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AIFM3 Has Pro-apoptotic, Whereas LZTR1 Has Bilateral Functions in the Intrinsic Apoptosis Pathway

İçsel Apoptoz Yolağında AIFM3 Pro-apoptotik, LZTR1 Çift Yönlü Fonksiyona Sahiptir

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ABSTRACT

Leucine Zipper-Like Post-Translational Regulator 1 (LZTR1) is associated with several congenital diseases and cancers. The degradation of LZTR1 upon apoptosis induction in HeLa cells was reported. In addition, mass spectrometry analyses indicate that LZTR1 interacts with Apoptosis Inducing Factor Mitochondria Associated 3 (AIFM3) protein in HEK293 cells. However, the functions of LZTR1 and AIFM3 in apoptosis are not known. In this study, the characterization of the roles of LZTR1 and AIFM3 in the intrinsic apoptosis mechanism was aimed. For this purpose, firstly, FLAG-LZTR1 and Myc-AIFM3 expression plasmids had been prepared and stably FLAG-LZTR1-expressing HEK293 Flp-In cells were established. Myc-AIFM3 expression plasmids were transiently transfected into parental and stably FLAG-LZTR1-expressing HEK293 cells. Apoptosis was triggered by treating cells with hydrogen peroxide (H_2O_2), and the levels of apoptosis-associated target proteins were analyzed using western blotting. Our analyses firstly revealed that H_2O_2 treatment triggered intrinsic apoptosis through p53/p21/BCL2/Caspase 8/Caspase 9/Caspase 3 axis of the intrinsic apoptosis pathway in HEK293 cells. In addition, increased cleaved Caspase 9, Caspase 3, and PARP1 protein levels were observed in AIFM3-overexpressing cells. LZTR1 was found to have pro-apoptotic roles by increasing AIFM3 and cleaved Caspase 3 proteins and anti-apoptotic roles by decreasing cleaved Caspase 8, Caspase 9, and PARP1 proteins. In conclusion, the data obtained in this study indicate that AIFM3 has pro-apoptotic, whereas LZTR1 has both anti- and pro-apoptotic roles in the intrinsic apoptosis pathway.

Keywords: AIFM3, Apoptosis, LZTR1

ÖZ

Leucine Zipper-Like Post-Translational Regulator 1 (LZTR1), çeşitli konjenital hastalıklar ve kanserler ile ilişkilendirilmiştir. HeLa hücrelerinde apoptoz indüklendiğinde LZTR1'in parçalandığı bildirilmiştir. Ayrıca kütle spektrometrisi analizleri, LZTR1'in HEK293 hücrelerinde Apoptosis Inducing Factor Mitochondria Associated 3 (AIFM3) proteiniyle etkileştiğini göstermektedir. Bununla birlikte, LZTR1'in ve AIFM3'ün apoptozdaki işlevleri bilinmemektedir. Bu çalışmada, LZTR1'in ve AIFM3'ün içsel apoptoz mekanizmasındaki rollerinin belirlenmesi amaçlanmıştır. Bu amaçla, ilk olarak, FLAG-LZTR1 ve Myc-AIFM3 ekspresyon plazmidleri hazırlandı ve kalıcı olarak FLAG-LZTR1 ifade eden HEK293 Flp-In hücreleri oluşturuldu. Myc-AIFM3 ekspresyon plazmidleri geçici olarak parental hücrelere ve FLAG-LZTR1'i kalıcı olarak ifade eden HEK293 hücrelerine transfekte edildi. Hidrojen peroksit (H_2O_2) ile apoptozun tetiklendiği bu hücrelerde apoptoz ile ilişkisi bilinen hedef proteinlerin düzeyleri western blotlama yöntemi ile analiz edildi. Analizlerimiz, ilk olarak, H_2O_2 maruziyetinin HEK293 hücrelerinde p53/p21/BCL2/Kaspaz 8/Kaspaz 9/Kaspaz 3 aksı üzerinden içsel apoptozu tetiklediğini ortaya koydu. Ek olarak, AIFM3'i aşırı ifade eden hücrelerde kesilmiş Kaspaz 9, Kaspaz 3 ve PARP1 protein seviyelerini arttığı belirlendi. İlaveten, LZTR1'in AIFM3 ve kesilmiş Kaspaz 3 proteinlerini artırarak pro-apoptotik, kesilmiş Kaspaz 8, Kaspaz 9 ve PARP1 proteinlerini azaltarak anti-apoptotik rollerinin olduğu tespit edildi. Sonuç olarak, bu çalışmada elde edilen veriler AIFM3'ün içsel apoptoz yolağında pro-apoptotik, LZTR1'in ise hem anti- hem de pro-apoptotik rollere sahip olduğunu ortaya koymaktadır.

Anahtar Kelimeler: AIFM3, Apoptoz, LZTR1

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INTRODUCTION

Germline mutations of Leucine Zipper-Like Post-Translational Regulator 1 (*LZTR1*) are associated with Noonan syndrome, chromosome 22q11.2 deletion syndrome, and susceptibility to schwannomatosis type 2.[1-3] Somatic mutations in *LZTR1* gene were determined in glioblastoma multiforme (GBM) and hepatocellular carcinoma (HCC).[4,5] LZTR1 functions as a negative regulator of the Ras/MAPK pathway.[6-10] In addition, the tumor suppressor roles of LZTR1 by fine-tuning the Ras/MAPK pathway signaling have been reported for GBM, HCC, and acute myeloid leukemia (AML) cancers.[4,11-13] However, LZTR1's roles in the other pathways remain to be identified.

It is known that LZTR1 protein levels decrease upon induction of apoptosis.[14] However, additional functional data elucidating the roles of LZTR1 in apoptosis have not yet been reported. Additionally, mass spectrometry data indicate that LZTR1 interacts with Apoptosis Inducing Factor Mitochondria Associated 3 (AIFM3) protein in HEK293 cells.[15,16] It is known that AIFM3 has amino acid sequence homology to apoptosis-inducing factor (AIF) proteins, localizes to mitochondria in HEK293 cells, and its overexpression elevates the apoptosis by inducing cleavage of Caspase 3.[17] However, AIFM3's effects on the other members of the apoptosis signaling, either alone or with LZTR1, are not known.

Apoptotic cell death can be activated through extrinsic and intrinsic pathways.[18] While the extrinsic pathway is activated by death receptors through binding of specific ligands, the intrinsic apoptosis pathway is induced by lethal stimuli, such as DNA damage or oxidative stress. [18,19] In the presence of the excess lethal stimuli, it is known that cell cycle progression is blocked, activities of the pro-apoptotic and anti-apoptotic proteins are regulated, and the initiator and executioner caspases are activated to fulfill apoptotic cell demise.[19] In this study, the functional impacts of overexpressions of LZTR1 and AIFM3 alone or together on levels of the apoptosis pathway-related proteins in hydrogen peroxide (H_2O_2)-induced apoptotic cells were analyzed to characterize the roles of LZTR1 and AIFM3 in the intrinsic apoptosis pathway.

MATERIAL AND METHODS

Molecular Cloning and Sanger DNA Sequencing

The wild-type AIFM3 (NM_001018060.3) and LZTR1 (NM_006767.4) open reading frames (ORFs) were amplified from HEK293 cells by using Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific). AIFM3 ORF was amplified using 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCGGCTGCTTCTCC-3' (forward) and 5-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATCAGGATCCTTTCCCCGTAAG-3' (reverse) primers; whereas LZTR1 ORF was amplified using 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTGGACCGGGCAGCACGGGGGGGCAGATC-3' (forward) and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGATGTCGGCGCCCAGCTCTGCGCACTG-3' (reverse) primers. Initially amplified AIFM3 and LZTR1 ORFs were cloned into the pDONR201 Entry vector using Gateway BP Clonase II Enzyme mixture (Thermo Fisher Scientific). Subsequently ORFs were cloned into pcDNA3 Myc-DEST and pFRT_DESTFLAGHA (Addgene) expression vectors, respectively by using the Gateway LR Clonase II Enzyme mixture (Thermo Fisher Scientific) to obtain Myc-tagged AIFM3 and FLAGHA-tagged LZTR1 mammalian expression plasmids.[20] The clones were verified with Sanger sequencing using the Brilliant Dye Terminator kit (Nimagen) and a Genetic Analyzer (ABI Hitachi 3130). The obtained sequencing data were analyzed with Chromas v2.6 software.

Cell Culture

The HEK293 Flp-In cell line was bought from Thermo Fisher Scientific. Cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin and streptomycin (Thermo Fisher Scientific) as monolayers. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Plasmid DNA transfections were performed using calcium phosphate transfection.[21] To prepare stably LZTR1-expressing HEK293 Flp-In cells (FLAG-LZTR1^s), cells were first cultured in 10 cm^2 petri dishes with the antibiotic-free medium. Following that, 2 µg of pFRT_DESTFLAGHA-LZTR1 expression plasmid and 18 µg of pOG44 plasmid, which encodes Flp recombinase that carries out integration of FLAG-HA-LZTR1 sequence into Flip recombination target (FRT) cassette in the genome of HEK293 Flp-In cells, were transfected. After overnight culture, cells were passaged into new petri dishes, and cells that stably FLAG-LZTR1-expressing cells were selected by culturing them with 75 µg/mL Hygromycin B (Thermo Fisher Scientific) containing medium for 14 days. Subsequently, cell colonies were detached, diluted with complete medium, seeded as single-cell colonies, and cultured. Western blotting experiments were performed using an anti-FLAG tag antibody to determine stably FLAG-LZTR1-expressing cells. Selected clones were frozen with 20% FBS and %10 dimethyl sulfoxide (DMSO) (Merck) containing antibiotic-free DMEM. Transiently Myc-AIFM3 expressing cells were prepared by transfecting pcDNA3 Myc-DEST AIFM3 expression plasmid into FLAG-LZTR1^s or HEK293 Flp-In cells with calcium phosphate method. Empty vectors were transfected into separate cells as negative controls during all transfection experiments. Apoptosis was induced by treating cells with 0.4 mM H_2O_2 (Merck) for 10 hours.

Antibodies

Antibodies used in this study (catalog numbers are indicated in parenthesis): BCL2 (4223), phosphorylated (p)-BCL2 (Ser70) (2827), Caspase 8 (9746), cleaved Caspase 8 (Asp374) (98134), Caspase 9 (9508), cleaved Caspase 9 (Asp315) (20750), Caspase 3 (14220), PARP1 (9532), cleaved PARP1 (Asp214) (32563), and p53 (9282) antibodies were purchased from Cell Signaling Technology. Myc tag (ab9106), Beta Actin (ab8226), p-p53 (Ser15) (ab1431), p-p53 (Ser46) (ab76242), and Survivin (ab76424) antibodies were purchased from Abcam. FLAG tag (F3165) and Myc-tag (M4439) antibodies were purchased from Merck. Horseradish peroxidase (HRP) conjugated anti-mouse (170-5047) and anti-rabbit (170-5045) secondary antibodies were purchased from Bio-Rad.

Cell Lysis and Western Blotting

Prior to the western blotting analyses, cells were lysed using TNTE buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100 (Merck), 10% glycerol, 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenylmethane sulphonyl fluoride (PMSF), and 1X protease inhibitors cocktail tablet (Roche) on ice for 30 minutes. Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fischer Scientific). 30 µg proteins from each sample were loaded on 7.5%, 10%, or 12% SDS-PAGE gels according to molecular weights of proteins of interest. Indicated primary and secondary antibodies were used according to the manufacturer's instructions. Protein bands were visualized with the ChemiDoc MP Imaging System (Bio-Rad). When necessary, membranes were incubated with acetic acid and reprobed starting from primary antibody incubation to determine protein levels of a different protein in the same membrane.[22]

RESULTS AND DISCUSSION

In order to identify the functional impacts of LZTR1 and AIFM3 on the intrinsic apoptosis pathway, firstly, apoptosis was induced in HEK293 cells by treating Myc-AIFM3 transfected or untransfected parental or stably FLAG-LZTR1 expressing (FLAG-LZTR1s) cells with H_2O_2. After that, the levels of the target proteins were determined with the western blotting method. Treatment of HEK293 Flp-In cells with H_2O_2 triggered cleavage of PARP1 protein, a well-known apoptosis biomarker (Figure 1).[23] In addition, decreased levels of cleaved PARP1 proteins were seen in stably LZTR1-overexpressing cells, suggesting that LZTR1 might have a role in diminishing the cleavage of PARP1. Besides PARP1, it is also known that, in the presence of mild intracellular damage stimulus, p53 is phosphorylated at 15. Serine (Ser15) residue to down-regulate the expression of p21, which can trigger cell cycle arrest. However, lethal damages increase intracellular p53 protein levels and cause Ser46 phosphorylation of p53. Subsequently, the p53 protein, which contains Ser15 and Ser46 phosphorylation modifications, triggers apoptotic cell death.[24] Accordingly, the elevation of total p53, p-p53 Ser15, and p-p53 Ser46 protein levels, and decrease of p21 amounts were determined in H_2O_2-treated cells (Figure 1). In addition, supporting Nacak et al.'s findings, indicating that LZTR1 protein levels decrease in apoptosis-induced HeLa cells, the decrease of FLAG-LZTR1 protein levels upon H_2O_2-induced apoptosis was observed[14]. Additionally, although downregulation of Myc-AIFM3 protein levels was detected due to H_2O_2-induced apoptosis in HEK293 cells, the increase of Myc-AIFM3 protein levels in stably FLAG-LZTR1-expressing and H_2O_2-treated cells were observed (Figure 1). Altogether, these results indicate that treating HEK293 cells with 0.4 mM H_2O_2 for 10 hours induced p53-mediated apoptosis and LZTR1 has an anti-apoptotic effect by causing the decrease in cleaved PARP1 protein levels.

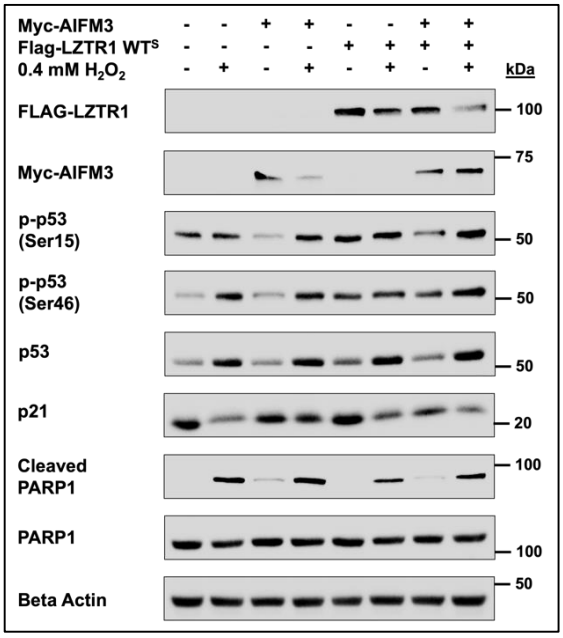

Figure 1. Western blotting results that show the effects of FLAG-LZTR1 and/or Myc-AIFM3 overexpressions on p53, p21, and PARP1 protein levels in H_2O_2-treated or untreated HEK293 Flp-In cells. Beta Actin levels were determined for loading control.

During the process of intrinsic apoptosis, it is known that activities of the anti-apoptotic BCL2 protein are inhibited via post-translational modification, the initiator Caspase 8 and Caspase 9 are activated by cleavage to initiate subsequent apoptotic events, and the executioner Caspase 3 is cleaved to implement apoptotic cell death.[19] Therefore, the levels of the above-mentioned apoptotic proteins were determined with the western blotting method to identify the roles of AIFM3 and LZTR1 in intrinsic apoptosis.

Our results revealed that p-BCL2 Ser70, which is required for the full activity of BCL2, protein levels decreased upon H_2O_2-treatment.[25] However, neither LZTR1 nor AIFM3 affected p-BCL2 Ser70 protein levels, although, the slight elevations in the total BCL2 protein levels were observed in FLAG-LZTR1-expressing cells (Figure 2).

In addition, prominent deregulations in the levels of cleaved caspases were determined in FLAG-LZTR1 and/or Myc-AIFM3-expressing cells. Firstly, our results showed that in HEK293 cells, Caspase 8 was cleaved

during H_2O_2-induced apoptosis, overexpression of LZTR1 decreased the amount of cleaved Caspase 8 proteins, and AIFM3 displayed a pro-apoptotic role by decreasing LZTR1's inhibitory effect on Caspase 8 cleavage (Figure 2). In addition, the cleaved Caspase 9 and Caspase 3 proteins were detected in H_2O_2-treated cells, which provides more evidence for the induction of apoptosis with H_2O_2 treatments in HEK293 cells (Figure 2). Furthermore, the decrease in cleaved Caspase 9 levels but the increase of the cleaved Caspase 3 protein levels were determined in FLAG-LZTR1-expressing cells compared to the untransfected cells, suggesting that LZTR1 displayed an inhibitory role in Caspase 9-mediated apoptosis but favored Caspase 3 activation with an unknown mechanism (Figure 2). Interestingly, a prominent increase of cleaved Caspase 9 levels in H_2O_2-treated Myc-AIFM3-expressing cells (Figure 2, lane four), but inhibition of Myc-AIFM3's effect on Caspase 9 cleavage by FLAG-LZTR1 were also observed (Figure 2, lane eight). Despite their opposite effects on Caspase 9 cleavage, our results indicated that both LZTR1 and AIFM3 upregulate Caspase 3 cleavage (Figure 2, lanes two, four, and eight).

In conclusion, the obtained results indicate that AIFM3 has pro-apoptotic effects on apoptosis by upregulating cleaved Caspase 3 and 9 levels, whereas LZTR1 has both pro-apoptotic effect by elevating cleaved Caspase 3 levels and anti-apoptotic effect by downregulating cleaved Caspase 9 levels.

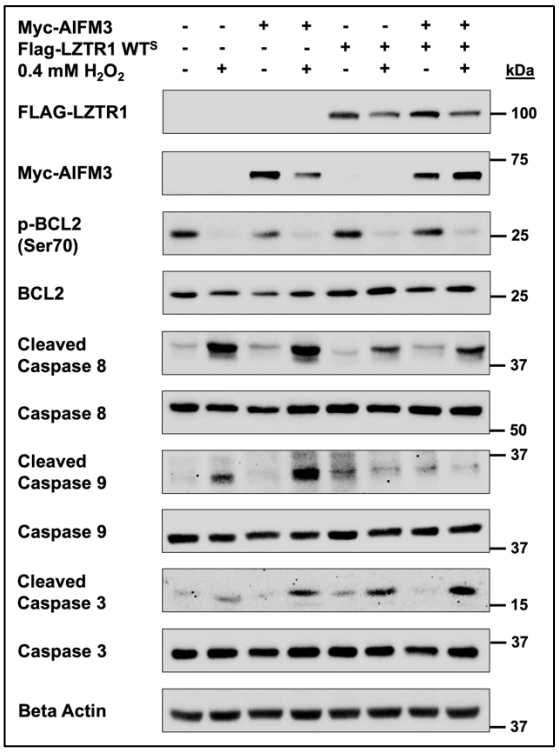

Figure 2. Western blotting results that show the effects of FLAG-LZTR1 and/or Myc-AIFM3 overexpressions on BCL2, Caspase 8, 9, and 3 protein levels in H_2O_2-treated or untreated HEK293 Flp-In cells. Beta Actin was detected for loading control.

CONCLUSION AND RECOMMENDATIONS

This study revealed that H_2O_2 treatment triggered apoptosis through p53/p21/BCL2/Caspase 8/Caspase 9/Caspase 3 axis in HEK293 cells. In addition, AIFM3's pro-apoptotic roles by upregulating the cleaved Caspase 9, Caspase 3, and PARP1 protein levels, LZTR1's pro-apoptotic effects by upregulating AIFM3 and cleaved Caspase 3 levels but its anti-apoptotic impacts by decreasing cleaved Caspase 8, Caspase 9, and PARP1 proteins were determined. The data obtained from this study strongly indicate that LZTR1 and AIFM3 play critical roles in the regulation of apoptosis. However, LZTR1's and AIFM3's effects on other apoptosis-related factors that have not been analyzed in this study should be determined with further studies. In addition, the molecules and mechanisms that regulate the functions of AIFM3 and LZTR1 in apoptosis pathway should also be investigated with additional studies.

Conflict of Interest

The authors have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

Authorship Contributions

G.Y.; Concept, design, supervising, financing and equipment, data collection and entry,

analysis and interpretation, literature search, writing, and critical review.

S.K.; Concept, data collection and entry.

T.D.; Analysis and interpretation, critical review.

B.T.; Analysis and interpretation, critical review.

E.K.; Analysis and interpretation, critical review.

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