Akt/PKB isoforms expression in the human lumbar herniated disc: correlation with clinical and MRI findings

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Received: 20 November 2010 / Revised: 19 April 2011 / Accepted: 6 May 2011 / Published online: 18 May 2011
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Abstract Intervertebral disc (IVD) degeneration suggests a complex process influenced by genetics, lifestyle and biomechanics, which accounts for the development of low back pain (LBP) and lumbar radiculopathy, a major cause of musculoskeletal disability in humans. The family of Akt/PKB kinases is a principal mediator in the signal transduction pathways, which contribute to transcriptional regulation, cell growth, proliferation, apoptosis, and survival ability. The purpose of this study was to evaluate the transcriptional profile of the AKT family genes in human herniated discs and the involvement of the PI3K-Akt signaling pathway in human IVD degeneration. Real-time PCR analysis was used to assess the mRNA expression pattern of the three Akt/PKB isoforms in 63 herniated and

10 control disc specimens. Our results showed a significant positive correlation between AKT1 and AKT3 mRNA in herniated discs suggesting a synergistic action between these isoforms in disc herniation. Interestingly, AKT2 mRNA was up-regulated in patients with acute pain during the first 12 months, indicating that AKT2 transcriptional activation may be associated with acute rather than chronic inflammation and phagocytosis. Finally, Akt1/PKB transcription presented a stepwise activation as disc herniation deteriorated. Our findings provide evidence on the transcriptional activation of the Akt/PKB pathway indicating that it is involved in lumbar disc degeneration. There is need for further studies to elucidate the exact role and down-stream signaling action of Akt/PKB isoforms in the pathogenesis of lumbar disc herniation.

Keywords Akt/PKB · mRNA expression · RT-PCR · Lumbar disc herniation

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Introduction

Intervertebral disc (IVD) degeneration is a multifactorial process influenced by genetics, lifestyle, and biomechanics and is defined as an aberrant cell-mediated response to progressive structural failure [1]. Degenerative changes and herniation contribute to the development of low back pain (LBP) and lumbar radiculopathy, a major cause of musculoskeletal disability in humans [2]. In current medical practice, 70–80% of the adult population reports at least one episode of LBP at some point in life [3]. However, only 10% of these episodes are accompanied by radiculopathy. In the American population, the incidence of symptomatic lumbar disc herniation is 1–2% and approximately 200,000 lumbar discectomies are performed

annually [4]. Nevertheless, information regarding the European population is limited.

The normal adult IVD is an avascular tissue consisting of a large amount of extracellular matrix (ECM) with low cell density (about 1% of the total volume). The transport of small solutes such as glucose, lactic acid, and oxygen in the inner parts of both the annulus fibrosus and nucleus pulposus is accomplished mainly by diffusion. The disc cells are responsible for preserving ECM integrity [5]. The control of homeostasis between matrix synthesis and degradation by a small number of cells (compared to the matrix volume) with poor nutrient supply is challenging. Changes in the metabolic balance of IVD cells affect the quality and quantity of the ECM and, consequently, its functional properties, providing a key point for disc degeneration [6]. Microscopically, disc degeneration is characterized by cell necrosis and apoptosis, cell proliferation, mucous degeneration, granular changes and ingrowths of nerves and blood vessels [1, 5]. Cell proliferation leads to cluster formation particularly in the nucleus pulposus [7].

The Akt/PKB signaling pathway is now recognized as one of the most critical cell survival determinants [8, 9]. Numerous studies during the last decade have shown that the family of Akt/PKB kinases (serine/threonine kinases) is a principal mediator in the signal transduction pathways activated in response to growth factors or insulin, which contribute to numerous cellular functions such as transcriptional regulation, cell growth, nutritional metabolism, proliferation, apoptosis, and survival ability. Three Akt/PKB genes have been identified in mammals: Akt1/PKB α , located at chromosome 14q32; Akt2/PKB β , located at chromosome 19q13; and Akt3/PKB γ , located at chromosome 1q44. The first two isoforms are expressed in all cell types and tissues, whereas PKB γ /Akt3 presents a more specific and tissue context expression. The three Akt/PKB isoforms consist of a conserved domain structure and have 80% similarity in protein sequence identity. However, Akt1-deficient mice show developmental defects, Akt2-deficient mice have defects in glucose homeostasis, and Akt3-deficient mice show defects in brain and neuronal system development [10]. Several studies have reported increased Akt activity in tumors of the breast, prostate, ovary, and pancreas [11].

Akt/PKB is activated by site-specific phosphorylation and full activation requires phosphorylation on residues Thr308 and Ser473 [8, 9]. Numerous stimuli, such as growth factors and inflammation, may activate Akt/PKB. For example, in herniated IVDs the exposed nucleus pulposus is misinterpreted as a foreign body and attracts the migration of macrophages to the epidural space, initiating a complicated inflammatory response [12]. Multiple signaling pathways, including the phosphoinositol-3-kinase

(PI3K)-Akt pathway, mediate inflammatory cytokine production in macrophages in the herniated disc. A previous study demonstrated that PDGF, bFGF and IGF-I can stimulate the proliferation of porcine IVD cells in vitro via the activation of the ERK and Akt signaling pathways [13]. However, the role of the PI3K-Akt pathway in human IVD degeneration or herniation has yet to be investigated.

The purpose of the current study was to examine the mRNA expression profile of the Akt/PKB family genes in lumbar IVD herniation, to correlate it with clinical and MRI features and to investigate their role in alterations of the ECM architecture and in the pathogenesis of IVD herniation.

Materials and methods

Patients

Tissue samples were collected from 63 consecutive patients (33 males and 30 females) with an average age of 47.3 years (27–68 years) who were subjected to open lumbar discectomy in our department during a recruitment period of 1 year (2008). The diagnosis of lumbar IVD herniation was based on history, clinical examination, and MRI findings. All the patients reported radiculopathy prior to surgery. Ten patients presented with progressive motor weakness and two patients were operated emergently for cauda equine syndrome. Thirty-seven patients reported experiencing maximum radicular pain before surgery. Exclusion criteria included spinal stenosis, spondylolisthesis, scoliosis, or systemic inflammatory disorders. After excision, an experienced pathologist, in addition to the clinicians present, examined the tissue samples and identified the pulposus sections to limit the percentage of endplates or annulus. The samples were then immediately refrigerated at -80°C until RNA extraction.

An MRI examination was routinely performed before surgical intervention to be used for the classification of IVD herniation. We used the recommendations of the combined task force from the North American Spine Society, the American Society of Spine Radiology, and the American Society of Neuroradiology, based on the imaging characterization of Milette [14]. Preoperative pain intensity was recorded using the visual analogue scale score (VAS). Patient employment was defined as heavy when it included manual labor with repetitive loading of the spine. Patient clinical and MRI characteristics are summarized in Table 1a.

Ten cadaveric IVD tissue samples were also excised within 12 h of the patient succumbing to the disease to be used as controls of normal Akt/PKB mRNA expression. Donors were aged between 20 and 46 years and had a

Table 1 Patient clinical characteristics

Characteristic	No. of patients
a. IVD herniation group	
Age (years)	
Mean (range)	47.3 (27–68)
Sex	
Male/female	33/30
Body mass index (BMI)	
Mean (range)	27.2 (21.07–33.05)
Level of IVD herniation	
L3–L4	11
L4–L5	24
L5–S1	28
IVD herniation classification	
Protrusion	23
Extrusion	33
Sequestration	7
Duration of symptoms	
<3 months	15
3–12 months	20
>12 months	28
Pain intensity (VAS)	
0–5	18
5–7	14
8–10	31
Employment	
Heavy/light	36/27
Smoking habits	
Smokers/non-smokers	26/37
<10 cig per day	6
10–20 cig per day	8
>20 cig per day	12
b. Control group	
Age (years)	
Mean (range)	36.8 (20–46)
Sex	
Male/female	6/4
Body mass index (BMI)	
Mean (range)	27.0 (21.9–33.0)
Employment	
Heavy/light	7/3
Smoking habits	
Smokers/non-smokers	4/6

negative history of low back pain, spinal trauma, or systemic inflammatory disease. There was no significant difference in terms of age, gender, body mass index, employment and smoking habits between the control (Table 1b) and IVD herniation groups (min $p > 0.24$). All the patients or relatives gave an informed written consent,

approved by the University Ethics Committee, to participate in the study. The study was in accordance with the Declaration of Helsinki Guidelines.

RNA extraction

Total RNA was isolated from frozen tissues using TRIzol reagent (Life Technologies Ltd., UK) according to the manufacturer's instructions. Tissue specimens were homogenized in TRIzol[®] reagent (Invitrogen, Carlsbad, CA) using a power homogenizer. RNA concentration and purity were determined on a UV spectrophotometer (Hitachi Instruments Inc., USA) by absorbance measurements (260-nm absorbance and 260/280-nm absorbance ratio). RNA integrity was examined by 1% agarose gel electrophoresis and ethidium bromide staining.

Reverse transcription and real-time PCR

Reverse transcription reactions for the preparation of first-strand cDNA from 2 µg of total RNA were performed according to the manufacturer's protocol using the AffinityScript[™] Multi Temperature cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Random hexamers were used as amplification primers. To remove the RNA template, cDNA was incubated with *E. coli* RNaseH, and stored at -20°C until use.

Real-time PCR reactions were carried out using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) with SYBR[®] Green I Master Mix (Stratagene, USA) according to the manufacturer's instructions. Data were collected and analyzed using Mx3000P real-time PCR software version 2.00, Build 215 Schema 60 (Stratagene). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in order to normalize AKT-1, -2, and -3 mRNA expression levels. The sequences of all the primer pairs used are depicted in Table 2. All primer pairs were designed to span at least one intron in order to avoid the amplification of contaminating genomic DNA along with the cDNA. To ensure the accuracy of quantification measurements and to construct a standard curve for the quantification process, a representative pool of all samples was diluted in a series of seven $2\times$ dilutions and run in the same plate. After initial denaturation at 95°C for 10 min, samples were subjected to 45 amplification cycles that comprised denaturation at 95°C for 30 s, annealing at the appropriate temperature for each primer pair for 30 s (Table 2) and elongation at 72°C for 30 s, followed by a melt curve analysis. Data collection was performed during both annealing and extension, with two measurements at each step and at all times during melt curve analysis. To verify the results of the melt curve analysis, PCR products were analyzed by electrophoresis on 2% agarose gels,

Table 2 Primer sequences used for quantitative real-time RT-PCR

Gene	Primer pair sequence (5'–3')	Annealing temperature (°C)	Product size (bp)
AKT-1	TCTATGGCGCTGAGATTGTG	58	145
	CTTAATGTGCCCCTCTTGT		
AKT-2	TGAAAACCTTCTGTGGGACC	60	172
	TGGTCCTGGTTGTAGAAGGG		
AKT-3	GGCGAGCTGTTTTCCATTTG	58	88
	GGCCATCTTTGTCCAGCATTAG		
GAPDH	GGAAGGTGAAGGTCGGAGTCA	60	101
	GTCATTGATGGCAACAATATCCACT		

stained with ethidium bromide, and photographed on a UV light transilluminator. Two negative controls were included in each PCR reaction, one with no cDNA template and one with no reverse transcription treatment. Akt/PKB isoforms transcription levels were calculated using the $2(-ddCt)$ method. Procedures were repeated with a cDNA template synthesized three times from the same RNA. The mRNA levels of each sample for every AKT isoform tested represent the mean value of data acquired from three independent RT-PCR experiments. Reproducibility of the real-time PCR results for the same samples was 99%.

Statistical analysis

The one-sample Kolmogorov–Smirnov test was employed to assess the normality of the distribution of the mRNA expression values for the genes studied. The mRNA expression of the three AKT isoforms in the control and herniated sample groups, as well as in groups of different clinicopathological features and MRI findings, were compared using non-parametric procedures (Kruskal–Wallis and Mann–Whitney test) based on the results of the Kolmogorov–Smirnov test. The Spearman rank correlation (non-parametric) test was employed to examine the Akt/PKB mRNA correlation pairwise in the groups of normal and herniated discs. Probability values or differences less than 0.05 were considered to be statistically significant. Statistical calculations were performed using SPSS software, version 11.

Results

AKT mRNA levels in normal and pathological discs

Similar mRNA expression levels of the three AKT genes were observed in control and herniated discs (Fig. 1). The mean levels and standard errors of the mean (SEM) in each group are shown in Table 3.

mRNA AKT genes co-expression analysis pairwise

In the group of control discs, no correlations were observed among the three AKT genes (AKT1, AKT2, and AKT3) (Spearman correlation). However, in the group of pathological discs a significant positive correlation was observed between AKT1 and AKT3 ($P < 0.001$, Spearman correlation) (Table 4).

Correlation of AKT gene transcript levels with clinicopathological features

Smoking

The smoking habits of patients showed no correlation with AKT gene transcript levels. Interestingly, tobacco use was found to be associated with MRI findings ($P = 0.008$, Spearman correlation). Specifically, disc sequestration was observed mainly in non-smoking patients while disc protrusion was observed in an equal number of smoking and

Fig. 1 Normalized transcript levels of **a** AKT-1, **b** AKT-2, and **c** AKT-3 in control and herniated disc specimens. No statistically significant differences were observed between the two groups. NS non-significant

Table 3 AKT isoforms mRNA expression in control and herniated lumbar discs

	Control discs (<i>N</i> = 10)	Herniated discs (<i>N</i> = 63)	<i>P</i> value*
AKT-1	0.90 ± 0.37	0.96 ± 0.98	NS
AKT-2	1.82 ± 0.89	1.22 ± 0.28	NS
AKT-3	0.92 ± 0.43	0.60 ± 0.10	NS

Data are presented as Mean ± SEM (standard error of the Mean)

* Mann–Whitney test

Table 4 Spearman correlation tables demonstrating the co-expression profile of AKT isoforms mRNA in control discs (a) and herniated discs (b)

	AKT-1	AKT-2	AKT-3
a. Control discs			
AKT-1			
Spearman's rho	1.000		
Sig. (2-tailed)			
AKT-2			
Spearman's rho	0.609	1.000	
Sig. (2-tailed)	0.200		
AKT-3			
Spearman's rho	0.185	0.213	1.000
Sig. (2-tailed)	0.726	0.686	
b. Herniated discs			
AKT-1			
Spearman's rho	1.000		
Sig. (2-tailed)			
AKT-2			
Spearman's rho	0.011	1.000	
Sig. (2-tailed)	0.934		
AKT-3			
Spearman's rho	0.529**	0.055	1.000
Sig. (2-tailed)	<0.001	0.671	

** Correlation is significant at the 0.01 level (2-tailed)

non-smoking patients ($P = 0.042$, χ^2 test) (Table 5). Disc extrusion was noted more frequently among smokers ($P = 0.025$, Fisher's exact test) as compared to disc sequestration.

Employment–Occupation

Patient employment or occupation did not affect significantly Akt/PKB transcript levels based on sedentary or light ($N = 27$) and heavy physical work ($N = 36$).

Duration of symptoms

AKT2 mRNA expression correlated significantly with the duration of patient symptoms ($p = 0.029$, Mann–Whitney

Table 5 Distribution of patients with disc herniation in respect of smoking habits and MRI findings

	Smoking habits		Total
	Non-smoking	Smoking	
MRI findings			
Protrusion	18	5	23
Extrusion	17	16	33
Sequestration	2	5	7
Total	37	26	63

Fig. 2 Normalized transcript levels of AKT-2 in patients with chronic pain (>12 months) were lower compared to patients with acute pain ($P = 0.029$, Mann–Whitney test)

test). Specifically, patients suffering for more than 12 months exhibited lower AKT2 mRNA levels, whereas patients with acute pain during the first 3–12 months demonstrated higher AKT2 mRNA levels (Fig. 2). Similar results were obtained in patients suffering for 6–12 months ($N = 14$) as compared to patients in pain for more than a year ($p = 0.048$).

Pain intensity

All the patients reported radiculopathy in their medical history. Akt/PKB gene mRNA expression was not significant between patients with low (VAS < 5), moderate (5–7) or intense pain (8–10).

Age and sex

Neither age nor sex correlated with AKT gene transcript levels.

MRI findings

A trend was observed in AKT1 mRNA levels to differ in discs with different MRI findings (sequestration, protrusion

and extrusion) ($P = 0.081$, Kruskal–Wallis test). Most importantly, however, AKT1 mRNA levels were significantly higher in sequestered compared to extruded discs ($P = 0.037$, Mann–Whitney test). Furthermore, significantly elevated AKT1 transcript levels were demonstrated in sequestered discs as compared to protruded discs ($P = 0.048$, Mann–Whitney test) (Fig. 3).

Discussion

During the last decade, there has been extensive research regarding disc degeneration. Nevertheless, there is no consensus on what constitutes disc degeneration or how to distinguish between disc aging, healing or remodeling. It is considered that a significant function of the disc cells is the maintenance and repair of the ECM to preserve structural disc integrity. Disc degeneration is characterized by cellular alterations such as disc-cell proliferation, cell-cluster formation, and cell death due to both apoptosis and necrosis [15, 16]. Current research has focused on the Akt/PKB signaling pathway since it comprises one of the most critical cell survival mediators. Its activation not only renders cells resistant to apoptotic stimuli but also contributes to several cellular functions such as proliferation, transcriptional regulation, and cell growth. To our knowledge, the present study is the first to investigate the expression of the Akt/PKB pathway in the pathogenesis of disc herniation by examining the mRNA expression profile of the three AKT genes in lumbar herniated and control discs.

AKT mRNA levels in normal and pathological discs

Akt isoforms have a considerable functional overlap [10]. Nevertheless, it is unknown whether any synergistic action exists among the three isoforms. Both the control and pathological specimens showed similar mRNA expression levels of the three AKT genes in the present study. However, our pairwise co-expression analysis demonstrated a significant positive correlation between AKT1 and AKT3 in the group of herniated discs, suggesting a synergistic

action between them, along with disc degeneration. Of note, this is the first time to our knowledge that the molecular profile of herniated discs is obtained and compared to that of normal discs. Our findings indicated that AKT1 mRNA correlated with AKT3 only in herniated discs. Our results provide evidence that alterations of the transcriptional profile of multiple genes rather than of one molecule alone appear to be involved in human lumbar disc herniation. However, mRNA alterations are not necessarily translated to the protein level; moreover, it is the activation of Akt that plays a role in cell response to exogenous stimuli. Therefore, further studies are needed to elucidate the exact role and actual function of Akt isoforms in the pathogenesis of ID herniation.

Furthermore, disc degeneration and herniation is known to be associated with marked neovascularization and increased innervation. In fact, both vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) can activate the Akt isoforms. Akt1 is associated with angiogenesis while Akt3 is correlated with neuronal cell metabolism [4]. We can only speculate whether neovascularization and neo-innervation comprise simultaneous processes during disc degeneration and herniation.

Correlation of AKT gene transcript levels with clinicopathological features

Smoking

In the intervertebral discs of rats, changes in gene expression by passive cigarette smoking precede the histological changes of disc degeneration [17]. At a low concentration, nicotine may stimulate nicotinic receptors, but at higher concentrations, it could have a direct toxic effect on nucleus pulposus (NP) cells. It is well known that nicotine inhibits collagen synthesis in NP and is responsible for the replacement of collagen type II with the inappropriate and stiffer collagen type I. Although nicotine may further reduce collagen content in the annulus fibrosus, it may also predispose the annulus to traumatic injury and degenerative changes such as those that occur in disc

Fig. 3 Normalized transcript levels of **a** AKT-1, **b** AKT-2, and **c** AKT-3 in respect of different MRI findings: bulging, protrusion, extrusion of the herniated disc specimens. AKT1 mRNA levels were significantly higher in extrusion discs compared to discs characterized by protrusion or bulging ($P = 0.037$, $P = 0.048$, Mann–Whitney test, respectively)

herniation [18]. Interestingly, after cessation of smoking the fibrous matrix begins to fill cracks and tears in the annulus fibrosus. However, since misalignment is not repaired, it has been noted that structural damage to the annulus fibrosus caused by smoking is irreversible [19]. In our evaluation the patients' smoking habits showed no correlation with AKT gene transcript levels. It could be suggested that a larger number of non-smoking patients ($N = 37$) and light smokers ($N = 6$) compared to heavy smokers ($N = 20$) would have strengthened our analysis. However, a more reasonable speculation would be that the toxic effect of nicotine on NP cells is exerted through different molecular mechanisms that do not necessarily include the transcriptional deregulation of the Akt/PKB kinases.

Interestingly, the smoking habits of patients with herniated discs were found to be associated with MRI findings. Specifically, disc sequestration was mainly associated with non-smoking patients, while disc extrusion was mainly associated with smokers, reinforcing the hypothesis that smokers are prone to higher structural damage of the lumbar discs.

Occupation

Occupational loading is believed to affect the risk of disc degeneration in the lumbar spine. According to previous reports, the risk for posterior bulging in MRI was found to be higher among construction carpenters. However, the decreased signal intensity compatible with disc degeneration is not related to occupation [20]. In the present study, the Akt/PKB gene transcript levels evaluated were not affected by patient occupation or employment.

Pain intensity

In the present study, pain intensity assessed by VAS was not found to be associated with Akt/PKB gene mRNA expression. This is not surprising since the extent of the disc herniation is poorly correlated with pain intensity. Of note, although all the patients included in the study reported radiculopathy in their medical history, a significant proportion of them made no reference to maximum pain at the time of the operation. Moreover, disc herniation associated with radiculopathy may be both a mechanical and biochemical disorder [21].

Duration of symptoms

Transcriptional AKT2 up-regulation was demonstrated by our findings in the group of patients with acute pain during the first 3–12 months, compared to patients suffering for

more than 12 months. Although we cannot determine whether nucleus pulposus, annulus fibrosus, chondrocyte-like cells of endplates or macrophages are the originating source of the Akt2/PKB- β mRNA levels exhibited in the present study, and a small contamination of the samples cannot be excluded, we can speculate an underlying association with the process of acute rather than chronic inflammation and phagocytosis in the herniated disc. A major limitation of this finding, however, is the subjectivity of each patient's definition of pain intensity and duration, which weakens the above hypothesis.

The exposed nucleus pulposus during disc herniation is misinterpreted as a foreign body with inflammatory properties leading to the process of phagocytosis due to the attraction of macrophages in the epidural space. The (PI3K)-Akt pathway mediates inflammatory responses, while the activation of Akt/PKB has been implicated in macrophage phagocytosis of foreign bodies [22, 23]. Our hypothesis is reinforced by the findings of Shiratsuchi and Basson who report that changes in tissue pressure during inflammation may regulate macrophage phagocytosis by the activation of PI-3K/Akt2/PKB β , but not Akt1/PKB α and Akt3/PKB γ [24].

MRI findings

The present study showed that AKT1 mRNA up-regulation is exhibited in sequestered compared to extruded as well as protruded discs. Higher AKT1 mRNA levels were also observed in extruded compared to protruded discs, however, the difference was not statistically significant. Consequently, Akt1/PKB transcription presents a stepwise activation as disc herniation deteriorates. This is not surprising since it is expected that the larger the disc herniation the higher the proliferation and tendency for enhanced disc-cell motility. Akt/PKB, a PI3 kinase-activated protein kinase, is known to be a principal mediator of cell survival that controls cell proliferation as well as the regulation of cell motility and migration [25]. Our results demonstrated an increase in Akt1/PKB α mRNA expression in the advanced stages of disc herniation (sequestration and extrusion), suggesting either increased survival and motility of the nucleus pulposus cells at the late stages of IVD degradation or an effort towards survival due to a hostile environment. This is also supported by previous reports demonstrating that Akt1/PKB α is directly involved with motility and invasion, possibly through the expression of MMP-9 [26] and MMP-2 [27]. The activation of only the Akt1/PKB α isoform, according to our findings, in correlation with the severity of disc herniation (as indicated by MRI) could be explained by the fact that Akt1/PKB α accounts for approximately 75% of the total Akt/PKB activity [27, 28].

Conclusion

Our results showed a significant positive correlation between AKT1 and AKT3 mRNA only in herniated discs suggesting a possible synergistic action between these isoforms in disc herniation. Smoking habits, occupation, and pain intensity were not found to be associated with Akt/PKB transcript levels. Interestingly, AKT2 mRNA was up-regulated in patients with acute pain during the first 12 months, indicating that AKT2 transcriptional activation could be associated with acute rather than chronic inflammation and phagocytosis. Finally, Akt1/PKB transcription presented a stepwise activation with disc herniation deterioration. Our findings provide evidence on the transcriptional activation of the Akt/PKB pathway in the process of disc degeneration indicating that it is involved in lumbar disc degeneration. There is need for further in-depth studies to elucidate the exact role and actual function of the Akt/PKB isoforms in the pathogenesis of lumbar disc herniation.

Conflict of interest None.

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