



# Investigation of Autophagy-Related Gene Expressions in the Rat Model of Parkinson Disease

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## Abstract

**Objectives:** Parkinson disease (PD) is characterized by protein aggregations in the cytoplasm of the dopaminergic neurons due to cellular stresses. In response to these stresses, autophagy is a conservative mechanism, and dysregulation of it results in protein aggregation. Despite the accepted prominent role of it in PD, autophagy associated-gene expression dysregulation involved in the autophagosome formation has remained largely unknown. In this study, the autophagy-related gene expressions in the rat model of PD were investigated.

**Materials and Methods:** Male Wistar rats were divided into control, sham and PD experimental model groups. By injection of 6-hydroxydopamine (6-OHDA) into the striatum, the rat model of PD was induced. The apomorphine-induced rotation test was done 1 week before (baseline) and 4 weeks after surgery and also Nissl staining was performed for the brain sections. Then, rat substantia nigra pars compacta (SNpc) was extracted and RT-PCR was performed to detect the expression of FOXO3A gene and the autophagy-related genes (ATG). Furthermore, using Western blotting, we investigated the protein levels of ATG101.

**Results:** Apomorphine-induced rotation test indicated significant contralateral rotations in the rat model group. Using RT-PCR, in the induction group, ATG101 was not expressed and ATG13, ATG14L, and VPS34 genes were downregulated in comparison with the control groups. Furthermore, Western blotting showed that ATG101 protein was not expressed in the model group.

**Conclusions:** The results showed that deregulation of ATG101 expression, as a factor involved in the initial stages of the autophagy, occurs in the rat model of PD.

**Keywords:** 6-Hydroxydopamine, ATG 101, Autophagy, Oxidative stress, Parkinson disease

## Introduction

Parkinson disease (PD) is the most common movement-associated disease and the second most common neurodegenerative disorder. The affected patients suffer from postural instability, resting tremor, bradykinesia, and muscle rigidity. It is believed that the PD-associated motor dysfunctions are due to the dopaminergic neuronal loss in the substantia nigra pars compacta (SNpc) (1). A variety of pathologic mechanisms, including oxidative stress, protein aggregation, and intracellular neuronal alpha-synuclein inclusions, known as the Lewy bodies, mitochondrial dysfunction, and ubiquitin-proteasome pathway abnormality, have been associated with the degeneration of dopaminergic neurons. Interestingly,

recent studies have shown that structural aberrant forms of alpha-synucleins are associated with familial forms of PD, as mutant forms of alpha-synuclein are more prone to oligomerization. These observations point to the crucial roles of protein aggregation in the pathogenesis of this disease (2,3). Cellular defects and stem cell therapy approaches (4-11) are attractive options for neurodegenerative diseases, however these cells rapidly die because of excessive autophagy and apoptosis. Autophagy plays a housekeeping role by clearing damaged cellular organelles, such as intracellular pathogens, mitochondria, endoplasmic reticulum, peroxisomes and by removing misfolded and also aggregated proteins. Indeed, recent studies have revealed that autophagy

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deregulation is evident in the brain of PD patients (12). Autophagy is orchestrated by specific proteins known as autophagy-related (ATG) genes and proteins. Furthermore, ATG gene knock-down resulted in the neurodegeneration and the emergence of cytoplasmic inclusions filled with ubiquitinated proteins (13, 14). Recent advances around inherited mutations causing the familial forms of PD has increased our insights into the role of ATG genes. Mutations in these genes have been related to the accumulation of protein, and mitochondrial and autophagic-lysosomal protein degradation dysfunction. Particularly, upon mitochondrial membrane depolarization, PINK1 (a serine/threonine kinase with a mitochondrial targeting sequence) accumulation at the outer mitochondrial membrane and Bnip3-like protein X (NIX/Bnip3L) protein translocation to the mitochondria increases. Then, NIX and PINK1 recruit parkin, leading to the parkin-dependent and voltage-dependent anion channel (VDAC) ubiquitination in the mitochondria. NIX binds to LC3, which additionally binds to p62. VDAC ubiquitination also recruits p62, while NIX recruits gamma-aminobutyric acid receptor-associated protein (GABARAP). These autophagy effectors remove depolarized mitochondria via mitophagy (mitochondrial autophagy) (15). Despite the prominent role of autophagy in PD, its associated-gene expression dysregulation involved in the autophagosome formation has remained largely unknown. In this study, we investigated the ATG expression levels in the 6-OHDA-induced rat model of PD. To this end, PD was induced using 6-OHDA in Wistar rats. We then performed apomorphine-induced rotation test 1 week before (baseline) and 4 weeks after surgery and Nissl staining was done for brain sections as well. Finally, we assessed mRNA expression of FOXO3A, VPS34, ATG101, ATG14L, ATG13 and LC3 genes and protein levels in rat SNpc by RT-PCR and western blotting, respectively.

## Materials and Methods

### Animals

Thirty male Wistar rats were used in this study (200-250 g). During the examinations, the rats were maintained under a light-dark cycle (12 h-12 h) and given food and water ad libitum.

### Rat Model of Parkinson Disease Development

The animals were randomly divided into 3 groups as follows: control, sham (injection of the ascorbate-saline solution into the left striatum) and lesion model (injection of 6-OHDA dissolved in the ascorbate-saline solution into the left striatum) groups. Each rat was anesthetized by intraperitoneal injection of 100 mg/kg ketamine combined with 20 mg/kg xylazine. Then, the rats were placed in the stereotaxic instrument. The left striatum region of the nigrostriatum was targeted at the following coordinates (relative to bregma): anterior-posterior (A/P): +0.2 mm; medio-lateral (M/L): 3 mm and dorso-ventral (D/V): 4.5

mm (from the dura) with a flat skull position according to the rat brain atlas by Paxinos and Watson (16). Then, 12.5 µg of 6-OHDA (Sigma, Germany) was dissolved in 5 µL of 0.2% ice-cold ascorbate-saline solution and used within 3 hours in order to create the rat model of PD. The injection was made using a 5 µL-Hamilton syringe at a rate of 1 µL/min, and the needle was left in place for 5 minutes after the injections (17). The sham group was injected with 0.2% ascorbate-saline solution. The control group did not receive any injections. For minimizing mortality, post-surgery care was done during the first week after the surgery.

### Behavioral Test

Apomorphine-induced rotation test was performed 1 week before (baseline) and 4 weeks after the surgeries. The rats were injected intraperitoneally with 0.5 mg/kg of apomorphine hydrochloride (Sigma, Germany) dissolved in 0.9% saline solution. After injection of apomorphine, the total number of contralateral 360 rotations was counted in a cylindrical clear chamber for the rats (a diameter of 35 cm and a height of 37 cm) for 60 minutes (18).

### Histological Examinations of Rat Brains

Following the behavioral testing, for Nissl staining, the rats were deeply anesthetized with halothane and transcardially perfused with 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate-buffer saline (PBS, Gibco, pH 7.4) for 30 minutes. Their brains were carefully removed from the skull and then carefully post-fixed for 10 hours in the same solution. Striatum and substantia nigra were dissected out and collected in PBS. Coronal tissue sections with the diameter of 10 µm were made from the substantia nigra at intervals of 2.4 to 2.9 mm from the interaural point in accordance with the Paxinos atlas. Coronal paraffin-embedded sections from rostral to caudal of the SNpc in each animal were then transferred onto gelatinized slides and stained with 0.1% cresyl violet (Sigma, Germany). The neurons in the dense part of the substantia nigra were counted in sections aligned with 4 levels of Paxinos atlas (i.e., 2.96, 3.2, 3.8, and 4.2) as compared with the center of interaural line (with the magnification of ×200, ×100). At each level, at least 2 sections were counted and the neurons with the cytoplasmic domain were also counted.

### Gene Expression Investigation

Total cellular RNA was extracted from the ipsilateral substantia nigra of the rat brains using high pure RNA isolation kit (Roche, Germany) followed by DNase I treatment (EN0521; Ferments, USA) and then cDNA synthesis (k1622; Thermo Scientific cDNA synthesis kit, USA). RT-PCR reactions were performed in accordance with the instructions provided by the kit. A summary of primers is presented in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous housekeeping control gene.

**Table 1.** Oligonucleotide Primers used in RT-PCR

Gene	Primer (5'--> 3')	Sequence Size
FoxO3a	F: GTCCATCATCCGTAGCGAAC R: TTTTCTCTGTAGGTCTTCGGTC	216 bp
Atg101	F: TGGGATCTCAGGGTCTTG R: AACCAAGACAAACTCACACC	227 bp
Atg13	F: ACTCTCCCTCCCTCTTTCTC R: TACAGCCTTTGGGAGATGATG	198 bp
VPS34	F: ACAGGCAAACCTTCCATATAG R: CATCAACCATCAAGGAGAAC	218 bp
Atg14L	F: TTGTCACCGTATCTTCCTGTTG R: CCAGCCGTCCTCTCTCTC	203 bp
LC3	F: TGTTAGGCTTGCTCTTTTGG R: GCAGAGGAAATGACCACAGAT	219 bp
GAPDH	F: ATCTGACATGCCGCTGGAG R: AAGGTGGAAGAATGGGAGTTGC	154 bp

### Protein Expression Investigation

Four weeks post-6-OHDA injection, the ipsilateral substantia nigra was isolated and homogenized in lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and supplemented with protease inhibitor mixture (Roche, Germany), centrifuged at 15 000 g for 20 minutes at 4°C and the supernatant containing the soluble proteins was collected. Protein concentrations were estimated using BioRad protein assay kit (BioRad, USA). Fifty micrograms of protein extract were mixed with loading buffer and boiled (70-80°C) for 8 minutes. Protein extracts were separated by 12% SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham, USA). The PVDF membrane was blocked in 5% skim milk (non-fat dry milk) or 5% horse serum in TBST (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.1% Tween 20) for 30 minutes at room temperature. After blocking, the membrane was probed with ATG101 primary antibody for 24 hours (Cat No: ab105387, Abcam, UK) and detected with HRP (horseradish peroxidase)-conjugated secondary antibody (Cat No: ab6721, Abcam, UK) for 1 hour at room temperature and developed using enhanced chemiluminescence (ECL) reagents (Amersham, USA) according to the manufacturer's instructions.

### Statistical Analysis

All data were expressed as the mean  $\pm$  standard deviation (SD). One-way ANOVA followed by a Tukey post hoc test was used to analyze the results of apomorphine-induced rotational behavior. Statistical significance was considered for  $P$  value  $< 0.05$ .

## Results

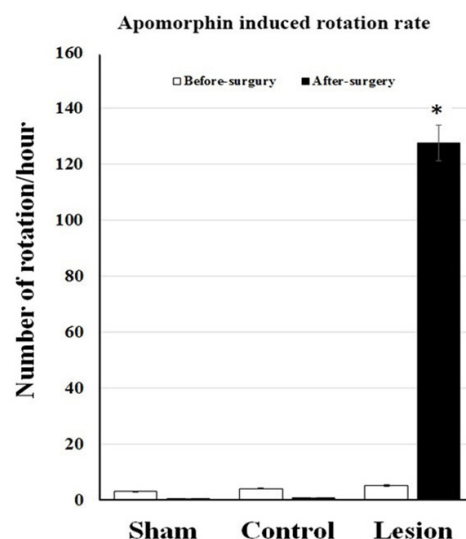
### Apomorphine-Induced Contralateral Rotations

Apomorphine-induced contralateral rotation test was done 1 week before surgery (baseline) in the 3 groups of rats. No significant difference was observed in the average number of contralateral rotations among 3 groups of rats. This test was also performed 4 weeks post-surgery to

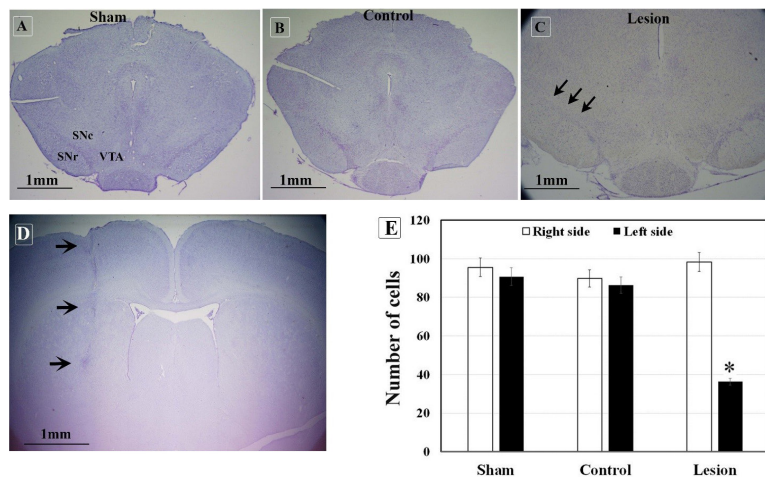
evaluate the average number of contralateral rotations in all rats. There was no significant difference in the control group 1 week before and 4 weeks after the surgeries. No significant difference was also observed in the sham group. However, apomorphine-induced contralateral turning showed a significant difference in the average number of contralateral rotations in the rat models of PD after 4 weeks compared to 1 week before the surgeries ( $P < 0.0001$ , Figure 1).

### Nissl Staining of the Brain Tissues

Photomicrographs of Nissl-stained rat midbrain from different groups of sham, control, and PD model were prepared (Figure 2). Nissl-positive cells were seen in the substantia nigra of both hemispheres of the sham and control groups as well as non-lesioned hemisphere (contralateral) of the PD rat model group. However, in the



**Figure 1.** The Average Numbers of Apomorphine-induced Rotation Tests in Sham, Control, and Parkinson's Disease (PD) Model Groups 1 Week Before and 4 Weeks After Surgery. The PD rat model group showed a significant difference in the average numbers of contralateral rotations after 4 weeks; \*  $P$ -value  $< 0.05$ .



**Figure 2.** Nissl Staining. Nissl-stained rat midbrains from different experimental groups: (A) sham, (B) control, (C) Parkinson's disease (PD) rat model, (D) injection site in the striatum and (E) Neuronal counting diagram in Nissl staining. (A) and (B) are representative of Nissl-stained sections illustrating the non-lesioned hemispheres of sham and control groups. (C) Nissl-positive cells in substantia nigra in the lesioned hemisphere. Arrows in C show a significant acceleration of cell loss in the 6-OHDA-treated rats and reduced nigral cells 30 days post-lesion induction. (D) arrows show injection site in the left striatum (E) For each rat, the midbrain sections (2.97, 3.2, 3.8, 4.2 mm; interaural, according to Paxinos-Watson planes) were examined by counting the substantia nigra neurons on each side using light microscopy at X200 magnifications. It was shown that the lesioned hemisphere of the PD model group showed a significant acceleration of cell loss and reduced number of nigral cells ( $P < 0.05$ ). VTA, ventral tegmental area; SNpc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata.

lesioned hemisphere of the PD model group, a significant acceleration of cell loss and reduced number of nigral cells 30 days post-lesion induction with 6-OHDA was occurred ( $P < 0.05$ ).

#### Assessment of Gene Expressions

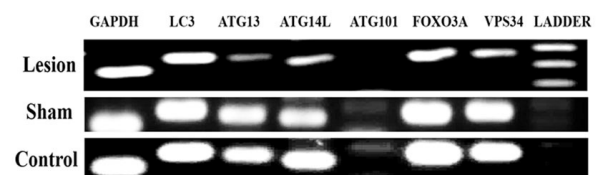
Evaluation of the ipsilateral substantia nigra gene expression showed that while GAPDH, FOXO3A, VPS34, ATG14L, ATG13 and LC3 genes were expressed in the PD rat model group, ATG101 did not. Moreover, sham and control groups expressed all of the aforementioned genes (Figure 3). However, semi-quantitative data from the gels, using ImageJ software 1.46r, revealed that ATG13, ATG14L, VPS34, and FOXO3A were downregulated in the rat model of PD (Figures 3 and 4).

#### ATG101 Western Blotting

It was shown that in the ipsilateral substantia nigra of both hemispheres of the sham and control groups, ATG101 was expressed at the protein level. However, in the ipsilateral substantia nigra (of the lesioned hemisphere) of the PD rat model group, the results showed no ATG101 protein expression in the 6-OHDA-treated rats 30 days post induction (Figure 5).

#### Discussion

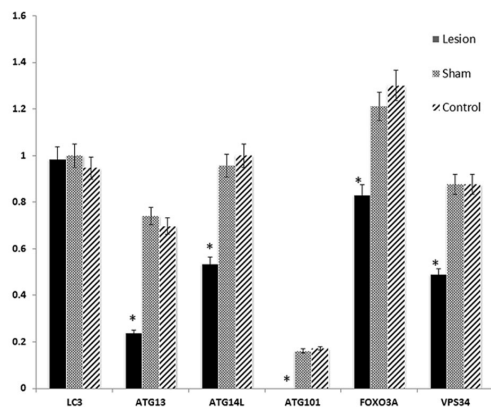
In the present study, the results showed that ATG101 gene was not expressed and ATG13, ATG14L, and VPS34 gene expressions were downregulated in the rat model of PD in comparison with the control group. It is well known that 6-OHDA is one of the toxins for modeling dopaminergic



**Figure 3.** RT-PCR Analysis of FOXO3A, VPS34, ATG14L, ATG13, LC3 and GAPDH Genes in Parkinson's Disease (PD) Rat Model (Lane 1), Sham (Lane 2), and Control (Lane 3) Groups. Evaluation of gene expression by RT-PCR method showed that while the PD model group expressed the genes of FOXO3A, VPS34, ATG14L, ATG13, LC3, and GAPDH, this group did not express ATG101 at the lesioned-hemisphere. Gene expression was seen in all of the above genes in sham and control groups. GAPDH served as an internal control gene.

neuron degeneration in PD. In the present study, unilateral 6-OHDA was used to create rat model of PD. Its injection into the striatum causes damage to the striatal terminals which is followed by a delayed dopaminergic neuron loss in SNpc. It is believed that 6-OHDA neurotoxicity is related to its pro-oxidant activity, as it accumulates in the cytosol of the neurons and easily oxidizes to form  $H_2O_2$ . Moreover, 6-OHDA can be accumulated in the mitochondria where it interacts with complex I and inhibits its activity (16). The percentage of dopaminergic neuronal loss obtained by this procedure is less marked compared to other procedures. Because of delayed, progressive dopaminergic neuronal loss of substantia nigra, unilateral injection into the striatum is more similar to the gradual neurodegeneration in human PD. Our results from Nissl staining provided evidence for the cell





**Figure 4.** Semi-quantitative RT-PCR Results. Data from the gels using ImageJ software revealed that ATG13, ATG14L, VPS34, and FOXO3A were downregulated in the rat model of Parkinson's disease; \* $P < 0.05$ .



**Figure 5.** ATG101 Protein Expression Analysis by Western Blotting. It was shown that in the ipsilateral substantia nigra of both hemispheres of the sham and control groups, ATG101 protein was expressed. However, in the ipsilateral substantia nigra (of the lesioned hemisphere) of the rat model of Parkinson's disease (PD), the results showed no ATG101 protein expression in the 6-OHDA-treated rats 30 days post induction.

loss in the 6-OHDA-induced PD rat model. The unilateral injection of 6-OHDA into the left striatum of the rats can cause the degeneration of many dopaminergic neurons and reduction in dopamine levels in the left striatum over time. This unilateral lesion induces turning behavior in response to dopaminergic agonists such as apomorphine, as it induces a pronounced rotation directing ipsilateral to the lesion side. In this study, after 4 weeks, the rats in the PD model group showed significant contralateral rotations as compared to 1 week before the surgeries. Thus, behavioral tests showed that the model was developed successfully. However, after isolation of substantia nigra from the 3 groups, semi-quantitative analysis of the RT-PCR gel results showed that ATG101 gene was not expressed and ATG13, ATG14L, and VPS34 were downregulated in the induction group in comparison with 2 other control groups. All of these genes are directly involved in the regulation and actually in the initiation phase of autophagy in the dopaminergic neurons. ATG proteins have been identified in yeast and less than half of these are thought to be involved in canonical autophagy, which is a highly conserved pathway shared by all organisms. Among higher eukaryotes, these genes are highly conserved, except for ATG1 complex. In *Saccharomyces cerevisiae*, it consists of 5 protein components including ATG1, ATG13, ATG17, ATG29, and ATG31, whereas

its mammalian counterpart, the UNC-51-like kinase, known as ULK complex, comprises FIP200, ATG101, ATG13 and either ULK1 or its homolog ULK2 (the Atg1 homologs). ATG101 directly binds to ATG13 but not to FIP200 (19, 20). The results of the recent study suggest that both of ATG13-binding surfaces and the WF finger of ATG101 are required for autophagy and ATG101 has other pivotal roles in addition to the stabilization of ATG13. Furthermore, recent data suggest that the presence of ATG101 is inevitable for FIP200 localization and ULK complex to the autophagosome formation site, but it is also important for FIP200 constitutive turnover at this site. Dysfunction of ATG101 protein causes the accumulation of FIP200 even under growing conditions due to a blockage in the basal level of autophagy pathway. Moreover, a recent study showed that ATG101 protein is crucial for LC3 recruitment into the autophagosome formation site. The WF finger and ATG13-binding surface of ATG101 protein play crucial roles in recruiting the downstream ATG proteins (21-23). Our results also showed that ATG13, ATG14L, VPS34, and FOXO3A were downregulated in the rat model of PD. It is evident that these genes have a pivotal role in the accomplishment of autophagy. As abovementioned, ATG13 cooperates with ATG101 to initiate the autophagy process. A recent study showed that FOXO3A directs a protective autophagy program in hematopoietic stem cells (24). FOXO3A is critical for maintaining a gene expression program that poises hematopoietic stem cells for rapid induction of autophagy upon starvation. On the other hand, 6-OHDA treatment increases protein oxidation, as indicated by carbonyl group accumulation. Oxidative stress, induced by 6-OHDA, reduces proteasome activities in PC12 cells. However, a recent study revealed that exposure to increased doses of 6-OHDA did not change the mRNA levels of RC6 (the 20S) and PA28 (11S regulatory subunit of 26S) transcripts, whereas the expression of the p112 (19S ATP-dependent regulatory subunit of 26S) subunit showed a moderate, but significant increase (25). However, the results of the present study supported the autophagy-related gene expression alteration. In the induction group, ATG101 gene was not expressed and ATG13, ATG14L, and VPS34 genes were downregulated in comparison with the control groups. Moreover, Western blotting showed that in the ipsilateral substantia nigra of both hemispheres of the sham and control groups, ATG101 was expressed at the protein level. However, in the ipsilateral substantia nigra (of lesioned-hemisphere) of the PD model group, the results showed no ATG101 protein expression in the 6-OHDA-treated rats 30 days post-lesion induction. In addition, in our recent study, ATG10 gene expression dysregulation was shown and discussed (26). This gene is involved in the initiation phase of autophagy and in the current study, other important ATGs were investigated which were critical for a dopaminergic cell to fulfil

autophagy at a proper level.

The novelty of this study is that despite discussions on the prominent role of autophagy in PD in former studies, its associated-gene expression dysregulation involved in the autophagosome formation was remained largely unknown in the course of disease pathogenesis. In this study, the autophagy-related gene expressions in the PD rat model were investigated. It seems that the main consequence of ATG101 protein loss would be a defective autophagosome formation and shutdown of the autophagy process. This gene is involved in the ULK complex formation and its downregulation comprises ULK complex actions on VPS34 activation, causing autophagy malfunction and intracellular dysfunctional protein aggregation. Thus, ATG101 downregulation may play an important role in the pathogenesis of PD. Using hormones and drugs are effective in cellular protection (27, 28), extracellular hemostasis (29), and neurite outgrowth (30), and in the autophagy-related disorders using such drugs may be promising in the near future. The results of the current study showed that autophagy deregulation occurs in the initial stages of the process in the rat model of PD. More investigations are needed to reveal the full spectrum of gene expression levels involved in the defective autophagy induced-PD.

#### Conflict of Interests

Authors have no conflict of interests.

#### Ethical Issues

The animal studies were carried out in accordance with the Helsinki Declaration on animal experimentation. The study was also approved by the Ethics Committee of Qazvin University of Medical Sciences (Code of Ethics: IR.QUMS.REC.1394.151).

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