1381.53	3.5356	0.4128	RALDFAVGEYNK
T6	52 - 71	2287.47	3.9341	0.5461	ALDFAVGEYNKASNDMYHSR
T7	80 - 96	1920.21	4.4485	0.4379	KQIVAGVNYFLDVELGR
T8	121 - 140	2277.66	3.2104	0.5249	AFCSFQIYAVPWQGTMTLSK
T9	81 - 96	1792.04	4.6148	0.6157	QIVAGVNYFLDVELGR

(a) Sequest parameter: Xcor, cross-correlation value; Delta, delta correlation value

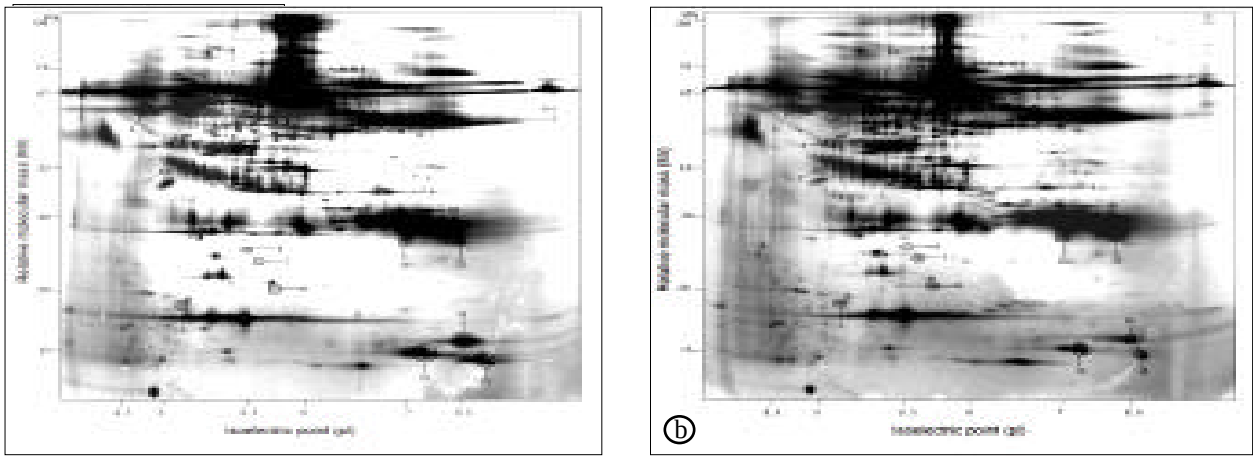

Figure 1 - Dimensional gel electrophoresis patterns of cerebrospinal fluid (CSF) from Sciatica patient **(a)** and control **(b)** Circles denote the proteins that were up-regulated in CSF of sciatica patient and squares denote proteins that were down-regulated.

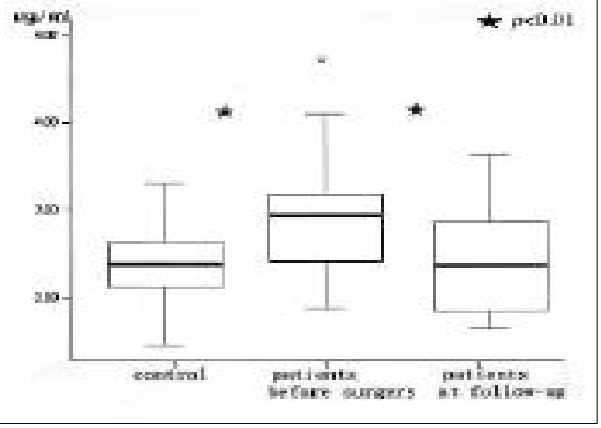

Figure 2 - Boxplot of cerebrospinal fluid levels of cystatin C from healthy controls, patients with sciatica before surgery and at the time of follow-up. The box lines represent the lower quartile, median and upper quartile values. The whiskers (lines extending from each end of the box) show the extent of the rest of the data. Outliers are data with values beyond the ends of the whiskers.

at follow-up. At first, the level of cystatin C in the CSF was much higher than the control group ($p<0.01$). After surgery, the levels went down notably ($p<0.01$). No statistical significance was observed between the healthy volunteers and the patients after the diskectomy follow-up. At the time of the follow-up, we evaluated the degree of pain by means of clinical examination. In comparison with the pre-surgical pain, there was a marked decrease of pain in all patients. Twenty-six patients showed a marked diminishing of pain and 4 patients had minimal residual pain in the affected the leg.

Discussion. So far, there are no reports of comparative studies on proteomical analysis of CSF in pain research. There are a few studies on CSF in the patients with sciatica caused by herniated lumbar intervertebral disk, in which they used ELISA, radioimmunoassay or luminescence immunoassay for measuring the concentration of proteins. The selected proteins in these studies were among those identified in CSF. There are hundreds of proteins in the CSF, not yet identified whose changes are unknown. As to which protein has the most important influence on sciatica, the question remains open.

In the present study, we used the comparative proteomical method to analyze the changes of all proteins in the CSF of the patients with lumbar intervertebral disk herniation. The study demonstrated significant changes in the expression of 15 proteins in the sciatica patients as compared to the control group. We assume that the alteration of these proteins in the CSF possibly relates to the persistent compression of the herniated lumbar disk. We can further hypothesize that these proteins play an important role in the transmission of nerve injury, and may potentially serve as markers for neurogenetic pain. The mass spectrogram's identification revealed that the protein with the most significant alteration was cystatin C. The ELISA verified that the CSF concentrations of cystatin C in the patients with sciatica changed significantly compared with those in people without pain ($p<0.01$).

Cystatin C is a low molecular weight basic protein that belongs to the cystatin superfamily, also known as neuroendocrine basic polypeptide, γ-trace or post γ-globulin, before it was sequenced,[10] and its molecular identity established.[11,12] In vivo, cystatin C, as a cysteine proteinase inhibitor, exists in various human body fluids, including CSF, blood, saliva and urine,[13,14] and can regulate the activity of endogenous and exogenous cysteine. In CSF, the choroid plexus mainly synthesizes cystatin C, and localized in the glial cells and neurons as well. In contrast to albumin, cystatin C's content is higher in CSF than in plasma, and we therefore consider it as a so-called CSF-specific or CSF-enriched protein.[15] However, we have long recognized cystatin C as a component of normal CSF. Until recently, it was found to be related to cerebral amyloid angiopathy, and taken as a diagnostic marker for Creutzfeldt-Jakob disease, in which its content in CSF increases significantly.[16]

Various stimuli, such as axotomy,[17] treatment with transforming growth factor-b[18] or dexamethasone[19] upregulates cystatin C gene expression. Reports state significant elevation in levels of cystatin C in the CSF of human patients experiencing persistent pain compared to normal controls.[20] In a rat spinal cord study, Yang et al[21] found that the expression of cystatin C mRNA in the corresponding lumbar spinal cord region increased markedly after stimulation of the spinal cord for 24 hours by experimental peripheral inflammation (hind paw injection of carrageenan), indicating that the dorsal horn cells of the spinal cord can synthesize cystatin C. Available studies on the spinal cord and brain suggest that the elevation of cystatin C could occur in either astrocytes or neurons.[22] Interestingly, either peripheral inflammation by formalin or zymosan,[23] or nerve injury by sciatic nerve ligation,[24] could lead to the increase of the activity of microglia and astrocytes in the spinal cord. Furthermore, astrocytes are markedly activated in an osteosarcoma pain model.[25] It is therefore concluded that spinal astrocytes secrete cystatin C and upregulate its gene expression.

In this study, all the patients in the case group had a pain history of more than 5 months. The long-term and persistent compression and pain stimuli may induce the dorsal horn cells in the spinal cord to synthesize and release cystatin C, which then overflowed into the CSF and increased its content. After surgery, the compression, and pain stimuli disappeared with decreased synthesization of cystatin C, so its level in the CSF gradually returned to normal. We can reasonably believe that the increase of cystatin C in the CSF plays an important role in the pathophysiological process of sciatica caused by lumbar intervertebral disk herniation. In future research, we would further investigate cystatin C's role in the course of nerve injury. We would carry out testing to see whether we could reduce the degree of sciatica by decreasing the concentration of cystatin C in the CSF.

Acknowledgments. We gratefully acknowledge the technical assistance of Xiaoli Zhang, Wei Jiang, PhD. This work was supported by grants from the National Natural Science Foundation of China, Shanghai Municipal Commission for Science and Technology.

References

1. Gertzbein SD, Tile M, Gross A, Falk R. Autoimmunity in degenerative disc disease of the lumbar spine. ***Orthop Clin North Am*** 1975; 6: 67–73.

2. McCarron RF, Wimpee MW, Hudkins PG, Laros GS. The inflammatory effect of nucleus pulposus. A possible element in the pathogenesis of low-back pain. ***Spine*** 1987; 12: 760–764.
3. Olmarker K, Blomquist J, Stromberg J, Nannmark U, Thomsen P, Rydevik B. Inflammatogenic properties of nucleus pulposus. ***Spine*** 1995; 20: 665–669.
4. Olmarker K, Nordborg C, Larsson K, Rydevik B. Ultrastructural changes in spinal nerve roots induced by autologous nucleus pulposus. ***Spine*** 1996; 21: 411–414.
5. Kanninen K, Goldsteins G, Auriola S, Alafuzoff I, Koistinaho J. Glycosylation Changes in Alzheimer's disease as revealed by a proteomic approach. ***Neurosci Lett*** 2004; 367: 235–240.
6. Dumont D, Noben JP, Raus J, Stinissen P, Robben J. Proteomic analysis of cerebrospinal fluid from multiple sclerosis patients. ***Proteomics*** 2004; 4: 2117-2124.
7. Jiang L, Lindpaintner K, Li HF, Gu NF, Langen H, He L, et al. Proteomic analysis of the cerebrospinal fluid of patients with schizophrenia. ***Amino Acids*** 2003; 25: 49-57.
8. Spengler DM. Lumbar discectomy. Results with limited disc excision and selective foraminotomy. ***Spine*** 1982; 7: 604–607.
9. Sanchez JC, Rouge V, Pisteur M, Ravier F, Tonella L, Moosmayer M, et al. Improved and simplified in-gel sample application using reswelling of dry immobilized pH gradients. ***Electrophoresis*** 1997; 18: 324-327.
10. Grubb A, Lofberg H. Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. ***Proc Natl Acad Sci USA*** 1982; 79: 3024–3027.
11. Abrahamson M, Grubb A, Olafsson I, Lundwall A. Molecular cloning and sequence analysis of cDNA coding for the precursor of the human cysteine proteinase inhibitor cystatin C. ***FEBS Lett*** 1987; 216: 229–233.
12. Brzin J, Popovic T, Turk V, Borchart U, Machleidt W. Human cystatin, a new protein inhibitor of cysteine proteinases. ***Biochem Biophys Res Commun*** 1984; 118: 103–109.
13. Cole T, Dickson PW, Esnard F, Averill S, Risbridger GP, Gauthier F, et al. The cDNA structure and expression analysis of the genes for the cysteine proteinase inhibitor cystatin C and for beta 2-microglobulin in rat brain. ***Eur J Biochem*** 1989; 186: 35–42.
14. El Gawad GA, Abrahamsson K, Norten L, Hijalmas K. Cystatin C- A superior marker of glomerular filtration rate in cystatin children and adolescents with continent ileal reservoir. ***Saudi Med J*** 2003; 24: S46.
15. Aldred AR, Brack CM, Schreiber G. The cerebral expression of plasma protein genes in different species. ***Comp Biochem Physiol B Biochem Mol Biol*** 1995; 111: 1-15.
16. Sanchez JC, Guillaume E, Lescuyer P, Allard L, Carrette O, Scherl A, et al. Cystatin C as a potential cerebrospinal fluid marker for the diagnosis of Creutzfeldt-Jakob disease. ***Proteomics*** 2004; 4: 2229-2233.
17. Miyake T, Gahara Y, Nakayama M, Yamada H, Uwabe K, Kitamura T. Up-regulation of cystatin C by microglia in the rat facial nucleus following axotomy. ***Brain Res Mol Brain Res*** 1996; 37: 273–282.
18. Solem M, Rawson C, Lindburg K, Barnes D. Transforming growth factor beta regulates cystatin C in serum free mouse embryo (SFME) cells. ***Biochem Biophys Res Commun*** 1990; 172: 945–951.
19. Bjarnadottir M, Grubb A, Olafsson I. Promoter-mediated, dexamethasone- induced increase in cystatin C production by HeLa cells. ***Scand J Clin Lab Invest*** 1995; 55: 617-623.
20. Mannes AJ, Martin BM, Yang HY, Keller JM, Lewin S, Gaiser RR, et al. Cystatin C as a cerebrospinal fluid biomarker for pain in humans. ***Pain*** 2003; 102: 251-256.
21. Yang HY, Wilkening S, Iadarola MJ. Spinal cord genes enriched in rat dorsal horn and induced by noxious stimulation identified by subtraction cloning and differential hybridization. ***Neuroscience*** 2001; 103: 493–502.
22. Taupin P, Ray J, Fischer WH, Suhr ST, Hakansson K, Grubb A, et al. FGF-2-responsive neural stem cell proliferation requires CCg, a novel autocrine/paracrine cofactor. ***Neuron*** 2000; 28: 385–397.
23. Sweitzer SM, Colburn RW, Rutkowski M, Deleo JA. Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. ***Brain Res*** 1999; 829: 209-221.
24. Coyle DE. Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. ***Glia*** 1998; 23: 75-83.
25. Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML, et al. Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. ***J Neurosci*** 1999; 19: 10886–10897.