Int J Clin Exp Pathol 2015;8(12):16097-16103
www.ijcep.com /ISSN:1936-2625/IJCEP0014013

Original Article

RASSF7 expression and its regulatory roles on apoptosis in human intervertebral disc degeneration

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Received August 5, 2015; Accepted November 17, 2015; Epub December 1, 2015; Published December 15, 2015

Abstract: Apoptosis plays an important role in intervertebral disc degeneration (IDD). Overwhelming evidence indicates that RASSF7 is essential for cell growth and apoptosis. Recently, it has been noted that the JNK signaling can be negatively regulated by suppressing phosphorylated-MKK7 activation during pro-apoptosis. We aimed to investigate the RASSF7 expression level in human degenerative nucleus pulposus (NP) cells and non-degenerative NP cells and the link between RASSF7-JNK with NP cells apoptosis. We harvested NP tissues from 20 IDD patients as disease group and 8 cadaveric donors as normal controls. We detected RASSF7 expression by Real-time-PCR and western blotting. Consequently, we found that the expression of RASSF7 was higher in non-degenerative group than in degenerative group ($P<0.05$). Overexpression of RASSF7 in degenerative NP cells led to decreased apoptosis rate than that in scramble group ($P<0.05$). Collectively, our findings suggest that RASSF7 plays an important role in human IDD and RASSF7 might be potentially developed as a curative agent.

Keywords: Intervertebral disc degeneration, apoptosis, RASSF7, nucleus pulposus, JNK

Introduction

As the most disabling condition globally, low back pain (LBP) is one of the biggest problems for public health systems in the world [1]. However, many factors have been identified as possible causes of LBP [2-5], one of which is intervertebral disc degeneration (IDD) [6]. IDD occurs frequently in adults and is tightly linked with low back pain and sciatica [7-11], which are the most common diseases resulting in morbidity with overwhelming socioeconomic consequences [1, 12-14]. Hitherto, the underlying machinery of IDD has been largely unknown. We have noted that Fas-mediated apoptosis in human IDD can be promoted by the down regulation of miR-155 [15-17]. Furthermore, we have addressed the expression profiles of long noncoding RNAs and mRNAs of human IDD, in particular the mRNAs pertaining to apoptosis [18]. Amongst the deregulated apoptosis-pertinent mRNAs, RASSF7 (NM_001143993) down-regulated up to 70 fold in IDD ($P=0.0000141$).

RASSF comprises a conserved motif named the RalGDS/AF6-type Ras association domain. RASSF7 is a newly defined RASSF member. Initially, RASSF7 was noted as HRAS cluster 1 on human chromosome 11p15.

In the past decade, apoptosis in IDD has ever become a research focus [13, 19]. Accumulating evidence suggests that apoptosis plays an essential role in pathogenesis of IDD in terms of *in vitro*, *in vivo*, studies [15, 20-22]. As for the apoptosis pathways, c-Jun N-terminal kinase (JNK) enzymes are important modulators of apoptosis in stress-activated signaling [23-27].

It is well established that Ras-association domain family proteins are involved in the regula-

Table 1. Demographic data of cadaveric donors and patients

Nucleus pulposus tissues	Age	Gender	Level	Degree*
Normal control (cadaveric donors)				
1	44	M	L4/5	I
2	38	M	L4/5	I
3	37	F	L4/5	I
4	45	M	L4/5	I
5	47	M	L4/5	I
6	39	F	L4/5	I
7	45	M	L4/5	I
8	39	M	L4/5	I
IDD Group (IDD patients)				
9	47	F	L4/5	IV
10	52	M	L4/5	IV
11	43	F	L4/5	V
12	36	F	L4/5	IV
13	42	M	L4/5	V
14	50	F	L5/S1	IV
15	45	F	L5/S1	IV
16	41	M	L4/5	IV
17	38	M	L4/5	IV
18	35	F	L5/S1	V
19	39	M	L4/5	IV
20	42	F	L5/S1	IV
21	52	M	L4/5	IV
22	42	M	L4/5	IV
23	46	M	L4/5	IV
24	38	F	L4/5	IV
25	49	F	L4/5	IV
26	36	M	L5/S1	IV
27	43	F	L4/5	IV
28	51	M	L4/5	IV

*Pfirrmann's grading system.

tion of JNK pathway [28]. The vertebrate RASSF consist of the classical RASSF members (RASSF1-6) and the newly defined N-terminal (NT) RASSF members (RASSF7-10) [29]. As the most investigated N-terminal RASSF protein, RASSF7 negatively modulates pro-apoptotic JNK signaling by suppressing phosphorylated-MKK7 activation [25].

However, there have been no studies reporting RASSF7 and its attendant roles in human IDD until now. Therefore, our research was aimed to investigate the role of RASSF7 in human NP cells and its putative roles in IDD.

Materials and methods

Sample collection

The Ethics Review Board of Xijing hospital, Xi'an, P. R. China (No. 20090611-3, No. 20111103-7) approved the study. As well, we signed informed consents with each patient and cadaveric donors' relatives. NP tissues from IDD patients were collected as degenerative group during discectomy [n=20; mean age: 43.4, SD: ±5.4; (range 35-52)] and normal cadaveric donors as control group [n=8; mean age: 41.8, SD: ±3.6; (range 37-47)] as we previously reported (**Table 1**). All the samples from patients with IDD were strictly selected to eliminate sequestration type of disc herniation. IDD degrees were classified by 3 experienced clinical observers who were blinded to the groups by Pfirrmann's MRI grading system [30]. We carefully dissected all samples under magnification and thereafter managed them in terms of specific conditions.

NP cell cultures

Following cautiously washed, redundant tissues including annulus fibrosus from outside of NP and cartilaginous endplate were detached. The rest of NP tissues underwent digestion for 40 min in PBS with 0.25% pronase at 37°C (Gibco-BRL, Carlsbad, CA, USA). Subsequently, further digestion was performed in PBS with 0.025% collagenase type II (Invitrogen) for 4 h. Following washed triple times with PBS, the digest was filtered via a nylon mesh with pore-size of 45-μm. Cells were cultured for three weeks in DMEM/F12-based culture medium, consisting of 15% fetal bovine serum (FBS; Gibco-BRL) and 1% penicillin/streptomycin (Invitrogen) in a 5% CO_2 incubator. The culture medium was renewed twice a week. NP cells with passage 1 or 2 were adapted for further studies.

RNA isolation and qRT-PCR

NP tissues were harvested from cadavers and IDD patients. We extracted total RNA by Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was acquired by using High-Capacity cDNA

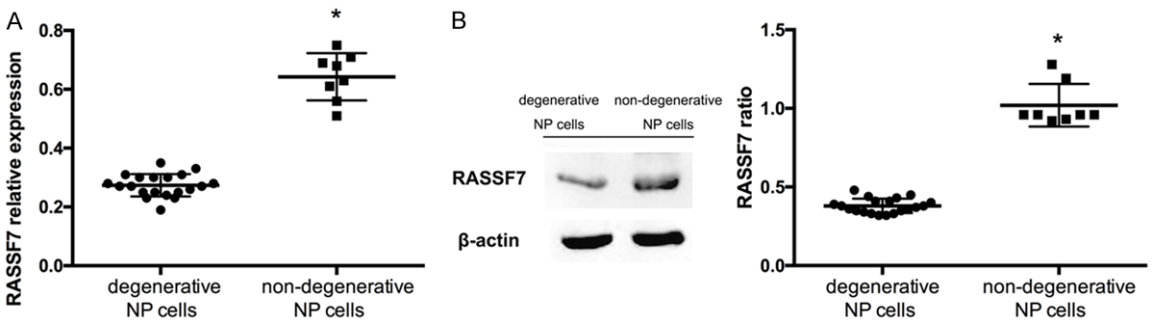

Figure 1. RASSF7 expression in degenerative NP cells. A. qRT-PCR analysis of RASSF7 expression. B. Western blotting detection of RASSF7 expression.

Archive Kit (ABI, Foster City, CA, USA). Primers for human RASSF7 and human GAPDH were purchased from Invitrogen (Invitrogen, NY, USA). NanoDrop (NanoDrop, Wilmington, DE, USA) was used to detect the concentrations of RNA. The RASSF7 expression levels were normalized to GAPDH mRNA controls. All RT reactions, including GAPDH controls, were run in triplicate in a GeneAmp PCR 9700 Thermocycler (ABI). The relative amounts of RASSF7 mRNA were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The primers used were as follows: RASSF7 forward: 5'-CAAAGGCCACGACTGCCTGTT-3', reverse: 5'-GGCACAGGCAACATGACAGAGA-3'; GAPDH forward: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-TGGTGAAGACGCCAGTGGA-3'.

Western blotting analysis

RASSF7 expression was detected by western blotting analysis in IDD group and control group. Following solubilizing cells in 2X SDS buffer, electrophoresized using 10% gel, proteins were transferred to PVDF membrane. The membranes were incubated for 1 h at room temperature with rabbit-anti-human antibody for RASSF7 (Abcam, USA). Mouse antibody to β-actin (Sigma, Saint Louis, USA) was used as control. IRDye 800 anti-rabbit or anti-mouse IgG antibody (LI-COR Biosciences, Nebraska, USA) was used to label antibodies. LI-COR Odyssey Imaging System was used to analyze expression levels.

Up-regulation of RASSF7 with lentiviral vector

We purchased lentiviral vectors encoding RASSF7 with green fluorescent protein (GFP) labeling Genechem (Genechem, Shanghai, P. R. China). We used a scrambled sequence as control. *In vitro* cultured NP cells were inoculated (Density: 1.5×10^5 cells/well) into a 24-well plate. The final volume of complete medium was 250 μL. The multiplicity of infection (MOI) for viral solutions was 10. Following incubation for 10 h and 96 h of recovery, culture flasks were investigated under fluorescent microscopy to confirm viral transfection.

Flow cytometry (FCM) analysis

FCM of NP cells 1 d following transfection was performed with APC Annexin V/7-AAD (BD Biosciences, San Diego, CA, USA) staining using standard protocol. Triple repeats were performed for each experiment.

Statistical analysis

For comparison between parameters of 2 groups, student's t-test was used. A *P* value less than 0.05 was considered as statistically significant. The SPSS 12.0 statistical package (SPSS, Chicago, IL, USA) was used for statistical analyses.

Results

Different levels of RASSF7 expression in human degenerative and normal NP cells

The mRNA expression level of RASSF7 in IDD was considerably lower than that of normal control (**Figure 1A**). Furthermore, the intensity of RASSF7 in IDD group was lower than that in control as a result of western blotting (**Figure 1B**). Quantitatively, the average RASSF7 percentage in IDD was mean: 0.243, SD: 0.03; whereas the average RASSF7 ratio in control was mean: 0.64, SD: 0.07 ($P<0.05$). Taken together, these results indicate the existence of

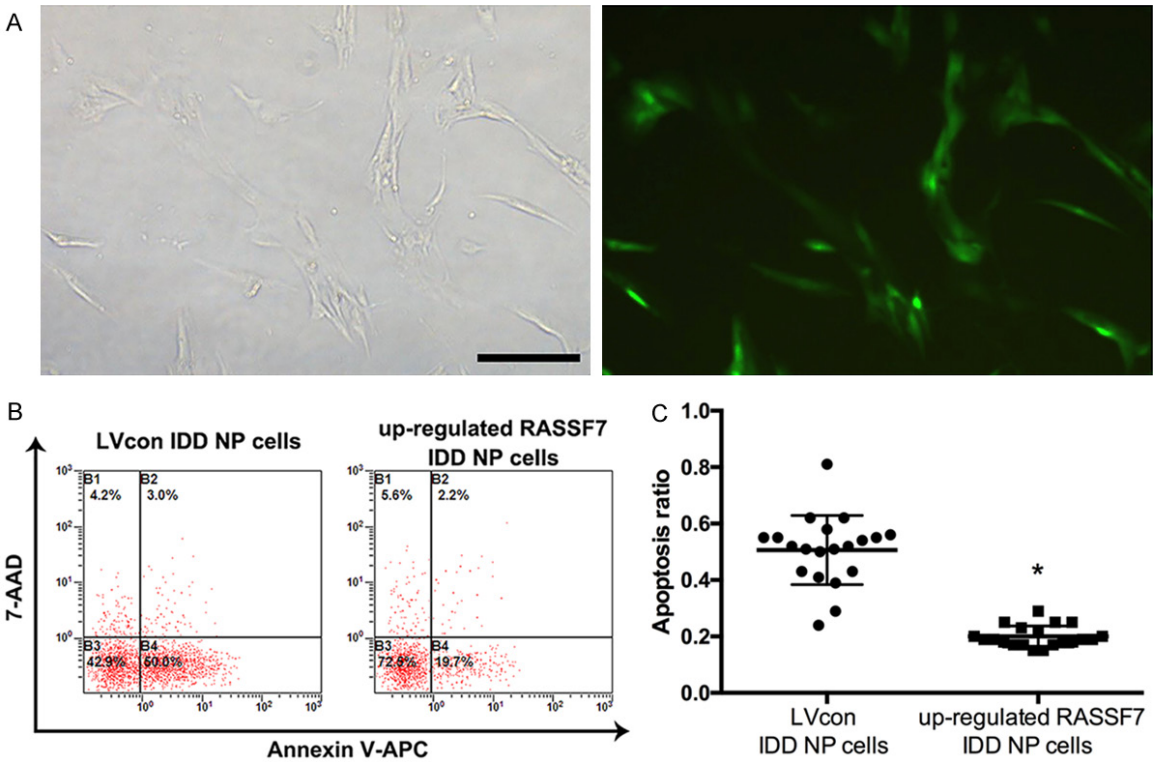

Figure 2. Apoptosis rate detection after up-regulated RASSF7 in IDD NP cells. A. Brightfield (left) and fluorescent (right) microscopy of degenerative NP cells tranfected with lentivirus labeled with green fluorescent protein. Scale bars =20 μm. B. Diagram of APC AnnexinV-/7-AADFCM of transfected NP cells. The graphs stand for typicalresults of apoptosis; values symbolize the average values of three experiments. C. Comparison of apoptotic cells between up-regulated RASSF7 groups and control goup (*P<0.05).

RASSF7 in human NP. Moreover, RASSF7 was down-regulated in human degenerative NP at both the mRNA and the protein level.

Up-regulation of RASSF7 in degenerated NP cells can lead to decreased apoptosis

The lentiviral transfection with RASSF7 led to GFP expression at high level (**Figure 2A**). FCM resulted in the apoptosis of transfected NP cells (**Figure 2B**). The apoptotic rate in up-regulated RASSF7 group was mean: 0.197, SD: 0.038. Approximately mean 0.50, SD: 0.114 apoptosis rate was detected in control group (**Figure 2C**). Our study found that up-regulated RASSF7 resulted in decreased apoptotic rate of degenerative NP cells (P<0.05).

Discussion

This study presents the first line of evidence unraveling the function of RASSF7 in human IDD. We detected the expression level of RASSF7 in human NP tissues and clarified its attendant roles in IDD for the first time. Importantly, *in vitro* modulation of RASSF7 expression in human NP cells can influence apoptosis, which might provide novel insights on the etiology of human IDD in terms of apoptosis machinery. IDD is a complicated pathological process, including various degenerative events such as cell death, miRNAs, gene polymorphisms and unbalanced immune privilege.

There are a number of lines of evidence indicating that apoptosis plays an essential role in pathogenesis of IDD [13, 19, 20]. Recently, researchers found that JNK enzymes, as the modulators of apoptosis, can be regulated by RASSF7 repressing the activity of phosphorylated-MKK7 to affect cell apoptosis [25]. RASSF7, previously known asHRC1 (HRAS1 cluster 1) and C11orf13, is a component of the N-terminal RASSF family [31]. Previous studies have shown that RASSF7 has putative roles in the regulation of cell growth and apoptosis. According to related studies, RASSF7 protein has been noted in various human cell lines [28, 32], and was found to elevate levels in the

hypoxic tissue of epithelial tumors [28, 33, 34]. As the intervertebral disc is the most avascular structure, NP tissue is in hypoxic conditions.

JNK enzymes are important modulators of apoptosis. JNK activation promotes the stability and transcriptional vitality of JNK substrates, subsequently mediating cell apoptosis via the MAPKKKs-MKK4/MKK7-JNK activation pathway. Takahashi et al. found that exclusive RASSF7 inhibition leads to anti-apoptotic regulation and increases cell protection from inappropriate JNK activation [25, 35]. Recent studies delineate that JNK activation in later phase (1-6 h) mediates pro-apoptotic signaling [36, 37]. NP cells play an essential role in the resistance against mechanical loadings by the synthesis of ECM to maintain the stability of intervertebral discs. Cell loss due to apoptosis might lead to IDD pathological process. Based on the high expression level of RASSF7 in non-degenerative NP cells, we propose that RASSF7 modulates the apoptosis of NP cells though RASSF7-MKK7-JNK pathway.

We over-expressed RASSF7 in degenerative NP cells using lentiviral vectors. The apoptotic rate of degenerative NP cells with upregulated RASSF7 is significant lower than that in scramble group. It should be stressed that the mechanisms of RASSF7-JNK links in human NP were clarified, which might cast light on the expression of RASSF7 in IDD. Previously, the function of RASSF7 especially in cell death has been noted. RASSF7 knockdown in neural tube cells presumably contributes to nuclear fragmentation and cell death [28, 38]. Takahashi et al. suggested that RASSF7 negatively regulates pro-apoptotic JNK signaling. In supplement with these studies, our findings showed that the apoptotic rate of degenerative NP cells was decreased after up-regulation of RASSF7, which might be due to apoptosis inhibition through RASSF7-JNK signaling.

Collectively, our study suggests that RASSF7 plays an important role in human IDD and that RASSF7 could be developed as a curative agent.

Acknowledgements

This work was supported by Chinese National Natural Science Foundation Grants (No. 8127-0028 and 81572182). We thank Jin-Tao Hu for her assistance in FCM analysis; Dan Li for helping obtain cadaveric specimens.

Disclosure of conflict of interest

None.

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