ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

INVESTIGATION OF THE EFFECTS OF *ATP13A2* MUTATIONS ON INTRACELLULAR IRON ACCUMULATION

M.Sc. THESIS

Ezgi ERTEREK

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Program

JULY, 2023

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

INVESTIGATION OF THE EFFECTS OF *ATP13A2* MUTATIONS ON INTRACELLULAR IRON ACCUMULATION

M.Sc. THESIS

Ezgi ERTEREK
(521201113)

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Program

Thesis Advisor: Prof. Dr. Arzu KARABAY

JULY, 2023

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

ATP13A2 MUTASYONLARININ HÜCRE İÇİ DEMİR BİRİKİMİNE ETKİLERİNİN İNCELENMESİ

YÜKSEK LİSANS TEZİ

Ezgi ERTEREK
(521201113)

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Arzu KARABAY

TEMMUZ, 2023

Ezgi Erterek, an M.Sc. student of ITU Graduate School with student ID 521201113, successfully defended the thesis/dissertation entitled "INVESTIGATION OF THE EFFECTS OF *ATP13A2* MUTATIONS ON INTRACELLULAR IRON ACCUMULATION", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Arzu KARABAY** İstanbul Technical University	.....
Jury Members :	**Prof. Dr. Ayça SAYI YAZGAN** Istanbul Technical University	.....
	Prof. Dr. Meral YÜKSEL Marmara University	.....

Date of Submission : 16 June 2023
Date of Defense : 24 July 2023

To my beloved family,

FOREWORD

I met the members of CYTO during my undergraduate studies. I have learnt everything I know in the lab from them, and I am very grateful to each and every one of them. I would like to thank Prof. Dr. Arzu Karabay for giving me the opportunity to meet them and join the lab. I am grateful to her for always helping me in my work and always encouraging me to do more. I would also like to thank Benan Temizci who has always helped me in my undergraduate and graduate life. Of course, I would also like to thank Seren Küçükvardar.

I owe a lot to all the members of CYTO who have always supported me. But I would like to give special thanks to my dear family. I would like to dedicate this thesis to my whole family, especially to my elder brother Erman Benli, who never lacked his financial and moral support.

I would also like to thank my friend Zeynep for her moral support. It was very important for me that she was there for me when I was struggling.

I would like to thank the Research Fund of Istanbul Technical University (ITU-BAP, Project no: 43140) for the financial support for my master thesis project.

July, 2023

Ezgi ERTEREK
(Molecular Biologist)

TABLE OF CONTENTS

Page

ABBREVIATIONS

ATF4 **:** Activating transcription factor 4
ATP13A2 **:** ATPase type 13A2
cDNA **:** Complementary DNA
CHOP **:** C/EBP-homologous protein
DMSO **:** Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
eIF2α **:** Eukaryotic initiation factor-2α
ER **:** Endoplasmic Reticulum
HEK293T **:** Human Embryonic Kidney 293T
HSP **:** Hereditary Spastic Paraplegia
ICC **:** Immunocytochemistry
KLD **:** Kinase, Ligase, DpnI
KRS **:** Kufor-Rakeb Syndrome
MRI **:** Magnetic Resonance Image
mRNA **:** Messenger RNA
NBIA **:** Neurodegenaration with Brain Iron Accumulation
PCR **:** Polymerase Chain Reaction
PD **:** Parkinson's Disease
PERK **:** Protein kinase RNA-like ER kinase
qPCR **:** Quantitative PCR
RNA **:** Ribonucleic acid
ROS **:** Reactive Oxygen Species

SYMBOLS

A	**:** Adenine
α	**:** Alpha
β	**:** Beta
C	**:** Cytosine
°C	**:** Degree Celsius
g	**:** Gram
G	**:** Guanine
µ	**:** Micro
M	**:** Molar
s	**:** Second
T	**:** Tymine

LIST OF TABLES

Page

LIST OF FIGURES

Page

INVESTIGATION OF THE EFFECTS OF *ATP13A2* MUTATIONS ON INTRACELLULAR IRON ACCUMULATION

SUMMARY

Kufor-Rakeb Syndrome (KRS), characterised by spasticity, dementia and supranuclear gaze paresis, is a form of early-onset atypical Parkinson's disease. Autosomal recessive and loss-of-function mutations in the *ATP13A2* gene have been shown to cause KRS. The *ATP13A2* gene is located on chromosome 1 and encodes a large transmembrane protein. ATP13A2 belongs to the P5 type ATPase protein family and is mainly located in the membranes of lysosomes and late endosomes. By transporting polyamines that can chelate iron, ATP13A2 is involved in maintaining iron homeostasis in cells. Because of this function, *ATP13A2* mutations can lead to iron accumulation in cells. In addition, this accumulated iron can lead to cell death because iron deposition can induce endoplasmic reticulum (ER) stress in cells. However, it is controversial whether all *ATP13A2* mutations cause iron deposition in patients based on their magnetic resonance images. In this study, ATP13A2-related iron deposition was analysed at the cellular level. Iron accumulation and its effects on cell viability were studied for three different ATP13A2 mutations, namely frameshift (FS), deletion (del) and missense (MS). These mutations were identified in three different KRS patients. To analyze the effects of these mutations, MCF7 cells overexpressing either wt or mutant ATP13A2 proteins (FS-ATP13A2, del-ATP13A2 and MS-ATP13A2) and primary fibroblasts from patients subjected to $FeCl_3$-$6H_2O$ treatment were used. Iron ions were detected in the cells by Prussian blue staining, and it was observed that frameshift and deletion mutations of *ATP13A2* caused the most iron accumulation in the cells. However, missense *ATP13A2* also contributed to iron deposition compared to control cells expressing wt ATP13A2 protein. On the other hand, iron accumulation is known to lead to cell death via the ER stress pathway. Therefore, cell viability was analysed for these three ATP13A2 mutations. Consistently, most cell death was detected in cells expressing FS-ATP13A2 and del-ATP13A2 in both MCF7 cells and fibroblasts. In addition, the viability of cells overexpressing MS-ATP13A2 was also decreased in association with the iron accumulation. This suggests that mutations in *ATP13A2* contribute to iron accumulation in cells and that this iron accumulation can lead to cell death.

ATP13A2 MUTASYONLARININ HÜCRE İÇİ DEMİR BİRİKİMİNE ETKİLERİNİN İNCELENMESİ

ÖZET

Kufor-Rakeb Sendromu (KRS) erken başlangıçlı atipik Parkinson hastalığının bir şeklidir ve spastisite, demansi ve supranükleer bakış parezisi ile karakterizedir. KRS otozomal resesif bir hastalıktır ve *ATP13A2* genindeki fonksiyon kaybı mutasyonu KRS'ye neden olabilir. *ATP13A2* geni 1. kromozomda bulunur ve büyük bir transmembran proteinini kodlar. ATP13A2, P5-tipi ATPaz protein ailesine aittir ve esas olarak lizozomların ve geç endozomların zarlarında bulunur. Temel olarak üç fonksiyonel bölgesi vardır: ATPase bölgesi, nükleotit bağlanma bölgesi ve otofosforilayon bölgesi. ATP13A2, demirleri şelatlayabilen poliaminlerin taşıması sayesinde hücrelerde demir homeostazının korunmasına yardımcı olur. Bu fonksiyon nedeniyle, *ATP13A2* mutasyonları hücrelerde demir birikimine neden olabilir. Demir birikiminin hücrelerde endoplazmik retikulum (ER) stresini tetikleyebilmesi nredeniyle bu biriken demir hücre ölümüyle sonuçlanabilir. Ancak hastaların manyetik rezonans görüntülerine göre, tüm *ATP13A2* mutasyonlarının hastalarda demir birikimine neden olup olmadığı tartışmalıdır. Farklı tip *ATP13A2* mutasyonlarına sahip hastaların bir kısmının MR görüntülerinde demir birikimi gözlemlenirken, diğer bir kısım hastanın MR görüntüsünde demir birikimine rastlanmamıştır. Ayrıca, demir birikimi ve mutasyon çeşidi arasında bir bağlantı da bulunmamaktadır. Bu tez çalışmasında, çerçeve kayması, delesyon ve yanlış anlam olmak üzere üç farklı homozigot *ATP13A2* mutasyonlarının demir birikimi ve bu demir birikiminin hücre canlılığına etkileri araştırıldı. Bu mutasyonlar üç farklı akraba olmayan KRS hastasında saptandı. Çerçeve kaymasına neden olan mutasyon, hastalarda güdük protein oluşturacak mRNA üretimine neden olmaktadır ve yapılan önceki çalışmalar ile mRNA'sının nonsense-mediated decay (NMD) aracılı mekanizma ile degrade olduğu bilinmektedir. Delesyon mutasyonu ise, ATP13A2 proteinin otofosforilasyon bölgesine denk gelmektedir. DKTGT otofosforilayon motifinin hemen yanında bulunan iki amino asitin (Lösin ve Treonin) delesyonuna sebep olmaktadır. Ayrıca, otofosforilayon motifine ek olarak bu iki amino asitin de türler arasında korunmuş olduğu bilinmektedir. Yanlış anlam mutasyonu ise, 5. transmembrane bölgesine denk gelmektedir ve yine türler arasında korunmuş lösin amino asitinin prolin aminoasitine değişmesine neden olmaktadır. Transmembran bölgesine denk gelmesi nedeniyle, proteinin lokalizsyonunu etkileyebileceği ve yanlış lokalize olan ATP13A2 proteininin işlevini yerine getirememesine neden olabileceğinden dolayı demir birikime sebep olacağı düşünülmektedir. Farklı çerçeve kayması ve yanlış anlam mutasyonları ile yapılan çalışmalarda, hastaların magnetic rezonans görüntülemerinde bazal ganglialarında demir birikimi bazı hastalar için gözlenirken bazı hastalar için gözlenememiştir. Bu çalışmanın amacı ise, farklı *ATP13A2* mutasyonları ile ilişkili demir birikimi hücresel düzeyde analiz edilmesidir ve varsa biriken hücresel demirlerin hücre ölümüne neden olup olmadığının araştırılmasıdır. Bu amaç doğrultusunda, hastalardan iğne biyopsisi ile doku örnekleri alınarak primer fibroblast hücre hatları oluşturulmuştur. Aynı işlem sağlıklı bir bireyden doku alınarak

tekrarlandı ve sağlıklı bireyden elde edilen fibroblast hücreleri kontrol amaçlı kullanılmıştır. Fibroblast hücrelerine ek olarak, yabanıl tip veya mutant ATP13A2 proteinleri MCF7 hücrelerinde aşırı ifade edilerek, *ATP13A2* mutasyonlarının spesifik etkisi araştırılmıştır. ATP13A2 proteinlerinin MCF7 hücrelerinde aşırı ifade edilebilmesi için, moleküler klonlama yöntemi ile wt-ATP13A2, FS-ATP13A2, del-ATP13A2 ve MS-ATP13A2 konstraktları elde edilmiştir. Moleküler klonlamada, yabanıl tip ATP13A2 cDNA'sının elde edilmesi için HEK293T hücrelerinden RNA izole edilmiştir ve cDNA'ya dönüştürülmüştür. Çerçeve kayması mutasyonunu içeren ATP13A2 cDNA'sı ise hasta hücrelerinin RNA'sından elde edilmiştir. Yabanıl tip ve çerçeve kayması mutasyonuna sahip ATP13A2 cDNA'ları tasarlanan primerler kullanılarak çoğaltılmıştır. Ardından, ilgili restriksiyon enzimleri kullanılarak, cDNA'lar ve vektör kesilmiştir. Kesilen insert ve vektörler, ligasyon reaksiyonu ile birleştirilerek wt-ATP13A2 ve FS-ATP13A2 konstraktları elde edilmiştir. Ardından, del-ATP13A2 ve MS-ATP13A2 konstraktları için yönlendirilmiş mutagenez metodu kullanılmıştır ve tasarlanan primerler aracılığı ile ilgili mutasyonlar yabanıl tip ATP13A2 sekansı içerisine entegre edilmiştir. Moleküler klonlama sonrasında, insert sekanslar Sanger sekanslama tekniği ile kontrol edilmiştir. Daha sonra uygulanacak yöntemler için, öncelikle fibroblast hücrelerinde ATP13A2 mRNA seviyeleri ve MCF7 hücrlerinde aşırı ifade edilmiş ATP13A2 proteinlerinin varlığı incelenmiştir. ATP13A2 mRNA seviyeleri kıyaslandığında, çerçeve kayması mutasyonuna sahip ATP13A2 mRNA seviyesinin kontrol ve diğer mutasyonlara sahip ATP13A2 mRNA seviyelerinden önemli ölçüde az olduğu gözlenmiştir. Çerçeve kayması mutasyonuna sahip ATP13A2 mRNA'sının NMD mekanizması ile degrade olması, çerçeve kayması mutasyonuna sahip fibroblastlardaki ATP13A2 mRNA'sının daha az gözlenmesini açıklamaktadır. Ardından, ATP13A2 proteinlerinin, MCF7 hücrelerinde aşırı ifade edilmesi kontrol edilmiştir. Bunun için, western blot ve immunocytochemistry (ICC) analizi kullanılmıştır. ATP13A2 proteinlerinin başarılı bir şekilde MCF7 hücrelerinden aşırı ifade edildiği doğrulanmıştır. Daha sonra, fibroblast ve MCF7 hücrelerinde demir birikimi gözlemlenmiştir. Bu amaç doğrultusunda, yabanıl tip veya mutant ATP13A2 proteinlerini aşırı eksprese eden MCF7 hücreleri, $FeCl_3$-$6H_2O$ muamelesi sonrası kullanılırken, hasta fibroblastlarına $FeCl_3$-$6H_2O$ uygulaması yapılmamıştır. Hasta fibroblastları, hücrelerde demir birikimine neden olabilecek *ATP13A2* mutasyonlarını hali hazırda bulundurduğundan dolayı, hücrelerdeki demir birikimini gözlemlemek için daha fazla demir uygulamasına ihtiyaç olmadığı düşünülmüştür. Prusya mavisi boyama yöntemi ile hücrelerdeki demir iyonları tespit edilmiştir ve hücrelerde en fazla demir birikimine *ATP13A2*'nin çerçeve kayması ve delesyon mutasyonlarının neden olduğu görülmüştür. Bununla birlikte, yanlış anlamlı ATP13A2 proteini de, yabanıl tip ATP13A2 proteinlerini eksprese eden kontrol hücreleri ile karşılaştırıldığında demir birikimine katkıda bulunmuştur. Yapılan önceki çalışmılarımızda, demir birikiminin hücre ölümü ile sonuçlandığı bilindiğinden dolayı, hücrelerin farklı $FeCl_3$-$6H_2O$ konstrasyonlarındaki hücre canlılığı farkları analiz edilmiştir. Hem MCF7 hücrelerinde hem de fibroblastlarda FS-ATP13A2 ve del-ATP13A2'yi eksprese eden hücrelerde en fazla hücre ölümü tespit edilmiştir. Ek olarak, MS-ATP13A2'nin eksprese edildiği hücrelerin canlılıkları da demir birikimine bağlı olarak azaldığı bulunmuştur. Sonuç olarak, *ATP13A2*'nin farklı tip mutasyonlarının hücrelerde demir birikimine katkıda bulunduğu ve bu demir birikimlerinin hücre ölümüne neden olduğu anlaşılmıştır. Ek olarak, yapılan incelemeler doğrultusunda, FS-ATP13A2 ve del-ATP13A2 proteinlerinin, MS-ATP13A2 proteinine kıyasla, hücrelerde daha fazla demir birikimine ve buna bağlı daha fazla hücre ölümüne neden olduğu bulunmuştur ve bu bulguların, hastalarda

hastalığın başaldığı zaman ile tutarlı olduğu saptanmıştır. FS-ATP13A2 ve del-ATP13A2 mutasyonlarına sahip hastalarda hastalık yaklaşık olarak 10 yaşlarında ortaya çıkmıştır, MS-ATP13A2 mutasyonuna sahip hastada ise 40 yaşlarında ilk semptomlar görülmüştür. Ayrıca, yapılan araştırmalar sonucunda farklı *ATP13A2* mutasyonlarının demir birikimine neden olduğu saptandığından dolayı, KRS'nin NBIA hastalık grubuna dahil edilmesi gerektiği düşünülmektedir.

1. INTRODUCTION

1.1 Rare Neurodegenerative Diseases

Neurodegeneration identified as loss of structure and function of neurons causes neurodegenerative diseases (Cannon & Greenamyre, 2011). In such diseases, neurons which lose their functions ultimately die. Some of the neurodegenerative disorders appear sporadically, however some of them result from genetic pathological mutations (Price vd., 1998). In these conditions, the diseases are inherited from parents to their children. Since neurodegeneration progression is not reversible, it is considered that these diseases are incurable (Salman et al., 2021). However, some of the mental or physical symptoms can be relieved with the help of specific treatments. Molecular-based research may help to detect and improve these treatments for neurodegenerative diseases.

Any disease with prevalence less than 1/2000 is known as a rare disease in European countries (Aymé & Schmidtke, 2007). Besides they are generally debilitating and life-threatening, they usually affect multiple systems. The rare diseases have mostly genetic causes. When the disease is inherited as an autosomal recessive form, consanguineous marriages, which is common in Turkey, increase the risk of appearance (Aksu, 2019).

1.2 Kufor-Rakeb Syndrome

Kufor-Rakeb syndrome (KRS) is a rare neurodegenerative disease which is inherited in autosomal recessive manner (Ramirez et al., 2006). It is a juvenile-onset atypical form of Parkinson's disease (PD). The disease is named after Kufor Rakeb, which is a town in Jordan as it is first identified there. KRS was first reported by Najim Al-Din and colleagues in 1994. The disease is characterised with progressive PD symptoms, supranuclear gaze paresis, spasticity and dementia (Kaul et al., 2016). It is known that neurodegenerative diseases share some clinical features. Likewise, some of the symptoms of KRS are similar with Parkinson's disease, amyotrophic lateral sclerosis

(ALS), hereditary spastic paraparesis (HSP) and neuronal ceroid lipofuscinosis (NCL).

Moreover, there are debating outcomes about whether KRS is classified as Neurodegeneration with Brain Iron Accumulation (NBIA). Schneider and colleagues suggested that KRS should be classified as NBIA type 3 since they determined iron accumulation by MRI (Magnetic Resonance Imaging) T2 in the basal ganglia of a patient suffering from KRS (2010). There are additional findings that support Schneider and colleagues' proposal (Brüggemann et al., 2010; Martino et al., 2015). Otherwise, Fong et al. (2011), Santoro et al. (2011) and Erro et al. (2019) had detected no iron accumulation in the basal ganglia of different KRS patients based on their MRI T2. Hence, the fact that KRS is classified as NBIA has not been cleared, yet.

1.3 Molecular Mechanism of Kufor-Rakeb Syndrome

KRS is caused by the mutations of *ATP13A2* (*ATPase type 13A2*) gene, which is found in the p arm of chromosome 1. The *ATP13A2* gene was first associated with KRS, which is characterised by pyramidal degeneration, upward gaze palsy, and cognitive disorders in patients because of the detected autosomal recessive mutation (Ramirez et al., 2006). There are several recorded mutations of *ATP13A2* causing KRS, HSP or PD (Kırımtay, 2021).

The *ATP13A2* gene has 29 exons and encodes a protein which has 1180 amino acids. ATP13A2 protein is a large membrane protein belonging to the P5-type ATPase family. It has 10 transmembrane domains and both terminals are oriented to cytoplasm (Figure 1.1). Moreover, ATP13A2 protein has 3 functional domains which are found in cytoplasm; actuator, phosphorylation, nucleotide binding domains (Holemans et al., 2005). ATP13A2 protein is expressed co-translationally in the endoplasmic reticulum (ER) membrane, after that it is localised to membranes of lysosomes and late endosomes by vesicular transporting. The function of the ATP13A2 protein is transporting cations from cytoplasm to the extracellular environment (Ramirez et al., 2006). Hence, it prevents cation accumulation in cells. ATP13A2 can enable the transport of cations themselves or chelating agents of the cations (Tan et al., 2011; van Veen et al. 2020).

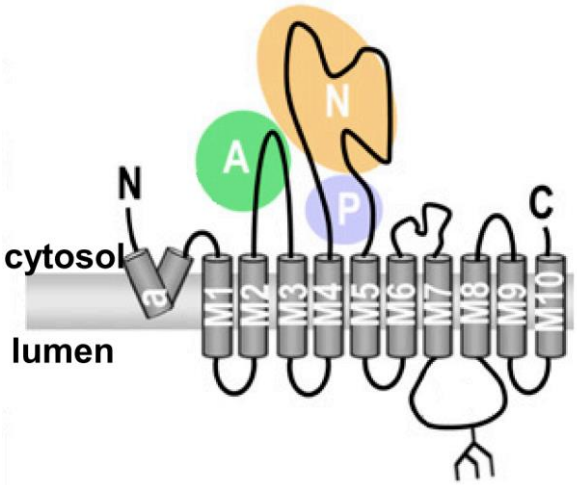

Figure 1.1: Schematic representation of ATP13A2 transmembrane protein. Transmembrane domains of ATP13A2 are shown with "M". Also, functional domains are found in cytosolic face of the protein, and they are indicated as A: ATPase domain, N: nucleotide binding domain and P: autophosphorylation domain.

Iron can be chelated by polyamines which are small ubiquitous molecules. Polyamines are essential for cellular activities such as cell growth (Gaboriau et al., 2004). ATP13A2 protein is mainly responsible for polyamine transport hence, it contributes to maintaining iron hemoastasis. When a polyamine binds to ATP13A2, it undergoes conformational change and transients to dephosphorylated state. In this state, ATP13A2 basicly opens outward and releases the polyamine (Im Sim et al, 2021). With the help of ATP13A2, polyamines can be transported from lysosomes and late endosomes to cytosol.

Iron is a cofactor needed for several biochemical processes such as oxygen transporting, cellular respiration, tricarboxylic acid cycle, lipid metabolism, gene regulation and DNA synthesis. On the other hand, it could also be dangerous as a cofactor of free-radical reactions. Therefore, the amount of iron ions must be controlled and regulated strictly in cells (Cairo et al., 2006).

ATP13A2 protein helps maintain iron homeostasis by transporting the chelating agents of iron ions (Spataro vd., 2019; Rinaldi vd., 2015). Therefore, the dysfunction of ATP13A2 can cause iron deposition in cells. Additionally, iron ions can be accumulated in the basal ganglia of patients due to unfunctional ATP13A2 protein in neurodegenerative disorders such as KRS.

Iron deposition can cause cell death via upregulating reactive oxygen species (ROS) (Tam et al., 2022). Autophagy caused by iron accumulation can be induced by activating transcription factor 4 (ATF4) pathway (Figure 1.2). The ATF4 pathway is initiated by phosphorylation of protein kinase RNA-like ER kinase (PERK). Activated PERK phosphorylates eukaryotic initiation factor-2α (elF2α). Phosphorylated elF2α leads to translocation of ATF4 to the nucleus and ATF4 initiates the expression of C/EBP-homologous protein (CHOP). In normal physiological conditions, CHOP is expressed at a limited level, and the increasing level of CHOP causes cells to die (Szegezdi et al., 2006).

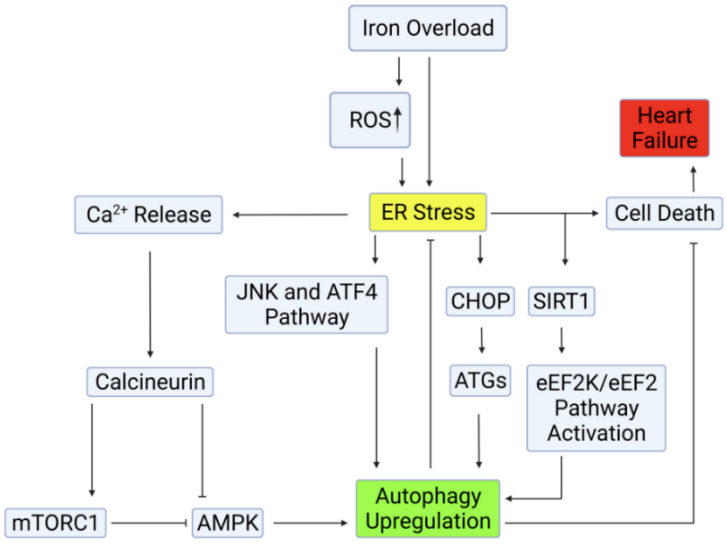

Figure 1.2: Pathway of cell death due to iron overload. Iron accumulation triggers ER stress and at the downstream, levels of ATF4 and CHOP increase (Tam et al., 2022).

1.4 Cases in the Study

In this study, three different *ATP13A2* mutations were investigated, and these *ATP13A2* mutations have been identified to cause KRS in three unrelated families.

1.4.1 Case 1 (Family A)

Case 1 was a 43-years-old male patient, born of second-degree consanguineous marriage. He developed frequent fallings and speech disorder at the age of 10. Then, he had difficulty in walking and loss of fine motor skills. After 12 years of age, different treatments were applied for pre-diagnosis of juvenile Parkinsonism until 25-years-old, however these were not beneficial for the patient. He has been confined to

bed since 30-years-old. Pedigree of the patient is seen in Figure 1.3. Additionally, the patient has a sister who shows similar symptoms.

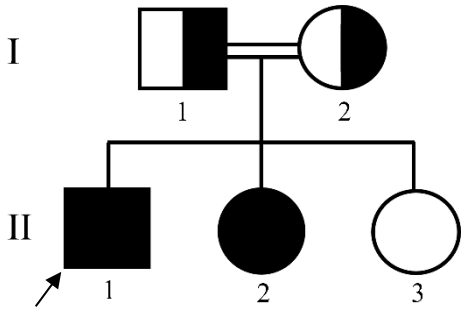

Figure 1.3: Pedigree of the patient A.II.1 who has frameshift *ATP13A2* mutation.

After whole exome sequencing analysis, homozygous c.G1426T, c.1429_1430insAAA and c.1422_1423delTG (p.P474fs) mutations were found in the *ATP13A2* gene (Figure 1.4a) in the patient and his sister, while a heterozygous form of the mutation was detected in the parents. Among these mutations, deletion leads to shifting frame in translation. Frameshifting related to the mutation is shown in Figure 1.4b. Since *ATP13A2* mutation of c.1422_1423delTG leads to a pre-termination stop codon in the exon 15, expressed mRNA is targeted by nonsense mediated decay (NMD) mechanism (Kırımtay et al., 2021). Therefore, ATP13A2 protein is not expressed in the patient cells.

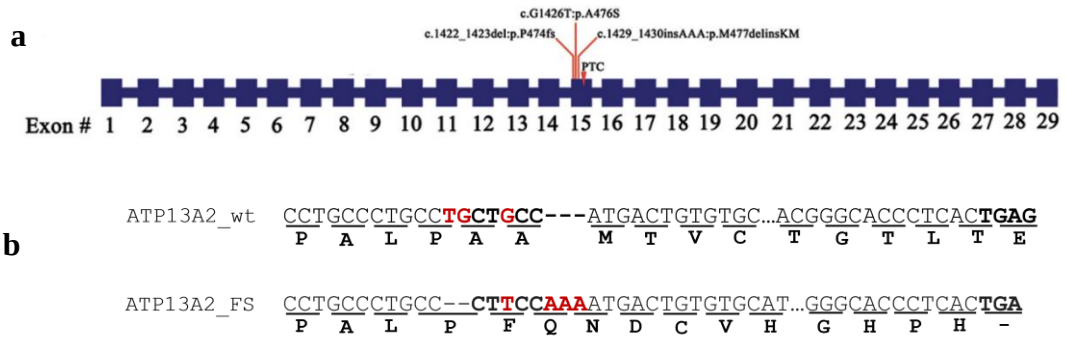

Figure 1.4: Represantation of the frameshift mutation. (a) All mutations in patient and his family are found in exon 15 and deletion mutation causes truncated protein formation due to formation of a premature-termination codon (PTC). (b) Shifting frame is represented after mutations are introduced.

On the other hand, all frameshift mutations do not cause iron deposition in the basal ganglia of patients. While three different frameshift mutations of which mRNAs were degraded by NMD caused iron deposition (Eiberg et al., 2012; Kırımtay et al, 2021), no iron deposition was observed for another one (Park et al., 2011). Additionally, there are also other case studies which showed iron deposition for various frameshift

mutations (Brüggemann et al., 2010; Martino et al., 2015; Schneider et al., 2010). In these studies, NMD was not investigated, hence it is unknown whether their proteins are expressed or not. Moreover, two different case studies showed that no iron deposition was found in the basal ganglia of patients who have different frameshift mutations of *ATP13A2* (Fong et al., 2011; Inzelberg et al., 2018). However, none of these studies searched for cellular level of iron accumulation.

1.4.2 Case 2 (Family B)

Case 2 was a 27-years-old male patient, born of first-degree cousin marriage. At 11 years of age, he suffered from left-sided tremor and stiffness. Then, mental retardation, pronounced parkinsonism and dystonia on the left side started at 13-years-old. Additionally, postural instability was observed in the patient (Figure 1.5).

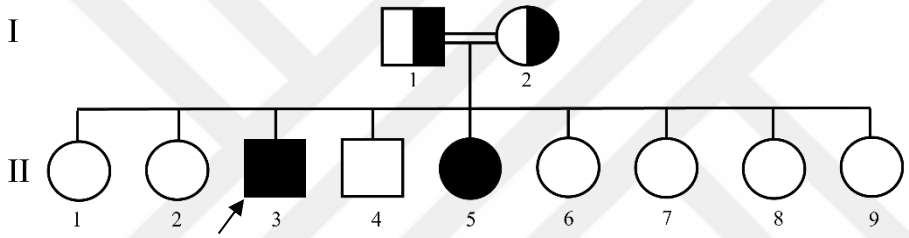

Figure 1.5: Pedigree of the patient B.II.3 who has deletion mutation in *ATP13A2*.

According to results of the whole exome sequencing, a predefined homozygous c.1551_1556delGTGAGG (p.L518_T519del) mutation in *ATP13A2* gene was determined in the patient. Consistently, a heterozygous form of the mutation was found in the parents. The region where predefined mutation is found is in the conserved region due to these amino acids being found in the phosphorylation domain of ATP13A2 protein (Figure 1.6).

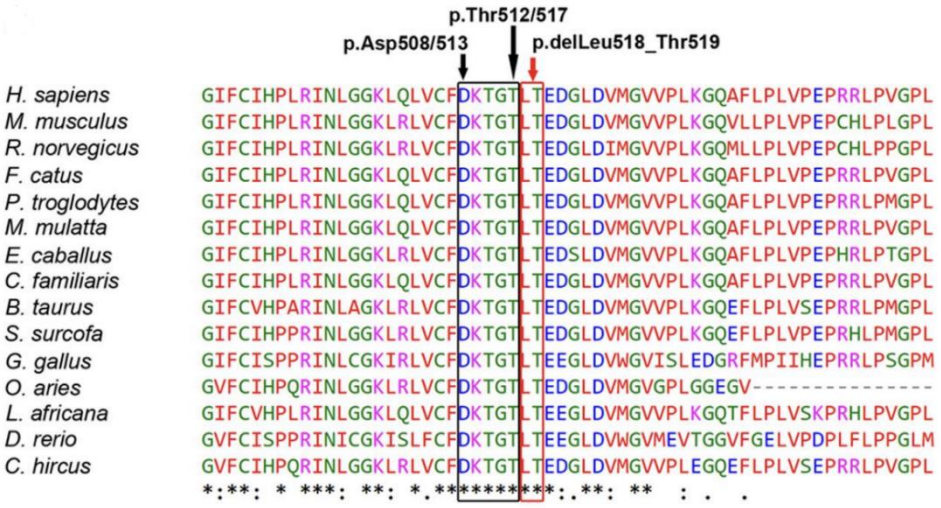

Figure 1.6: Conservative regions of ATP13A2 among species (Estrada-Cuzcano et al., 2017). Auto-phosphorylation motif (DKTGT) and adjacent amino acids are conserved in all given species. (* indicates conservative amino acids, : indicates almost conservative amino acids, . indicates nearly conservative amino acids)

These deleted two amino acids are found near the auto-phosphorylation motif (DKTGT) (Estrada-Cuzcano et al., 2017). Phosphorylation of 508D is necessary for the function of ATP13A2 (Holemans et al., 2015). However, there are not any studies which showed a relation between amino acids deletions of ATP13A2 and iron deposition since another deletion mutation has not been defined yet.

1.4.3 Case 3 (Family C)

Case 3 was a 48-years-old male patient who was also born of second-degree consanguineous marriage. His pedigree is shown in Figure 1.7. Disease-related symptoms started with gait disturbance and balance disorder at the age of 40. 3 years later, his speech was impaired, and he had to walk with support after 4 years. When considering all the symptoms, he was pre-diagnosed for clinical ataxia. His sister also suffered from similar symptoms. When whole exome sequencing was done, c.2816T>C (p.L939P) missense mutation was found in *ATP13A2* gene.

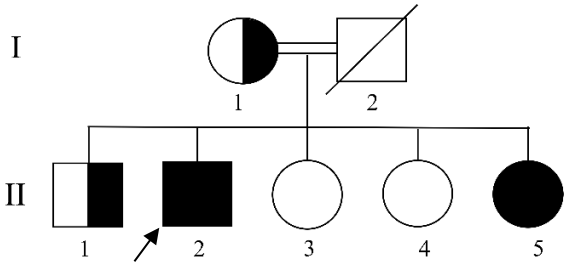

Figure 1.7: Pedigree of the patient C.II.2 who has missense *ATP13A2* mutation.

Detected missense mutation is found in the 5th transmembrane domain. Like deletion mutation in Family B, this one is also found in the conserved region (Figure 1.8). Localization of the protein can be affected by the mutations which are found in the transmembrane domains. Previously, a similar type of mutation was shown to cause ATP13A2 protein to locate in the ER (Park et al, 2011). Moreover, Park and colleagues stated that they did not see any sign of iron deposition. Addition to this, there are two other cases where no iron deposition was found in the patients' MRI for different missense mutations in*ATP13A2* (Erro et al., 2019; Santoro et al., 2011). Nevertheless, it was reported that another missense mutation in *ATP13A2* causes iron deposition in several brain areas of a patient (Chien et al., 2021).

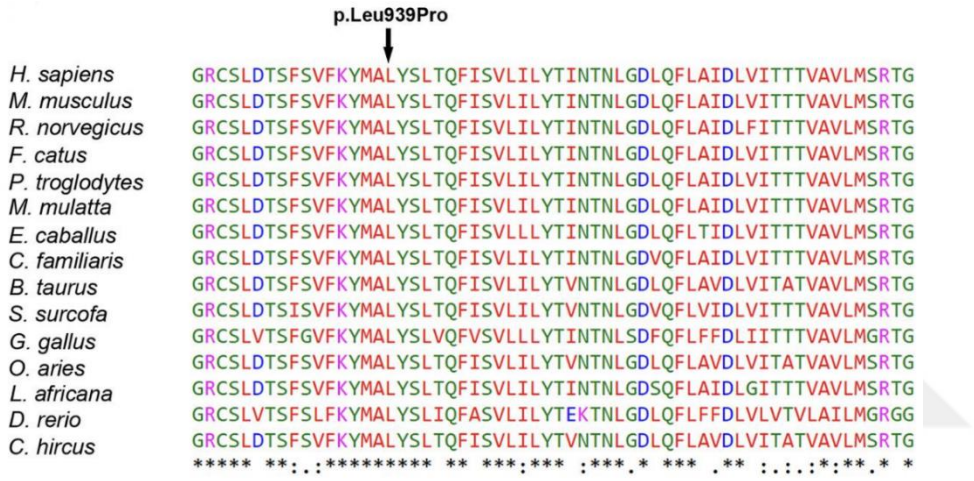

Figure 1.8: Conservative regions of ATP13A2 among species. Leucine in 939th position and neighboring amino acids are seen strongly conservative. (* indicates conservative amino acids, : indicates mostly conservative amino acids, . indicates nearly conservative amino acids)

1.5 Aim of the Study

The cellular effects of different *ATP13A2* mutations were investigated in this study. All mutations which were studied in this thesis are shown in Figure 1.9. Since ATP13A2 protein helps maintaining iron hemoastasis in cells, loss of function mutation in ATP13A2 can cause iron imbalance in cells. According to previous studies, the effect on iron accumulation of mutant ATP13A2 is not clear. Hence, the study with three different types of mutations can contribute to understand the effect of mutant ATP13A2 proteins on iron deposition in cells.

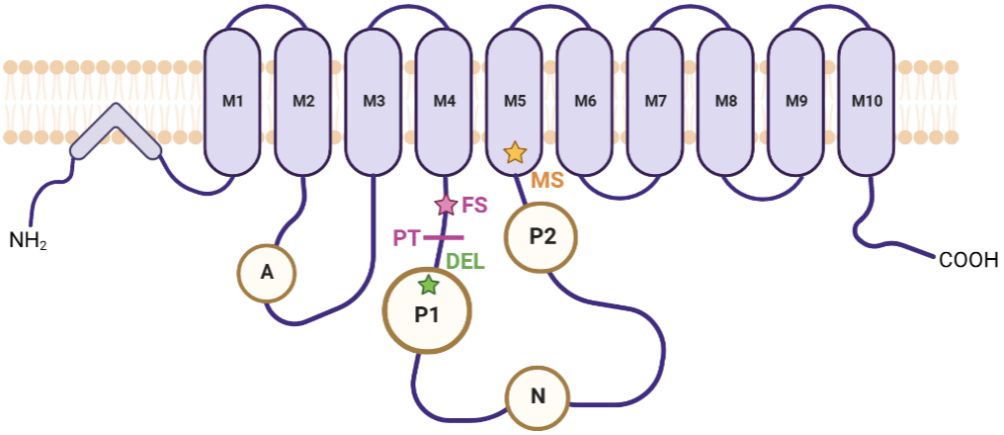

Figure 1.9: Locations of all studied *ATP13A2* mutations. While FS (pink) and Del (green) mutations are found in cytosolic domain of the protein, MS (yellow) mutation is found in transmembrane domain (M). FS mutation causes truncated protein expression due to formation of a premature termination codon (PTC). Del mutation is found in the phosphorylation domain (P1). Other domains are indicated as A and N for actuator and nucleotide binding domain, respectively.

The aim of this study was to understand the how different ATP13A2 mutant proteins contribute to iron accumulation and if the cell viability can be affected due to accumulated iron. It was studied because loss of function mutation in ATP13A2 causes KRS and whether this neurodegenerative disease can be classified as NBIA was not clear.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory equipment

Appendix A displays the laboratory equipment used in this study.

2.1.2 Commercial chemicals and kits

Appendix B displays the chemicals, buffers, enzymes and kits used in this study.

2.1.3 Preparation of buffers

Appendix C displays the preparation of buffers used in this study.

2.1.4 Vector and primers

Appendix D displays the vector and primers used in this study.

2.2 Methods

2.2.1 Tissue culturing

Tissues of patients which were taken with needle biopsy were washed in 3 ml PBS before breaking into pieces with a lancet. Half of the sheared tissue was incubated in dispase II at 37 ℃ for 1 hour and the remaining tissues in the 60 mm cell culture dish were dried at 37 °C for 1-2 hours. After incubation, dispase II was removed with centrifugation at 1000 rpm for 5 minutes after 5 ml addition of DMEM with 20% FBS and %1 penicillin-streptomycin. Pellet was dissolved in 4 ml of DMEM and transferred to a 60 mm cell culture dish. Also, 4 ml of DMEM was added to dried tissues. All cultured tissues were incubated at 37 °C with 5% CO_2.

2.2.2 Molecular cloning

To overexpress ATP13A2 proteins in MCF7 cells, constructs including wt and mutated ATP13A2 cDNA were prepared. These ATP13A2 cDNAs were inserted in pcDNA3.1(+)/myc-His A vector to obtain myc/His tagged ATP13A2 proteins.

Firstly, $1x10^6$ HEK293T cells were seeded in a 100 mm cell culture dish for cDNA of wt-ATP13A2. Also, cDNA of ATP13A2 with frameshift mutation was obtained from the fibroblast culture of the patient's cells. $0.2x10^6$ of fibroblast cells of A.II.1 were

seeded in 100 mm cell culture dish. After the cells reached to proper density, cells were scraped with 3 ml TBS. After centrifugation with 1500 rpm for 7 minutes, pellets of cells were used for RNA isolation which is made with RNA isolation kit (Macherey-Nagel; MN, 740955.50). Then, cDNAs of 1 mg total RNAs from healthy and three different patient samples were obtained with ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs; NEB, E6560) with Oligo d(T)23 VN primer.

cDNAs of *ATP13A2* were amplified with the help of PCR. Q5® High-Fidelity DNA Polymerase (NEB, M0491) was used and the reaction mix of PCR is shown in Table 2.1.

While cDNA of wt-*ATP13A2* was amplified with *ATP13A2* forward and wt-*ATP13A2* reverse primers with PCR conditions which is shown in Table 2.2, cDNA of FS-ATP13A2 was amplified with *ATP13A2* forward and FS-*ATP13A2* reverse primers according to PCR conditions which is shown in Table 2.3.

Table 2.1: PCR ingredients and their amounts.

Ingredients	Final Concentration	Volume in PCR
Q5® Reaction Buffer	1X	5 µl
dNTP	10 mM	0.5 µl
Forward Primer	10 µM	1.25 µl
Reverse Primer	10 µM	1.25 µl
cDNA	50 ng	1 µl
Q5® High-Fidelity DNA Polymerase	20 units/ml	0.25 µl
dH_2O	-	up to 25 µl

Table 2.2: PCR conditions for wt-ATP13A2.

Step	Temperature (°C)	Duration (s)	Cycle
Initial Denaturation	98	30	
Denaturation	98	10	35
Annealing	72	20	
Extension	72	110	
Final Extension	72	120	
Hold	4	∞	

Table 2.3: PCR conditions for FS-ATP13A2.

Step	Temperature (°C)	Duration (s)	Cycle
Initial Denaturation	98	30	
Denaturation	98	10	
Annealing	70	30	35
Extension	72	75	
Final Extension	72	120	
Hold	4	∞	

After amplification, PCR products were purified with PCR clean-up kit (MN, 740609.50) and the purified DNAs were restricted with double-digestion with relevant enzymes at 37 °C. The restriction procedure is shown in Table 2.4

Table 2.4: Double-digestion procedure.

Ingredients	Insert (x2 reaction)	Vector (x2 reaction)
DNA	700 ng	1 µg
XbaI	1 µl	1 µl
CutSmart Buffer	3 µl	4 µl
dH_2O	up to 30 µl	up to 40 µl
After 2 hours incubation at 37 °C		
EcoRI	1 µl	1 µl
CutSmart Buffer	0.5 µl	0.5 µl
dH_2O	up to 35 µl	up to 45 µl
Additional 1 hour incubation at 37 °C		

Digested products were run on 0.7% agarose gel at 90V for 45 minutes. DNA fragments which were found to be in proper size were extracted from agarose gel with gel extraction kit (MN, 740609.50).

Ligation reactions were prepared according to Table 2.5. While wt-*ATP13A2* was ligated with 1:2 (vector:insert) molar ratio, FS-*ATP13A2* was ligated to vector with 1:11 molar ratio. Prepared reactions were incubated at room temperature overnight.

Table 2.5: Ingredients of ligation reaction.

Ingredients	wt-*ATP13A2*	FS-*ATP13A2*
Vector	75 ng	50 ng
Insert	80 ng	150 ng
10X T4 DNA Ligase Reaction Buffer	2 µl	2 µl
T4 DNA Ligase (NEB, M0202)	1 µl	1 µl
20 mM ATP	1 µl	1 µl
dH_2O	up to 20 µl	up to 20 µl

5 µl of ligated products was transformed to 50 µl of competent *E. coli* DH5a. DNA and competent *E. coli* were incubated on ice for 30 minutes then heat shock was applied at 42 °C for 45 seconds. The mixture was immediately transferred onto ice for 2 minutes. After 450 µl LB Broth was added, it was incubated at 37 °C on a shaker with 180 rpm for 1 hour. Then, the cells were precipitated with a centrifuge at 5000 rpm for 7 minutes. Pellets were dissolved in approximately 100 µl of LB Broth and they were spread on LB Agar including 50 µg/ml ampicillin. LB Agar plates were incubated at 37 °C overnight. After overnight incubation, the presence of the insert in the vector was confirmed with colony PCR. Colonies were dissolved in 20 µl dH_2O and 5 µl of these were incubated at 85 °C for 10 minutes. After this incubation, PCR was prepared with the same conditions. Confirmed colonies were grown in 6 ml of LB broth with 100 µg/ml ampicillin at 37 °C overnight. After growth, bacteria were precipitated by centrifugation at3500 rpm for 8 minutes. The pellets were used for plasmid isolation with kit (MN, 740588.50).

The other constructs were prepared with Q5® Site-Directed Mutagenesis Kit (NEB, E0554). Primers of reactions were shown in Table 2.1 for deletion and missense mutations of *ATP13A2*. Ingredients of reactions and PCR conditions are shown in Table 2.6 and Table 2.7, respectively.

Table 2.6: Site-directed mutagenesis ingredients.

Ingredients	Final Concentration	Volume in PCR
Q5 Hot Start High-Fidelity 2X Master Mix	1X	12.5 µl
Forward Primer	10 µM	1.25 µl
Reverse Primer	10 µM	1.25 µl
DNA	22.5 ng	0.65 µl
dH_2O	-	up to 25 µl

Table 2.7: Site-directed mutagenesis PCR conditions.

Step	Temperature (Deletion mutation)	Temperature (Missense mutation)	Duration (s)	Cycle
Initial Denaturation	98 °C	98 °C	30	
Denaturation	98 °C	98 °C	10	25
Annealing	66 °C	63 °C	25	
Extension	72 °C	72 °C	230	
Final Extension	72 °C	72 °C	120	

After PCR, KLD treatments were prepared with 1 µl of PCR products with 2X KLD reaction mix and 10X KLD enzyme mix in 10 µl. The reaction was incubated at room temperature for 30 minutes. Then, 5 µl of KLD mixture was transformed to 50 µl of E. coli DH5α. Transformed bacteria were plated on LB agar including 50 µg/ml ampicillin. After incubation at 37 °C overnight, colonies were grown in 6 ml of LB broth including 50 µg/ml ampicillin. After that, plasmids were isolated with the plasmid isolation kit (MN, 740588.50).

2.2.3 Western blot

Overexpression of constructs in MCF7 cells were controlled with western blot. MCF7 cells were seeded in a 6-well cell culture plate as $0.5x10^6$ cells/well density. After 24 hours, 3 µg of plasmids were used to transfect the cells with 9 µg of PEI for 1:3 (DNA:PEI) ratio. After 48 hours, the cells were harvested by scraping and cell pellets were obtained by centrifugation. The cell pellets were used for total protein isolation with the RIPA buffer (Santa Cruz, sc-24948). Isolated proteins' concentrations were measured with the Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23227). 50 µg of proteins were loaded on the 10% acrylamide/bis-acrylamide gel (ingredients are given in Table 2.8) after they were denatured at 60 °C for 20 minutes. Then, proteins on the gel were transferred to the nitrocellulose membrane with a wet transfer system.

Table 2.8: Ingredients and their amounts of separating and stacking gels.

Ingredients	Separating gel (10%)	Stacking gel (5%)
40% acrylamide/bis-acrylamide	3.05 ml	0.7 ml
1.5 M Tris (pH 8.8)	3 ml	-
0.5 M Tris (pH 6.8)	-	1.95 ml
10% SDS	120 µl	52 µl
10% APS	120 µl	52 µl
TEMED	12 µl	5.2 µl
dH_2O	3.86 ml	1.85 ml

After that, the membrane was blocked with 5% milk powder prepared in TBS-T. ATP13A2 proteins were detected with ATP13A2 antibody (Santa Cruz, sc-293367) by treatment overnight at 4 °C. After overnight incubation, membranes were washed with TBS-T for 3 times. Then, the membrane was treated with a secondary antibody (LI-COR IRDye anti-mouse 800CW, 1:15000 diluted). Non-specific bindings were removed by washing with TBS-T for 3 times. Membrane was imaged with LI-COR Odyssey CLx and image was analysed with Image Studio Programme.

Overexpression of constructs in MCF7 cells were also controlled with western blot. MCF7 cells were seeded in a 6-well cell culture plate as $0.5x10^6$ cells/well density. After 24 hours, 3 µg of plasmids were used to transfect the cells with 9 µg of PEI for 1:3 (DNA:PEI) ratio. After 48 hours, the cells were harvested by scraping and cell pellets were obtained by centrifugation. Proteins were isolated from cell pellets and their concentrations were measured. 50 µg of proteins were mixed with a buffer including 8 M urea and 50 mM DTT as 1:1 ratio in volume. Heat denaturation was applied at 60 °C for 20 minutes. Then, proteins were transferred from gel to nitrocellulose membrane by wet transfer system. After blocking, the membrane was incubated in myc-mouse primary antibody at 4 °C overnight. Secondary antibody (LI-COR IRDye anti-mouse 680RD, 1:15000 diluted) was used for detection. Membrane image was obtained with LI-COR Odyssey CLx.

2.2.4 Quantitative PCR (q-PCR)

Primary fibroblast cells were seeded as $0.5x10^6$ in 100 mm cell culture dishes. After their density reached the optimum, the cells were collected by scraping. Subsequently, pellets of cells were used for RNA isolation (MN, 740955.50). After that, 500 ng of RNAs were used for cDNA isolation (NEB, E6560). Obtained cDNAs were used for real time PCR. Ingredients of PCR are seen in Table 2.9. Before that, primer-probe mix was prepared as 2X for *ATP13A2* and *β-actin*, separately. In 2X mix, there were 2 µmol probe and 5 µmol primer.

Table 2.9: Ingredients of real time PCR.

Ingredients	Final Concentration	Volume in PCR
Primer-Probe mix	1X	2 µl
LightCycler 480 Probes Master Mix	1X	10 µl
cDNA	25 ng/µl	5 µl
dH_2O	-	3 µl

2.2.5 Immunocytochemistry (ICC)

Overexpression of ATP13A2 proteins were also detected with fluoresant labelled antibodies via confocal microscopy. Firstly, coverslips were covered with 1 ml of poly-L-lysine for 1 hour in 12-well plate after coverslips were washed with ethanol and dried under UV light. Subsequently, MCF7 cells were seeded in 12-well plate as $0.3x10^6$ cells/well density. After 24 hours, cells were transfected with 1.5 µg of plasmids with 1:3 PEI. Cells were fixed at 48^{th} hour of transfection with the help of

4% paraformaldehyde for 15 minutes. Then, coverslips were washed with 1X PBS for 3 times. Cells were blocked with blocking buffer for 1 hour at room temperature before myc-tag primary antibody (rabbit) incubation overnight at 4 °C. Primary antibody was prepared in blocking buffer as 1:200 dilution. After overnight incubation, coverslips were washed before secondary antibody (rabbit-594) and phallocidin (in dilution 1:250 and 1:70, respectively) were applied to cells for 1 hour at room temperature. During washing after incubation, DAPI (in dilution 1:1000) was applied to cells for 10 minutes at room temperature. Then, coverslips were closed on the lam with mounting medium. Prepared coverslips were imaged under confocal microscope (Leica) with 63X objective.

2.2.6 Qualitative analysis of iron accumulation with prussian blue staining

Primary fibroblast cells were seeded in 6-well cell culture plate as $0.1x10^6$ cells/well density. After 48 hours, cells were washed with PBS for 3 times and fixed with 4% paraformaldehyde for 10 minutes. After fixation, cells were again washed with PBS for 3 times. Then, 1:1 mixture of 20% HCl (v/v) and 10% potassium ferrocyanide (w/v) were applied to cells for 30 minutes. After incubation, cells were washed with dH_2O for 3 times. Cells were treated with nuclear fast red as a counterstain for 8 minutes. Then, washed cells remaining in dH_2O were imaged with the brightfield microscope (63X objective). Images were analysed in the ZEN Blue Programme.

MCF7 cells were seeded in 60 mm cell culture dishes as $1x10^6$ density. The next day, 6 µg of plasmids were transfected to cells with 1:3 PEI. After 24 hours, the cells were lifted with trypsin and $0.5x10^6$ of them were recultured to a 6-well cell culture plate with 250 µM $FeCl_3$-$6H_2O$. After 24 hours of treatment, the cells were fixed with 4% paraformaldehyde and stained with the same procedure. Then, the cells were imaged with the brightfield microscope (63X objective).

2.2.7 Quantitative Analysis of iron accumulation with iron assay kit

The concentrations of iron in the MCF7 cells where wt and mutated ATP13A2 proteins were overexpressed were measured with iron assay. MCF7 cells were seeded in 100 mm cell culture dishes as $3x10^6$ density. Two cell culture dishes were used for each construct. Cells were transfected with 12 µg of plasmid with 1:3 DNA:PEI ratio. After 24 hours, cells were treated with 600 µM $FeCl_3$-$6H_2O$. After 24 hours of treatment, cells were lifted with trypsin, and they were counted. Then, collected cells were used

for measurement of iron with the iron assay kit (Abcam, ab83366). Then, iron amounts per $1x10^6$ cells were calculated.

2.2.8 MTT assay

To measure cell viability depending on $FeCl_3$-$6H_2O$ treatments, MTT assay was used. Fibroblast cells were seeded in 96-well cell culture plate as $0.005x10^6$ cells/well density. After 2 days, the cells were treated with increasing dosage of $FeCl_3$-$6H_2O$ in 100 µl DMEM for 24 hours. Then, 10 µl of 5 mg/ml MTT prepared in PBS was added to cells and the cells were incubated at 37 °C for 4 hours. After that, DMEM was removed, and the cells were dissolved in 200 µl of DMSO. Amounts of dissolved formazan salts were measured at 570 nm with the reference filter at 655 nm and the results were analysed.

MCF7 cells were seeded in a 6-well plate and the cells were transfected with 3 µg of plasmids in the next day. After 24 hours of transfection, transfected cells were replated to a 96-well plate as $0.01x10^6$ cells/well density with 500 µM $FeCl_3$-$6H_2O$. After 24 hours of treatment, 10 µl of 5 mg/ml MTT was added to cells and the cells were incubated at 37 °C for 4 hours. Then, DMEM was removed, and the formazan salts were dissolved in 200 µl of DMSO. The concentrations of formazan salts were measured in spectrophotometer and cell viability was analysed.

3. RESULTS

3.1 Sanger Sequencing Results of Cloned ATP13A2 cDNAs

Wild type and frameshift mutant ATP13A2 cDNA sequences were cloned into pcDNA3.1(+)/myc-His A expression vector. cDNA sequences were amplified with polymerase chain reaction with the help of the designed primers (Table A.1). Molecular cloning was carried out with EcoRI and XbaI restriction enzymes. cDNAs and the expression vector were restricted by using these enzymes. Subsequently, they were ligated with T4 ligaze enzyme to obtain constructs. After cloning was confirmed with the help of agarose gel electrophoresis, inserts were sequenced with Sanger sequencing method. Then, deletions and missense mutations on ATP13A2 were made to the wild type *ATP13A2* sequence on a plasmid containing wt ATP13A2 using site-directed mutagenesis. Appendix E displays the results of Sanger sequencings.

3.2 Detection of ATP13A2 mRNA Levels in Fibroblasts and Protein Levels in MCF7 Cells

ATP13A2 mRNA levels of healthy and patients' fibroblasts were analyzed as relatively (Figure 3.1). When mRNA levels of patient fibroblasts were compared with control fibroblast's level, in fibroblasts having FS mutation (A.II.1), it was found to be as almost 3 times lower. On the other hand, there was no significant difference of mRNA levels of the other patients' fibroblasts.

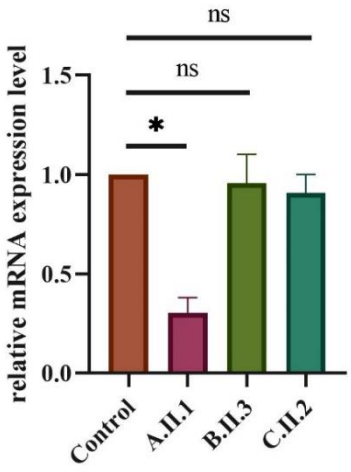

Figure 3.1: Relative ATP13A2 mRNA expression levels in fibroblasts. mRNA levels of patient fibroblasts were normalized to level of control fibroblasts. Data are represented as mean ± SEM. (A.II.1: patient having frameshift mutation, B.II.3: patient having deletion mutation, C.II.2: patient having missense mutation). Significancy was determined with t-test analysis. ** p< 0.01, *** p<0.001

Overexpression of ATP13A2 proteins in MCF cells were detected with western blot with the help of myc-tag primary antibody (Figure 3.2). Protein expression level of the cloned FS-ATP13A2 (shown as truncated ATP13A2) was seen to be the highest.

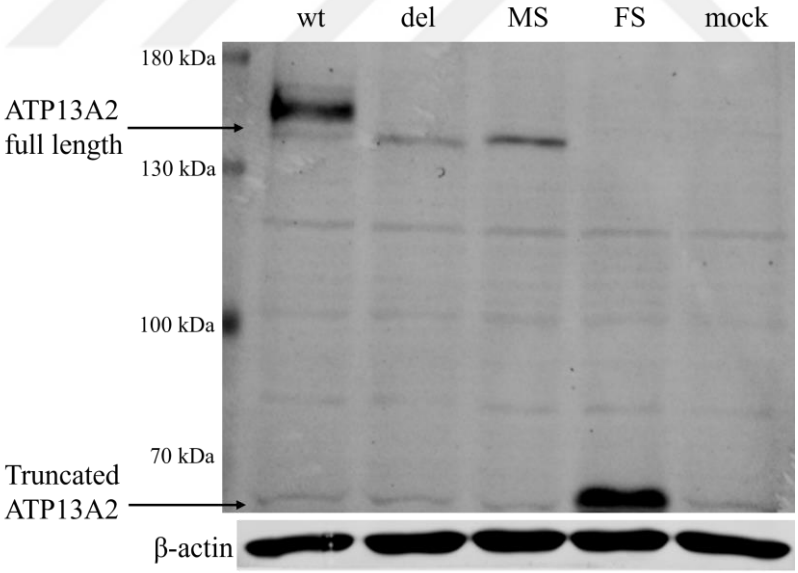

Figure 3.2: Western blot analysis of overexpressed ATP13A2 proteins. Proteins were detected with myc-tag primary antibody. Full length ATP13A2 was observed at almost 150 kDa while truncated form was observed at 55 kDa.

3.3 Observation of Overexpressed ATP13A2 Proteins in MCF7 Cells

Overexpression of wt or mutant ATP13A2 proteins in MCF7 cells was also confirmed with the help of ICC. In this method, myc tags, which were found C-terminal of each

overexpressed ATP13A2 proteins, were stained with fluorescent antibody (Figure 3.3). Myc-tags were shown as red. Also, actin filaments were stained with phallocidin. Actins were shown as green. According to ICC, wt or mutant ATP13A2 proteins were seen successfully overexpressed in MCF7 cells.

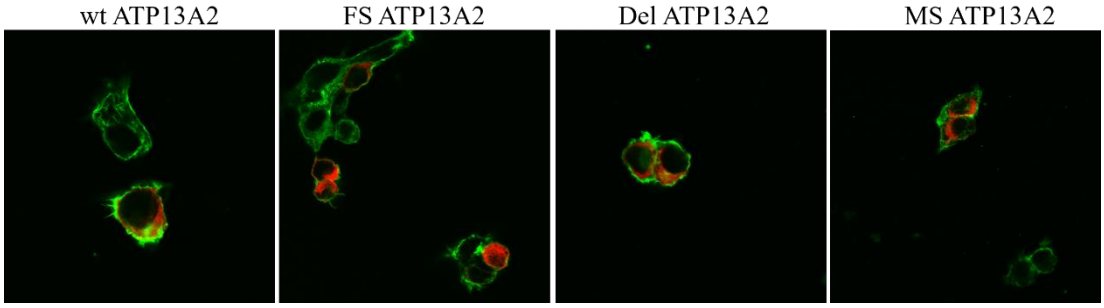

Figure 3.3 Immunocytochemistry analysis of overexpressed ATP13A2 proteins. Proteins were detected with myc-tag primary antibody. Red indicates myc-tagged ATP13A2 and green shows actin stained with phallocidin.

3.4 Accumulated Iron Comparision Among Different Mutant ATP13A2 Proteins Expressing Cells

In order to investigate cellular iron accumulation in the fibroblasts or MCF7 cells, Prussian blue staining was applied. After transfection, MCF7 cells were treated with 250 µM $FeCl_3$-$6H_2O$ for 24 hours. Then, iron ions in the cells were stained with potassium ferrocyanide and nuclear fast red (Figure 3.4). It was observed that there was more accumulated iron in the cells where frameshift or deletion mutant ATP13A2 proteins were overexpressed when compared with wt ATP13A2 overexpressing cells. Although wt ATP13A2 overexpressing MCF7 cells accumulated some iron after $FeCl_3$-$6H_2O$ treatment, this accumulation was not much when compared to mutant ATP13A2 proteins overexpressing cells.

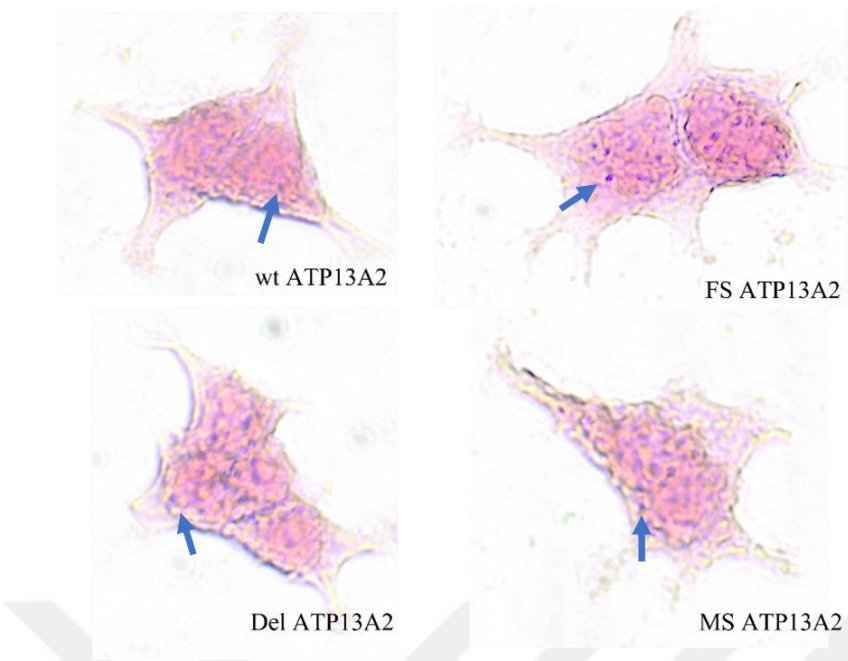

Figure 3.4 Stained intracellular irons in MCF7 cells where wt or mutant ATP13A2 proteins were overexpressed. Intracellular irons were detected with Prussian blue staining after 250 µM $FeCl_3$-$6H_2O$ treatment. Purplish blue points indicate stained iron accumulations which were showed with blue arrows. The cells were imaged with Leica MC170 HD Camera on Zeiss Axio Microscope.

On the other hand, patient fibroblasts were analysed without treatment with $FeCl_3$-$6H_2O$. After they were cultured in phenol red-free DMEM, iron ions were made visible with the help of Prussian blue staining (Figure 3.5). In contrast to overexpression results, no iron accumulation was observed in healthy primer fibroblast. Moreover, the amount of accumulated iron in primer fibroblasts of C.II.2 harboring missense mutation was the least among patients' fibroblasts. Iron deposition of primer fibroblasts of A.II.1 having frameshift mutation and B.II.3 having deletion mutation were seen to be almost similar.

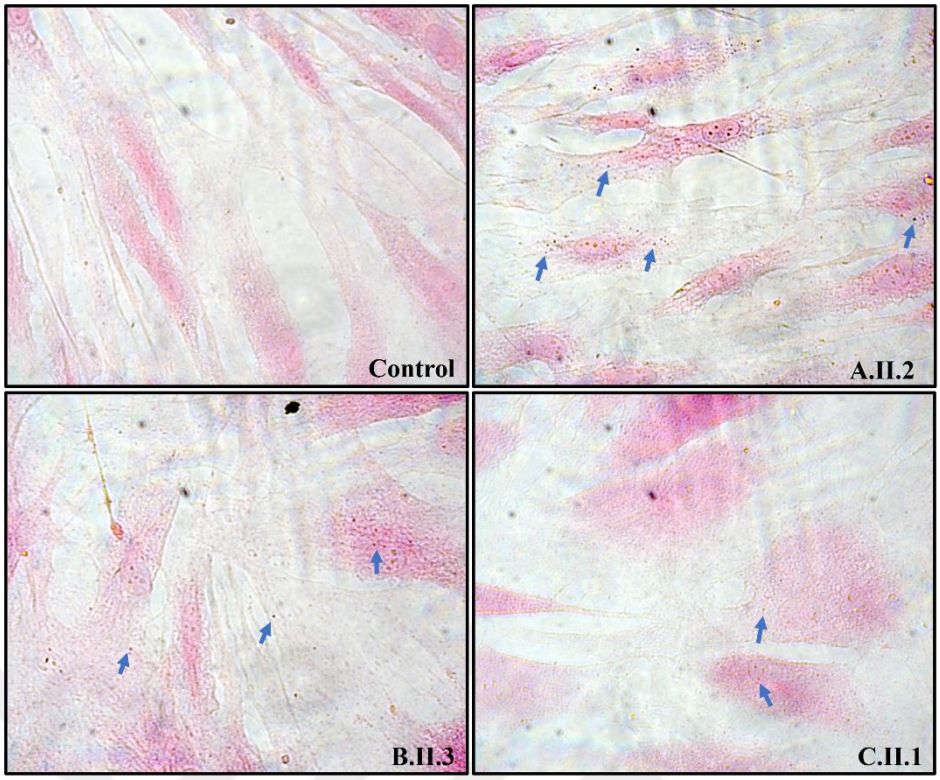

Figure 3.5: Stained intracellular iron in healthy and patient fibroblasts. Intracellular iron accumulations were detected with Prussian blue staining. Stained accumulated iron areas are shown as purplish-blue points and blue arrows indicate these accumulation points in fibroblasts. The cells were imaged with Leica MC170 HD Camera on Zeiss Axio Microscope. (A.II.1: patient having frameshift mutation, B.II.3: patient having deletion mutation, C.II.2: patient having missense mutation)

3.5 Measurement of Quantitative Iron Amount in ATP13A2 Overexpressing Cells

Cellular iron quantities were measured with the iron assay kit (ab83366). In order to measure iron deposition after overexpression of wt or mutant ATP13A2 proteins the cells were treated with 600 µM $FeCl_3$-$6H_2O$ for 24 hours. Quantitative comparison was calculated by normalising to $1x10^6$ cells, as this indicates accumulated iron ions in the cell population (Figure 3.6). After measurement, it was found that FS-*ATP13A2* causes the most iron deposition per $1x10^6$ cells. On the other hand, while MS-*ATP13A2* lead to more iron accumulation than wt-ATP13A2, the amount of accumulated iron in del-*ATP13A2* overexpressed cells was observed less than wt-*ATP13A2* overexpressed cells. However, the changes were not significant.

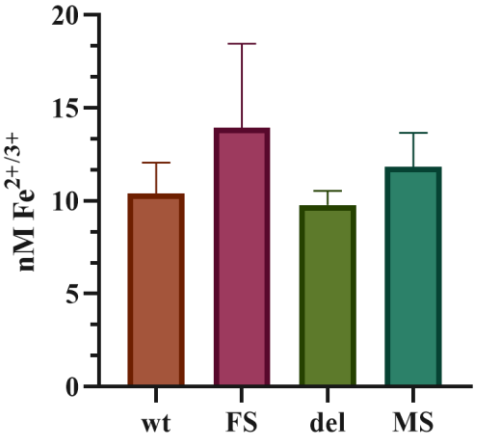

Figure 3.6: Quantitative iron concentrations in MCF7 cells where wt or mutant ATP13A2 proteins were overexpressed. Intracellular irons were measured with Iron Assay Kit (ab83366) and presented as per $1x10^6$ cells. Data are represented as mean ± SEM.

3.6 Effects of Iron Accumulation on Cell Viability

In order to observe the effects of accumulated iron on cell viability, MTT assay was used. Firstly, $FeCl_3$-$6H_2O$ concentration which causes more difference in cell viability between FS-ATP13A2 and wt-ATP13A2 overexpressing MCF7 cells were determined among three different concentrations (Figure 3.7a). For comparison, FS-ATP13A2 was preferred since it was known that this mutation causes cell death for patient's fibroblasts compared to healthy ones which express wild type ATP13A2 when fibroblasts were treated with $FeCl_3$-$6H_2O$ (Kırımtay et al., 2021). After concentration was determined as 500 µM $FeCl_3$-$6H_2O$, all transfected cells were treated for 24 hours before MTT reagent was added. Cell viability was normalised to wt-ATP13A2 overexpressing cells by accepting its viability as 100%. While the most cell death was observed in FS-*ATP13A2* overexpressing cells, MS-*ATP13A2* caused the least cell death among mutations (Figure 3.7b). However, all mutations resulted in significantly reduced cell viability.

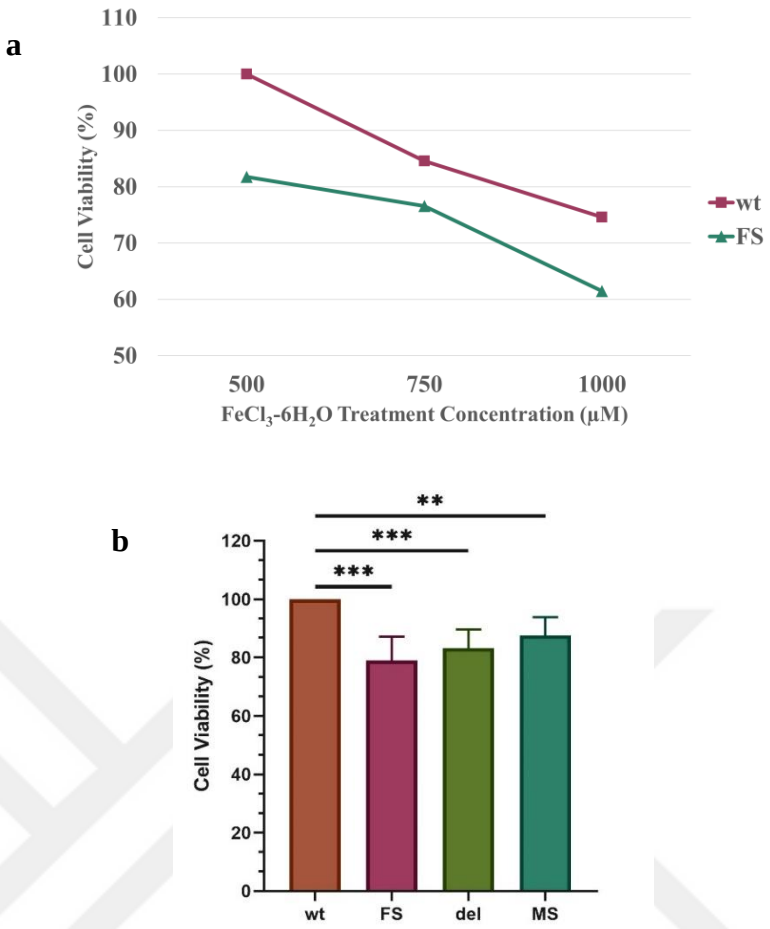

Figure 3.7: Cell viability of MCF7 cells where wt or mutant ATP13A2 proteins were overexpressed after $FeCl_3$-$6H_2O$ treatment. (a) Three different $FeCl_3$-$6H_2O$ concentrations were analysed with wt and FS ATP13A2 overexpressed cells in order to detect proper concentration. (b) Cell viability of MCF7 cells overexpressing wt or mutant ATP13A2 proteins after 500 µM $FeCl_3$-$6H_2O$ treatment. Data are represented as mean ± SEM. Significancy was determined with t-test analysis. ** $p< 0.01$, *** $p<0.001$

Besides, all fibroblasts were treated with different concentrations of $FeCl_3$-$6H_2O$ (Figure 3.8a). Cell viability of untreated condition was accepted as 100% for each type of primer fibroblasts (wt or mutant). Cell viabilities of patients' fibroblasts were less than healthy fibroblasts at each concentration except the concentration of 250 µM $FeCl_3$-$6H_2O$. At the final concentration, cell viability was compared to each other (Figure 3.8b). While healthy fibroblasts' viability was not decreased significantly, viability of all patients' fibroblasts was reduced significantly at 1000 µM $FeCl_3$-$6H_2O$ concentration. Furthermore, cell viability of fibroblasts harboring frameshift (A.II.1) and deletion mutations (B.II.3) were reduced more than the viability of fibroblasts having missense mutation (C.II.2).

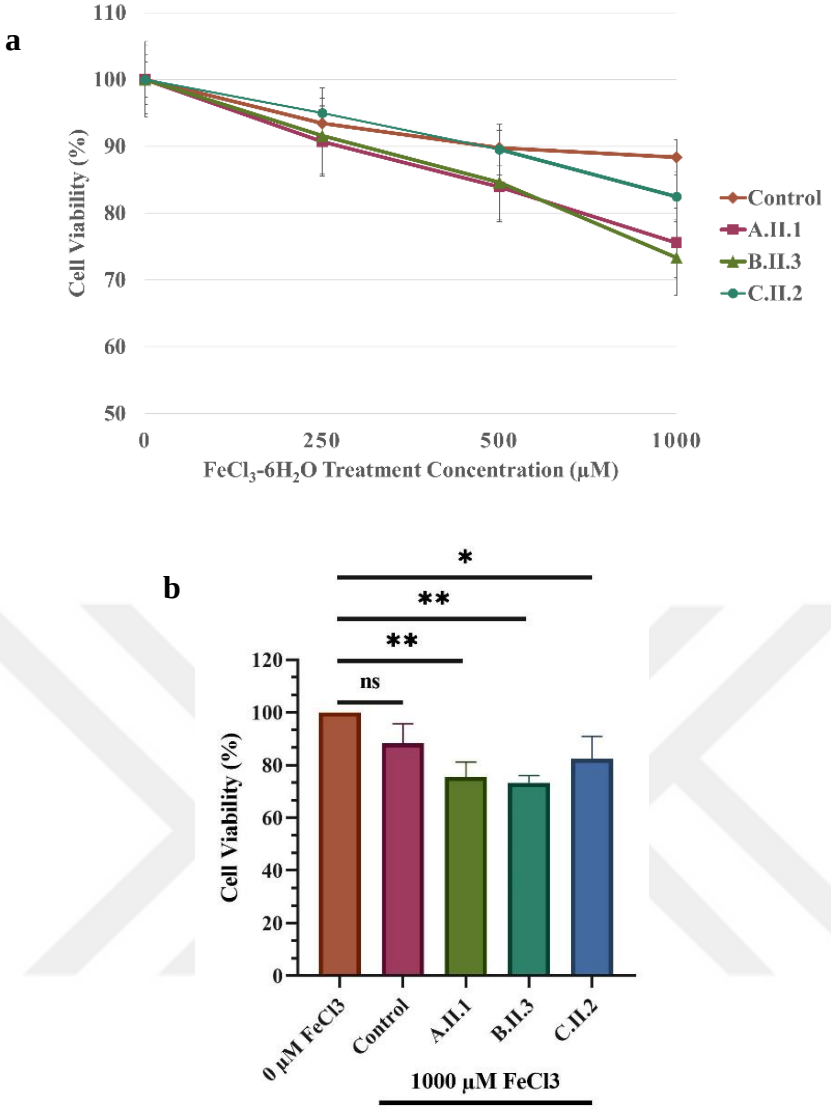

Figure 3.8: Cell viability of healthy and patient fibroblasts after $FeCl_3$-$6H_2O$ treatment. (a) Cell viability was determined after increasing concentrations of $FeCl_3$-$6H_2O$ treatment. (b) Cell viability was analysed at 1000 µM $FeCl_3$-$6H_2O$ concentration comparing with untreated conditions considered as 100% viability. Data are represented as mean ± SEM. Significancy was determined with t-test analysis. * $p< 0.1$, ** $p< 0.01$ (A.II.1: patient having frameshift mutation, B.II.3: patient having deletion mutation, C.II.2: patient having missense mutation)

4. DISCUSSION

Since ATP13A2 protein mediates iron homeostasis in cells, the mutations of *ATP13A2* may disrupt this balance. Accumulated iron could be harmful for cells, and it can force the cells to undergo apoptosis. In Kufor-Rakeb syndrome (KRS), patients may have homozygous or compound-heterozygous mutations in *ATP13A2* (Brüggemann et al., 2010). Although *ATP13A2*, which is a causitive gene of KRS, is related with iron homeostasis in cells, it is not clear whether KRS can clearly be classified as Neurodegeneration with Brain Iron Accumulation (NBIA). Different mutations of *ATP13A2* have different effects on iron deposition. In this study, three different *ATP13A2* mutations were investigated in the aspect of iron accumulation and the effects of the accumulated iron on cell viability. In accordance with this purpose, wild type ATP13A2 construct and, frameshift, deletion or missense mutant ATP13A2 constructs were used for overexpression in addition to primary fibroblasts of patients. These ATP13A2 constructs were prepared successfully with molecular cloning technique. While primary fibroblasts reflect the disease at the cellular level, overexpression helps to understand specific effects of the *ATP13A2* mutations.

When mRNA levels of *ATP13A2* were compared between healthy and patient fibroblasts, mRNA level of patient A.II.1 having frameshift mutation was observed significantly lower due to its mRNA being degraded by nonsense mediated decay mechanism (Kırımtay et al., 2020). The mRNA levels showed that other patients' fibroblasts had similar level of ATP13A2 mRNA with control fibroblasts. Additionally, overexpressed proteins were confirmed with western blot and immunocytochemistry (ICC). According to western blot, the expression level of FS-ATP13A2 protein was seen to be higher than wt-ATP13A2, del-ATP13A2 and MS-ATP13A2. However, protein isolation is harder for large transmembrane proteins because they have more hydrophobic surface area than smaller ones (Carpenter et al., 2008). Whilewt-ATP13A2, del-ATP13A2 and MS-ATP13A2 proteins have 10 tranemembrane domain, FS-ATP13A2 protein has 4 transmembrane domains. Hence, it is possible that the protein expression levels of different ATP13A2 constructs could

be similar but FS-ATP13A2 could have been isolated easier. On the other hand, FS-ATP13A2 construct could be more effective in transfection due to its smaller size. In case of more efficient transfection of FS-ATP13A2 construct, higher level of FS-ATP13A2 protein can be elucidated by effective transfection. Furthermore, MCF7 cells were efficiently transfected with these constructs according to ICC images. Also, untransfected, only actin filaments-stained cells were seen as Myc-tagged ATP13A2 were not overexpressed in these cells. So, in these cells.

ATP13A2 overexpressing MCF7 cells were treated with $FeCl_3$-$6H_2O$ before Prussian blue staining while patients' fibroblasts were not treated, since these fibroblasts already had the mutation that would cause the iron accumulation. Therefore, an extra stimulation was not required for them to accumulate iron. On the other hand, MCF7 cells were expressing mutant ATP13A2 proteins only for 48 hours and this duration may not have been enough for cells to deposit irons in detectable levels in the absence of $FeCl_3$-$6H_2O$ treatment. Consequently, accumulated iron level was detected in wt-ATP13A2 overexpressing MCF7 cells, however control fibroblast did not cause iron accumulation.

Additionally, iron assay was used to measure iron amounts quantitatively. Since an excessive number of cells was required in order to keep standards' range, primer fibroblasts could not be used for this quantitative measurement assay. ATP13A2 overexpressed MCF7 cells were considered as suitable to meet this number of cells. As a result of quantitative measurement, no significant change was detected in $Fe^{2+/3+}$ concentration. Though intracellular Fe^{2+} was detected in untreated MCF7 cells in another studies (Cao et al., 2020), no iron ion concentration could be detected in untreated MCF7 cells in this study. Therefore, MCF7 cells were treated with 600 µM $FeCl_3$-$6H_2O$ before cells were used for measurement. This high dose treatment of $FeCl_3$-$6H_2O$ could affect the iron accumulation in cells, however the possible-difference may not have been observed due to the use of a high $FeCl_3$-$6H_2O$ concentration treatment. On the other hand, it can be said that measured iron amounts in FS-ATP13A2 protein overexpressed cells was seen non-significant due to its high standard deviation.

On the other hand, it is known that accumulated iron could be harmful and deathly for cells (Kırımtay et al., 2020). This cell death could be caused by triggered endoplasmic

reticulum (ER) stress, as it is known that iron overload leads to an increase in reactive oxygen species (ROS) in cells and increased ROS triggers ER stress (Tam et al., 2022). Hence, iron deposition causes cell death. Therefore, changes in cell viability could provide an indication of the accumulated iron levels in the cells. According to the results of the MTT assay to measure the difference in cell viability, the viability of MCF7 cells overexpressing different type of mutant ATP13A2 proteins was decreased significantly after 500 µM $FeCl_3$-$6H_2O$ treatment. It is also a noteworthy finding that the least decrease was observed in MCF7 cells overexpressing MS-ATP13A2 protein among MCF7 cells and fibroblasts from patient C.II.2 harboring the missense mutation among fibroblasts. These cell viability results were consistent with Prussian blue staining in the aspect of that less iron accumulation was found in missense mutant ATP13A2 expressing cells. These results can be interpreted that the missense mutation of ATP13A2 led to less iron accumulation in cells compared to the other mutations.

Furthermore, these differences in iron accumulation and cell viability were consistent with the age of onset in the patients. In the patient with the missense mutation, symptoms of KRS started at the age of 40, but in the patients with the frameshift and deletion mutations, symptoms of KRS started at the age of 10.

As a result, these different *ATP13A2* mutations causes iron accumulation in cells. However, the type of mutation affects the dysfunction of the ATP13A2 protein and therefore the accumulation amount of iron in the cell.

On the other hand, *ATP13A2* mutations could cause tomislocalization of ATP13A2 proteins (Park et al., 2011). These mutant proteins may be stuck in ER due to the mutations. As further study, the localization of wt and mutant ATP13A2 proteins could be detected in cells. If the mutant ATP13A2 proteins get stuck in ER, it would also trigger ER stress (Yorimitsu et al., 2006). Therefore, ER stress proteins could be also studied in the absence of $FeCl_3$-$6H_2O$.

5. CONCLUSIONS

In this study, different ATP13A2 mutations were analyzed to understand their effects on iron accumulation in cells. According to findings, missense mutation in transmembrane domain was not as effective as frameshift or deletion mutations in autophosphorylation domain in the aspect of iron accumulation. However, decreasing in cell viability was detected evidently for all mutation types after treatment of $FeCl_3$-$6H_2O$. It shows that ATP13A2 protein is important to maintain iron balance for cells. Additionally, it can be concludedthat the KRS should be classified as NBIA disease group.

REFERENCES

Aksu, B. (2019). Rare Disease and Orphan Drug Situations in Turkey and around the World. *Aurum Journal of Health Sciences*, *1*(2), 95-109.

Aymé, S., & Schmidtke, J. (2007). Networking for rare diseases: a necessity for Europe. *Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz*, *50*(12), 1477-1483.

Brüggemann, N., Hagenah, J., Reetz, K., Schmidt, A., Kasten, M., Buchmann, I., ... & Klein, C. (2010). Recessively inherited parkinsonism: effect of ATP13A2 mutations on the clinical and neuroimaging phenotype. *Archives of neurology*, *67*(11), 1357-1363.

Cairo, G., Bernuzzi, F., & Recalcati, S. (2006). A precious metal: Iron, an essential nutrient for all cells. *Genes & nutrition*, *1*, 25-39.

Cannon, J. R., & Greenamyre, J. T. (2011). The role of environmental exposures in neurodegeneration and neurodegenerative diseases. *Toxicological Sciences*, *124*(2), 225-250.

Cao, X., Li, Y., Wang, Y., Zhang, X., Yu, T., Zhu, C., & Guan, J. (2020). Curcumin Suppresses Tumorigenesis via Promoting SLC1A5-mediated Ferroptosis in Breast Cancer.

Carpenter, E. P., Beis, K., Cameron, A. D., & Iwata, S. (2008). Overcoming the challenges of membrane protein crystallography. *Current opinion in structural biology*, *18*(5), 581-586.

Chien, H. F., Rodriguez, R. D., Bonifati, V., Nitrini, R., Pasqualucci, C. A., Gelpi, E., & Barbosa, E. R. (2021). Neuropathologic Findings in a Patient with Juvenile-Onset Levodopa-Responsive Parkinsonism Due to ATP13A2 Mutation. *Neurology*, *97*(16), 763-766.

Eiberg, H., Hansen, L., Korbo, L., Nielsen, I. M., Svenstrup, K., Bech, S., ... & Nielsen, J. E. (2012). Novel mutation in ATP13A2 widens the spectrum of Kufor-Rakeb syndrome (PARK9). *Clinical genetics*, *82*(3), 256-263.

Erro, R., Picillo, M., Manara, R., Pellecchia, M. T., & Barone, P. (2019). From PARK9 to SPG78: The clinical spectrum of ATP13A2 mutations. *Parkinsonism & related disorders*, *65*, 272-273.

Estrada-Cuzcano, A., Martin, S., Chamova, T., Synofzik, M., Timmann, D., Holemans, T., ... & Schüle, R. (2017). Loss-of-function mutations in the ATP13A2/PARK9 gene cause complicated hereditary spastic paraplegia (SPG78). *Brain*, *140*(2), 287-305.

Fong, C. Y., Rolfs, A., Schwarzbraun, T., Klein, C., & O'Callaghan, F. J. (2011). Juvenile parkinsonism associated with heterozygous frameshift ATP13A2 gene mutation. *European journal of paediatric neurology*, *15*(3), 271-275.

Gaboriau, F., Kreder, A., Clavreul, N., Moulinoux, J. P., Delcros, J. G., & Lescoat, G. (2004). Polyamine modulation of iron uptake in CHO cells. *Biochemical pharmacology*, *67*(9), 1629-1637.

Holemans, T., Sørensen, D. M., van Veen, S., Martin, S., Hermans, D., Kemmer, G. C., ... & Vangheluwe, P. (2015). A lipid switch unlocks Parkinson's disease-associated ATP13A2. *Proceedings of the National Academy of Sciences*, *112*(29), 9040-9045.

Im Sim, S., von Bülow, S., Hummer, G., & Park, E. (2021). Structural basis of polyamine transport by human ATP13A2 (PARK9). *Molecular cell*, *81*(22), 4635-4649.

Inzelberg, R., Estrada-Cuzcano, A., Laitman, Y., De Vriendt, E., Friedman, E., & Jordanova, A. (2018). Kufor-Rakeb syndrome/PARK9: one novel and one possible recurring Ashkenazi ATP13A2 mutation. *Journal of Parkinson's Disease*, *8*(3), 399-403.

Kaul, A., Franzese, K., & Cohen, J. (2016). Poster 298 ataxia and dysarthria in two siblings with Kufor-Rakeb syndrome: a case report. *PM & R: the journal of injury, function, and rehabilitation*, *8*(9S), S257.

Kırımtay, K. (2021). *Investigation of the effect of ATP13A2 (PARK9) frameshift mutation on the protein function* (Doctoral dissertation, Lisansüstü Eğitim Enstitüsü).

Kırımtay, K., Temizci, B., Gültekin, M., Yapıcı, Z., & Karabay, A. (2021). Novel mutations in ATP13A2 associated with mixed neurological presentations and iron toxicity due to nonsense-mediated decay. *Brain Research*, *1750*, 147167.

Martino, D., Melzi, V., Franco, G., Kandasamy, N., Monfrini, E., & Di Fonzo, A. (2015). Juvenile dystonia-parkinsonism syndrome caused by a novel p. S941Tfs1X ATP13A2 (PARK9) mutation. *Parkinsonism & Related Disorders*, *21*(11), 1378-1380.

Najim Al-Din, A. S., Wriekat, A., Mubaidin, A., Dasouki, M., & Hiari, M. (1994). Pallido-pyramidal degeneration, supranuclear upgaze paresis and dementia: Kufor-Rakeb syndrome. *Acta neurologica scandinavica*, *89*(5), 347-352.

Park, J. S., Mehta, P., Cooper, A. A., Veivers, D., Heimbach, A., Stiller, B., ... & Sue, C. M. (2011). Pathogenic effects of novel mutations in the P-type ATPase ATP13A2 (PARK9) causing Kufor-Rakeb syndrome, a form of early-onset parkinsonism. Human mutation, 32(8), 956-964.

Price, D. L., Sisodia, S. S., & Borchelt, D. R. (1998). Genetic neurodegenerative diseases: the human illness and transgenic models. *Science*, *282*(5391), 1079-1083.

Ramirez, A., Heimbach, A., Gründemann, J., Stiller, B., Hampshire, D., Cid, L. P., ... & Kubisch, C. (2006). Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. *Nature genetics*, *38*(10), 1184-1191.

Rinaldi, D. E., Corradi, G. R., Cuesta, L. M., Adamo, H. P., & de Tezanos Pinto, F. (2015). The Parkinsonassociated human P5B-ATPase ATP13A2 protects against the iron-induced cytotoxicity. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1848*(8), 1646-1655.

Salman, M. M., Al-Obaidi, Z., Kitchen, P., Loreto, A., Bill, R. M., & Wade-Martins, R. (2021). Advances in applying computer-aided drug design for neurodegenerative diseases. *International journal of molecular sciences*, *22*(9), 4688.

Santoro, L., Breedveld, G. J., Manganelli, F., Iodice, R., Pisciotta, C., Nolano, M., ... & Bonifati, V. (2011). Novel ATP13A2 (PARK9) homozygous mutation in a family with marked phenotype variability. *Neurogenetics*, *12*(1), 33-39.

Schneider, S. A., Paisan-Ruiz, C., Quinn, N. P., Lees, A. J., Houlden, H., Hardy, J., & Bhatia, K. P. (2010). ATP13A2 mutations (PARK9) cause neurodegeneration with brain iron accumulation. *Movement Disorders*, *25*(8), 979-984.

Spataro, R., Kousi, M., Farhan, S. M., Willer, J. R., Ross, J. P., Dion, P. A., ... & Katsanis, N. (2019). Mutations in ATP13A2 (PARK9) are associated with an amyotrophic lateral sclerosis-like phenotype, implicating this locus in further phenotypic expansion. *Human genomics*, *13*(1), 1-10

Szegezdi, E., Logue, S. E., Gorman, A. M., & Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO reports*, *7*(9), 880-885.

Tam, E., Reno, C., Nguyen, K., Cho, S., & Sweeney, G. (2022). Importance of Autophagy in Mediating Cellular Responses to Iron Overload in Cardiomyocytes. *Reviews in Cardiovascular Medicine*, *23*(5), 167.

Tan, J., Zhang, T., Jiang, L., Chi, J., Hu, D., Pan, Q., ... & Zhang, Z. (2011). Regulation of intracellular manganese homeostasis by Kufor-Rakeb syndrome-associated ATP13A2 protein. *Journal of Biological Chemistry*, *286*(34), 29654-29662.

van Veen, S., Martin, S., Van den Haute, C., Benoy, V., Lyons, J., Vanhoutte, R., ... & Vangheluwe, P. (2020). ATP13A2 deficiency disrupts lysosomal polyamine export. *Nature*, *578*(7795), 419-424.

Varone, E., Decio, A., Chernorudskiy, A., Minoli, L., Brunelli, L., Ioli, F., ... & Zito, E. (2021). The ER stress response mediator ERO1 triggers cancer metastasis by favoring the angiogenic switch in hypoxic conditions. *Oncogene*, *40*(9), 1721-1736.

Yang, L., Zhou, X., Sun, J., Lei, Q., Wang, Q., Pan, D., ... & Ding, Y. (2020). Reactive oxygen species mediate anlotinib-induced apoptosis via activation of endoplasmic reticulum stress in pancreatic cancer. *Cell death & disease*, *11*(9), 766.

Yorimitsu, T., Nair, U., Yang, Z., & Klionsky, D. J. (2006). Endoplasmic reticulum stress triggers autophagy. *Journal of Biological Chemistry*, *281*(40), 30299-30304.

APPENDICES

APPENDIX A: Laboratory equipment

APPENDIX B: Chemicals and buffers

APPENDIX C: Preparation of buffers

APPENDIX D: Vector and primers

APPENDIX E: Sequences of cloned ATP13A2 cDNAs

APPENDIX A: Laboratory equipments

Autoclave	
Brightfield Microscope	Zeiss, Axio
Cell Culture Plates	NEST
Centrifuge	Allegra 25R Centrifuge Beckman Coulter
Confocal Microscope	Leica TCS SP2
Distill Water	Machine Milli-Q Merc-Millipore
Electrophoresis Systems	Bio-Rad Mini-PROTEAN® Tetra Cell, Bio-Rad
Flat Shaker	Heidolph Doumax 1030
Freezer	Siemens and Uğur (-20 °C) & SANYO (-80 °C)
Heat Block	FOUR E'S Scientific
Ice Machine	Scotsman AF10
Incubator	ESCO
Magnetic Stirrer	ISOLAB Laborgeräte GmbH
Microcentrifuge	Sigma 1-14K
Microplate Reader	Bio-Rad Benchmark Plus
Microwave	Arcelik MD582
pH Meter	Mettler Toledo MP220
Pipettes	Eppendorf 2µ 20 µL 200 µl, 1000 µl
Refrigerator	Siemens (+4 °C)
Rotator	DLAB MX-RL-Pro
Serological Pipette	NEST 5, 10, 25 ml
Spinner	Weightlab intruments, CMV6000
Thermal Cycler	Bio-Rad T100
Transfer System	Bio-rad Mini Trans-Blot Cell System
Visualization	Li-COR Odyssey CLx
Vortex	Heidolph Reax Top
Weighing scale	Precisa 620C SCS Precisa BJ 610C

APPENDIX B: Commercial chemicals, buffers, enzymes and kits

CHEMICALS AND BUFFERS	
Acrylamide / Bisacrylamide Solution	Merck-Millipore
Adenosine triphospate (ATP)	Sigma-Aldrich
Agarose	Multicell
Ammonium Persulfate (APS)	Merck-Millipore
Bovine Serum Albumin (BSA)	Capricorn
Dithiothreitol (DTT)	Merck-Millipore
DMEM	Gibco
Ethylenediamine tetra acetic acid (EDTA)	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Capricorn
Glycine	Merck
Hydrochloric acids (HCl)	Merck-Millipore
Iron (III) Chloride (FeCl3)	Merck-Millipore
Methanol	Merck
MTT Reagent	Merck-Millipore
PBS Tablets	BioShop
Penisillin/Streptomycin (P/S)	Gibco
Potassium Ferrocyanide	Merck-Millipore
RIPA Buffer	SantaCruz
Sodium Chloride	Merck-Millipore
Sodium dodecyl sulfate (SDS)	Merc-Millipore
TAE Buffer (50X)	Bio-Rad
Tetramethylethylenediamine (TEMED)	Merck-Millipore
Tris Base	Sigma-Aldrich
Tween-20	Merck-Millipore
Urea	Sigma-Aldrich

ENZYMES AND KITS	
ACTB Primer and Probe	Roche
BCA Assay	Thermo
cDNA Synthesis Kit	New England Biolabs
Dispase II	Gibco
EcoRI Restriction Enzyme	New England Biolabs
Iron Assay Kit	Abcam
LightCycler® 480 Probes Master	Roche
NucleoSpin gel and PCR Clean-up kit	Macherey-Nagel
NucleoSpin plasmid kit	Macherey-Nagel

NucleoSpin RNA kit	Macherey-Nagel
Q5® High-Fidelity DNA Polymerase	New England Biolabs
Q5® Site-Directed Mutagenesis Kit	New England Biolabs
T4 Ligase Enzyme	New England Biolabs
Trypsine	Gibco
XbaI Restriction Enzyme	New England Biolabs

ANTIBODIES

4′,6-diamidino-2-phenylindole (DAPI)	Invitrogen
Anti-ACTB	Cell Signaling Technology, #4970
Anti-ATP13A2 antibody	Santa Cruz, sc-293367
Anti-Ero1-L*α* antibody	Cell Signaling Technology, #3264
Anti-myc tag antibody	Cell Signaling Technology, #2278
Anti-p-eIF2*α* antibody	Cell Signaling Technology, #9721
IRDye® 680RD Secondary Antibody	Cell Signaling Technology, #5470P
IRDye® 680RD Secondary Antibody	Cell Signaling Technology, #5366S
IRDye® 800CW Secondary Antibody	Cell Signaling Technology, #5257S
IRDye® 800CW Secondary Antibody	Cell Signaling Technology, #5151S
Phallocidin	Eugene
Rabbit-597 Secondary Antibody	Cell Signaling Technology, #8890S

APPENDIX C: Preparation of buffers

SDS-PAGE Running Buffer (10X)

Tris-base ($C_4H_{11}NO_3$)	8.3 g
Glycin ($C_2H_5NO_2$)	144 g
SDS ($NaC_{12}H_{25}SO_4$)	10 g
dH_2O	up to 1 liter

Transfer Buffer (1X)

Tris base ($C_4H_{11}NO_3$)	3 g
Glycine ($C_2H_5NO_2$)	14.4 g
dH_2O	up to 800 ml
Methanol 200 ml	200 ml

TBS (10X)

Tris,base ($C_4H_{11}NO_3$)	24 g
Sodium chloride (NaCl)	88 g
dH_2O	up to 1 liter

TBS-T (1X)

10X TBS Solution	100 ml
Tween-20	0.1 ml
dH_2O	up to 1 liter

Blocking Buffer for Western Blot

Milk powder	5 g
TBS-T	100 ml

Blocking Buffer for Immunocytochemisty

BSA	3 g
Triton X-100	0.1 ml
1X PBS	up to 100 ml

Lurtia-Bertani (LB) Broth

Tryptone	1 g
Sodium chloride	1 g
Yeast Extract	0.5 g
dH_2O	up to 100 ml

Lurtia-Bertani (LB) Agar

Tryptone	1 g
Sodium chloride	1 g
Yeast Extract	0.5 g
Agar	1.5 g
dH_2O	up to 100 ml

APPENDIX D: Vector and primers

Vector which was used in the study is shown in Figure A.1. pcDNA3.1(+)/myc-His A was used for cloning of ATP13A2 cDNAs. Designed primers are seen in Table A.1. Primers were purchased from PRZ Biotech.

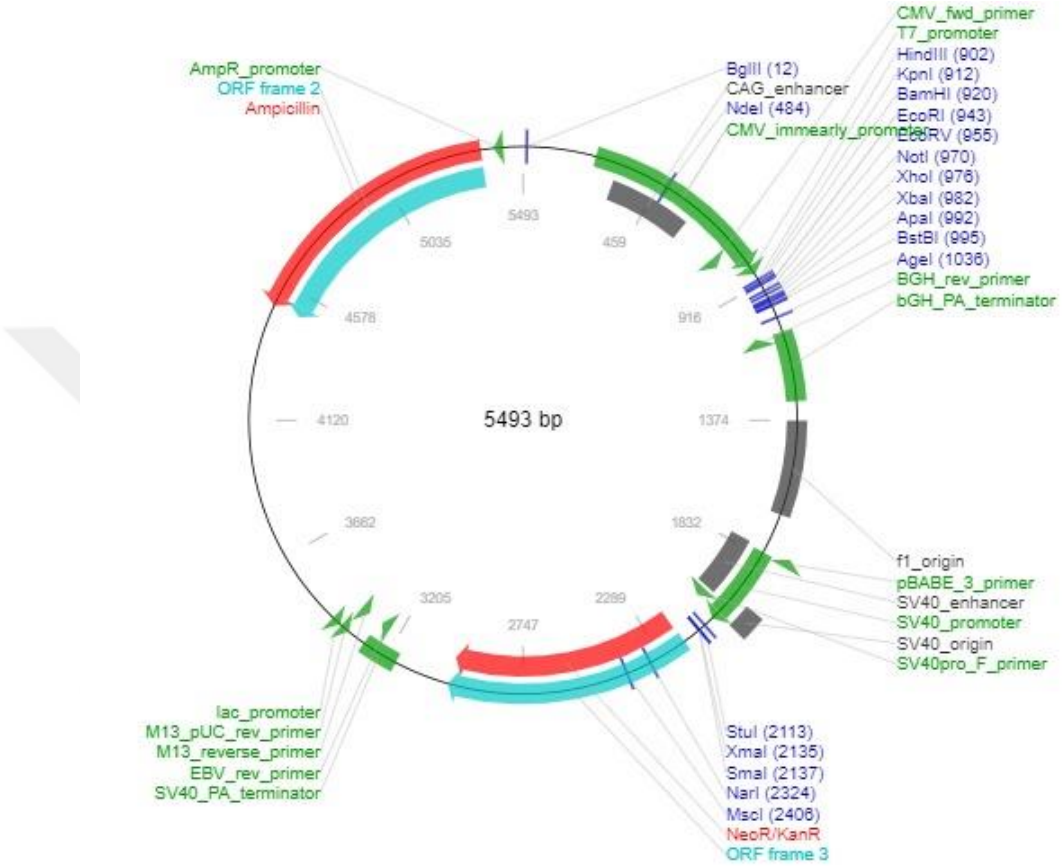

Figure A.1: Plasmid map of pcDNA3.1(+)/myc-His A. Plasmid consists of 5493 basepair and includes ampicillin selection gene. Restriction enzyme sites are shown in multiple cloning site. EcoRI and XbaI enzymes were used for cloning.

Table A.1 Sequences and relevant restriction enzymes.

Primer Name	Primer Sequence (5' → 3')	Restriction Enzyme
ATP13A2 Forward	GAAAGAATTCATGAGCGCAGACAGCAGCC	EcoRI
wt-ATP13A2 Reverse	GATCTAGACCTCAGGGGGCCGGCGGG	XbaI
FS-ATP13A2 Reverse	ATTCTAGAGTGAGGGTGCCCGTCTTG	XbaI
Del-ATP13A2 SDM Forward	TGAGGACGGCTTAGACGT	-
Del-ATP13A2 SDM Reverse	GTGCCCGTCTTGTCGAAA	-
MS-ATP13A2 SDM Forward	TACATGGCTCCGTACAGCCTG	-
MS-ATP13A2 SDM Reverse	CTTGAAGACGCTGAACGAAG	-

APPENDIX E: Sequences of cloned ATP13A2 cDNAs

In the sequences, silent mutations are shown as underlined. Related mutations are indicated as bold. Sequences in blue colour display myc-tag and sequences in purple colour shows 6x-his-tag sequences.

wt-ATP13A2 sequence:

```
ATP13A2_Isoform1
ATP13A2_wt_sequence

ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
************************************************************

ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
************************************************************

TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
************************************************************

CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
************************************************************

AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
************************************************************

CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
************************************************************

CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
************************************************************

GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
************************************************************

CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
************************************************************

GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
************************************************************

CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
************************************************************
```

```
ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
************************************************************

TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
************************************************************

CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
************************************************************

CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
************************************************************

GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
************************************************************

CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
************************************************************

AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTGCCGGAGGGG 1080
AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTTCCGGAGGGG 1080
************************************************** **********

CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
************************************************************

TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
************************************************************

TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
************************************************************

TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
************************************************************

ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
************************************************************

GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCCTGCTGCCATGACTGTGTGC 1440
GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCCTGCTGCCATGACTGTGTGC 1440
************************************************************

ACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTGCGC 1500
ACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTGCGC 1500
************************************************************

ATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGCACCCTCACTGAG 1560
ATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGCACCCTCACTGAG 1560
************************************************************
```

```
GACGGCTTAGACGTGATGGGGGTGGTGCCCCTGAAGGGGCAGGCATTCCTGCCCCTGGTC 1620
GACGGCTTAGACGTGATGGGGGTGGTGCCCCTGAAGGGGCAGGCATTCCTGCCCCTGGTC 1620
************************************************************

CCAGAGCCTCGCCGCCTGCCTGTGGGGCCCCTGCTCCGAGCACTGGCCACCTGCCATGCC 1680
CCAGAGCCTCGCCGCCTGCCTGTGGGGCCCCTGCTCCGAGCACTGGCCACCTGCCATGCC 1680
************************************************************

CTCAGCCGGCTCCAGGACACCCCCGTGGGCGACCCCATGGACTTGAAGATGGTGGAGTCT 1740
CTCAGCCGGCTCCAGGACACCCCCGTGGGCGACCCCATGGACTTGAAGATGGTGGAGTCT 1740
************************************************************

ACTGGCTGGGTCCTGGAGGAAGAGCCGGCTGCAGACTCAGCATTTGGGACCCAGGTCTTG 1800
ACTGGCTGGGTCCTGGAGGAAGAGCCGGCTGCAGACTCAGCATTTGGGACCCAGGTCTTG 1800
************************************************************

GCAGTGATGAGACCCCCACTTTGGGAGCCCCAGCTGCAGGCAATGGAGGAGCCCCCGGTG 1860
GCAGTGATGAGACCTCCACTTTGGGAGCCCCAGCTGCAGGCAATGGAGGAGCCCCCGGTG 1860
**********^*************************************************

CCAGTCAGCGTCCTCCACCGCTTCCCCTTCTCTTCGGCTCTGCAGCGCATGAGTGTGGTG 1920
CCAGTCAGCGTCCTCCACCGCTTCCCCTTCTCTTCGGCTCTGCAGCGCATGAGTGTGGTG 1920
************************************************************

GTGGCGTGGCCAGGGGCCACTCAGCCCGAGGCCTACGTCAAAGGCTCCCCGGAGCTGGTG 1980
GTGGCGTGGCCAGGGGCCACTCAGCCCGAGGCCTACGTCAAAGGCTCCCCGGAGCTGGTG 1980
************************************************************

GCAGGGCTCTGCAACCCCGAGACAGTGCCCACCGACTTCGCCCAGATGCTGCAGAGCTAT 2040
GCAGGGCTCTGCAACCCCGAGACAGTGCCCACCGACTTCGCCCAGATGCTGCAGAGCTAT 2040
************************************************************

ACAGCTGCTGGCTACCGTGTCGTGGCCCTGGCCAGCAAGCCACTGCCCACTGTGCCCAGC 2100
ACAGCTGCTGGCTACCGTGTCGTGGCCCTGGCCAGCAAGCCACTGCCCACTGTGCCCAGC 2100
************************************************************

CTGGAGGCAGCCCAGCAACTGACGAGGGACACTGTGGAAGGAGACCTGAGCCTCCTGGGG 2160
CTGGAGGCAGCCCAGCAACTGACGAGGGACACTGTGGAAGGAGACCTGAGCCTCCTGGGG 2160
************************************************************

CTGCTGGTCATGAGGAACCTACTGAAGCCGCAGACAACGCCAGTTATCCAGGCTCTGCGA 2220
CTGCTGGTCATGAGGAACCTACTGAAGCCGCAGACAACGCCAGTTATCCAGGCTCTGCGA 2220
************************************************************

AGGACCCGCATCCGCGCCGTCATGGTGACAGGGGACAACCTGCAGACAGCGGTGACTGTG 2280
AGGACCCGCATCCGCGCCGTCATGGTGACAGGGGACAACCTGCAGACAGCGGTGACTGTG 2280
************************************************************

GCCCGGGGCTGTGGCATGGTGGCCCCCCCAGGAGCATCTGATCATCGTCCACGCCACCCAC 2340
GCCCGGGGCTGTGGCATGGTGGCCCCCCCAGGAGCATCTGATCATCGTCCACGCCACCCAC 2340
************************************************************

CCTGAGCGGGGTCAGCCTGCCTCTCTCGAGTTCCTGCCGATGGAGTCCCCCACAGCCGTG 2400
CCTGAGCGGGGTCAGCCTGCCTCTCTCGAGTTCCTGCCGATGGAGTCCCCCACAGCCGTG 2400
************************************************************

AATGGCGTTAAGGATCCTGACCAGGCTGCAAGCTACACCGTGGAGCCAGACCCCCGATCC 2460
AATGGCGTTAAGGATCCTGACCAGGCTGCAAGCTACACCGTGGAGCCAGACCCCCGATCC 2460
************************************************************
```

```
AGGCACCTGGCCCTCAGCGGGCCCACCTTTGGTATCATTGTGAAGCACTTCCCCAAGCTG 2520
AGGCACCTGGCCCTCAGCGGGCCCACCTTTGGTATCATTGTGAAGCACTTCCCCAAGCTG 2520
************************************************************

CTGCCCAAGGTCCTGGTCCAGGGCACTGTCTTTGCCCGCATGGCCCCTGAGCAGAAGACA 2580
CTGCCCAAGGTCCTGGTCCAGGGCACTGTCTTTGCCCGCATGGCCCCTGAGCAGAAGACA 2580
************************************************************

GAGCTGGTGTGCGAGCTACAGAAGCTTCAGTACTGCGTGGGCATGTGCGGAGACGGCGCC 2640
GAGCTGGTGTGCGAGCTACAGAAGCTTCAGTACTGCGTGGGCATGTGCGGAGACGGCGCC 2640
************************************************************

AATGACTGTGGGGCCCTGAAGGCGGCTGATGTCGGCATCTCGCTGTCCCAGGCAGAAGCC 2700
AATGACTGTGGGGCCCTGAAGGCGGCTGATGTCGGCATCTCGCTGTCCCAGGCAGAAGCC 2700
************************************************************

TCAGTGGTCTCACCCTTCACCTCGAGCATGGCCAGTATTGAGTGCGTGCCCATGGTCATC 2760
TCAGTGGTCTCACCCTTCACCTCGAGCATGGCCAGTATTGAGTGCGTGCCCATGGTCATC 2760
************************************************************

AGGGAGGGGCGCTGTTCCCTTGACACTTCGTTCAGCGTCTTCAAGTACATGGCTCTGTAC 2820
AGGGAGGGGCGCTGTTCCCTTGACACTTCATTCAGCGTCTTCAAGTACATGGCTCTGTAC 2820
****************************  *****************************

AGCCTGACCCAGTTCATCTCCGTCCTGATCCTCTACACGATCAACACCAACCTGGGTGAC 2880
AGCCTGACCCAGTTCATCTCCGTCCTGATCCTCTACACGATCAACACCAACCTGGGTGAC 2880
************************************************************

CTGCAGTTCCTGGCCATCGACCTGGTCATCACCACCACAGTGGCAGTGCTCATGAGCCGC 2940
CTGCAGTTCCTGGCCATCGACCTGGTCATCACCACCACAGTGGCAGTGCTCATGAGCCGC 2940
************************************************************

ACGGGGCCAGCGCTGGTCCTGGGACGGGTGCGGCCACCGGGGGCGCTGCTCAGCGTGCCC 3000
ACGGGGCCAGCGCTGGTCCTGGGACGGGTGCGGCCACCGGGGGCGCTGCTCAGCGTGCCC 3000
************************************************************

GTGCTCAGCAGCCTGCTGCTGCAGATGGTCCTGGTGACCGGCGTGCAGCTAGGGGGCTAC 3060
GTGCTCAGCAGCCTGCTGCTGCAGATGGTCCTGGTGACCGGCGTGCAGCTAGGGGGCTAC 3060
************************************************************

TTCCTGACCCTGGCCCAGCCATGGTTCGTGCCTCTGAACAGGACAGTGGCCGCACCAGAC 3120
TTCCTGACCCTGGCCCAGCCATGGTTCGTGCCTCTGAACAGGACAGTGGCCGCACCAGAC 3120
************************************************************

AACCTGCCCAACTACGAGAACACCGTGGTCTTCTCTCTGTCCAGCTTCCAGTACCTCATC 3180
AACCTGCCCAACTACGAGAACACCGTGGTCTTCTCTCTGTCCAGCTTCCAGTACCTCATC 3180
************************************************************

CTGGCTGCAGCCGTGTCCAAGGGGGCGCCCTTCCGCCGGCCGCTCTACACCAATGTGCCC 3240
CTGGCTGCAGCCGTGTCCAAGGGGGCGCCCTTCCGCCGGCCGCTCTACACCAATGTGCCC 3240
************************************************************

TTCCTGGTGGCCCTGGCGCTCCTGAGCTCCGTCCTGGTGGGCCTTGTCCTGGTCCCCGGC 3300
TTCCTGGTGGCCCTGGCGCTCCTGAGCTCCGTCCTGGTGGGCCTTGTCCTGGTCCCCGGC 3300
************************************************************

CTCCTGCAGGGGCCGCTGGCGCTGAGGAACATCACTGACACCGGCTTCAAGCTGCTGCTG 3360
CTCCTGCAGGGGCCGCTGGCGCTGAGGAACATCACTGACACCGGCTTCAAGCTGCTGCTG 3360
************************************************************
```

```
CTGGGTCTGGTCACCCTCAACTTCGTGGGGGCCTTCATGCTGGAGAGCGTGCTAGACCAG 3420
CTGGGTCTGGTCACCCTCAACTTCGTGGGGGCCTTCATGCTGGAGAGCGTGCTAGACCAG 3420
************************************************************

TGCCTCCCCGCCTGCCTGCGCCGCCTCCGGCCCAAGCGGGCCTCCAAGAAGCGCTTCAAG 3480
TGCCTCCCCGCCTGCCTGCGCCGCCTCCGGCCCAAGCGGGCCTCCAAGAAGCGCTTCAAG 3480
************************************************************

CAGCTGGAACGAGAGCTGGCCGAGCAGCCCTGGCCGCCGCTGCCCGCCGGCCCCCTGAGG 3540
CAGCTGGAACGAGAGCTGGCCGAGCAGCCCTGGCCGCCGCTGCCCGCCGGCCCCCTGAGG 3540
************************************************************

TAG------------------------------------------------------------ 3543
---TCTAGAGGGCCCTTCGAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCATACC 3597


---------------------- 3543
GGTCATCATCACCATCACCATTGA 3621
```

FS-ATP13A2 sequence:

```
ATP13A2_Isoform1
ATP13A2_FS_sequence

ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
************************************************************

ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
************************************************************

TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
************************************************************

CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
************************************************************

AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
************************************************************

CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
************************************************************

CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
************************************************************

GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
************************************************************

CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
************************************************************
```

```
GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
************************************************************

CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
************************************************************

ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
************************************************************

TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
************************************************************

CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
************************************************************

CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
************************************************************

GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
************************************************************

CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
************************************************************

AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTGCCGGAGGGG 1080
AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTGCCGGAGGGG 1080
************************************************************

CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
************************************************************

TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
************************************************************

TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
************************************************************

TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
************************************************************

ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
************************************************************

GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCCTGCTGCC---ATGACTGTG 1439
GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCC--CTTCCAAAATGACTGTG 1440
****************************************  ** **   *********
```

```
TGCACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTG 1499
TGCACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTG 1500
************************************************************

CGCATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGCACCCTCACT 1559
CGCATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGCACCCTCAC- 1557
***********************************************************

GA---------------------------------------------------------- 1559
--AGAGGGCCCTTCGAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCATACCGGTC 1617


-------------------- 1559
ATCATCACCATCACCATTGA 1635
```

Del-ATP13A2 sequence:

```
ATP13A2_Isoform1
ATP13A2_del_sequence

ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
************************************************************

ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
************************************************************

TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
************************************************************

CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
************************************************************

AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
************************************************************

CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
************************************************************

CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
************************************************************

GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
************************************************************

CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
************************************************************

GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
************************************************************
```

```
CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
************************************************************

ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
************************************************************

TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
************************************************************

CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
************************************************************

CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
************************************************************

GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
************************************************************

CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
************************************************************

AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTGCCGGAGGGG 1080
AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTTCCGGAGGGG 1080
************************************************** **********

CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
************************************************************

TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
************************************************************

TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
************************************************************

TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
************************************************************

ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
************************************************************

GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCCTGCTGCCATGACTGTGTGC 1440
GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCCTGCTGCCATGACTGTGTGC 1440
************************************************************

ACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTGCGC 1500
ACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTGCGC 1500
************************************************************
```

```
ATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGCACCCTCACTGAG 1560
ATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGC------ACTGAG 1554
********************************************      ******

GACGGCTTAGACGTGATGGGGGTGGTGCCCCTGAAGGGGCAGGCATTCCTGCCCCTGGTC 1620
GACGGCTTAGACGTGATGGGGGTGGTGCCCCTGAAGGGGCAGGCATTCCTGCCACTGGTC 1614
************************************************** ******
CCAGAGCCTCGCCGCCTGCCTGTGGGGCCCCTGCTCCGAGCACTGGCCACCTGCCATGCC 1680
CCAGAGCCTCGCCGCCTGCCTGTGGGGCCCCTGCTCCGAGCACTGGCCACCTGCCATGCC 1674
************************************************************

CTCAGCCGGCTCCAGGACACCCCCGTGGGCGACCCCATGGACTTGAAGATGGTGGAGTCT 1740
CTCAGCCGGCTCCAGGACACCCCCGTGGGCGACCCCATGGACTTGAAGATGGTGGAGTCT 1734
************************************************************

ACTGGCTGGGTCCTGGAGGAAGAGCCGGCTGCAGACTCAGCATTTGGGACCCAGGTCTTG 1800
ACTGGCTGGGTCCTGGAGGAAGAGCCGGCTGCAGACTCAGCATTTGGGACCCAGGTCTTG 1794
************************************************************

GCAGTGATGAGACCCCCACTTTGGGAGCCCCAGCTGCAGGCAATGGAGGAGCCCCCGGTG 1860
GCAGTGATGAGACCTCCACTTTGGGAGCCCCAGCTGCAGGCAATGGAGGAGCCCCCGGTG 1854
************** ********************************************

CCAGTCAGCGTCCTCCACCGCTTCCCCTTCTCTTCGGCTCTGCAGCGCATGAGTGTGGTG 1920
CCAGTCAGCGTCCTCCACCGCTTCCCCTTCTCTTCGGCTCTGCAGCGCATGAGTGTGGTG 1914
************************************************************

GTGGCGTGGCCAGGGGCCACTCAGCCCGAGGCCTACGTCAAAGGCTCCCCGGAGCTGGTG 1980
GTGGCGTGGCCAGGGGCCACTCAGCCCGAGGCCTACGTCAAAGGCTCCCCGGAGCTGGTG 1974
************************************************************

GCAGGGCTCTGCAACCCCGAGACAGTGCCCACCGACTTCGCCCAGATGCTGCAGAGCTAT 2040
GCAGGGCTCTGCAACCCCGAGACAGTGCCCACCGACTTCGCCCAGATGCTGCAGAGCTAT 2034
************************************************************

ACAGCTGCTGGCTACCGTGTCGTGGCCCTGGCCAGCAAGCCACTGCCCACTGTGCCCAGC 2100
ACAGCTGCTGGCTACCGTGTCGTGGCCCTGGCCAGCAAGCCACTGCCCACTGTGCCCAGC 2094
************************************************************

CTGGAGGCAGCCCAGCAACTGACGAGGGACACTGTGGAAGGAGACCTGAGCCTCCTGGGG 2160
CTGGAGGCAGCCCAGCAACTGACGAGGGACACTGTGGAAGGAGACCTGAGCCTCCTGGGG 2154
************************************************************

CTGCTGGTCATGAGGAACCTACTGAAGCCGCAGACAACGCCAGTTATCCAGGCTCTGCGA 2220
CTGCTGGTCATGAGGAACCTACTGAAGCCGCAGACAACGCCAGTTATCCAGGCTCTGCGA 2214
************************************************************

AGGACCCGCATCCGCGCCGTCATGGTGACAGGGGACAACCTGCAGACAGCGGTGACTGTG 2280
AGGACCCGCATCCGCGCCGTCATGGTGACAGGGGACAACCTGCAGACAGCGGTGACTGTG 2274
************************************************************

GCCCGGGGCTGTGGCATGGTGGCCCCCCAGGAGCATCTGATCATCGTCCACGCCACCCAC 2340
GCCCGGGGCTGTGGCATGGTGGCCCCCCAGGAGCATCTGATCATCGTCCACGCCACCCAC 2334
************************************************************

CCTGAGCGGGGTCAGCCTGCCTCTCTCGAGTTCCTGCCGATGGAGTCCCCCACAGCCGTG 2400
CCTGAGCGGGGTCAGCCTGCCTCTCTCGAGTTCCTGCCGATGGAGTCCCCCACAGCCGTG 2394
************************************************************
```

```
AATGGCGTTAAGGATCCTGACCAGGCTGCAAGCTACACCGTGGAGCCAGACCCCCGATCC 2460
AATGGCGTTAAGGATCCTGACCAGGCTGCAAGCTACACCGTGGAGCCAGACCCCCGATCC 2454
************************************************************

AGGCACCTGGCCCTCAGCGGGCCCACCTTTGGTATCATTGTGAAGCACTTCCCCAAGCTG 2520
AGGCACCTGGCCCTCAGCGGGCCCACCTTTGGTATCATTGTGAAGCACTTCCCCAAGCTG 2514
************************************************************

CTGCCCAAGGTCCTGGTCCAGGGCACTGTCTTTGCCCGCATGGCCCCTGAGCAGAAGACA 2580
CTGCCCAAGGTCTTGGTCCAGGGCACTGTCTTTGCCCGCATGGCCCCTGAGCAGAAGACA 2573
************ ***********************************************

GAGCTGGTGTGCGAGCTACAGAAGCTTCAGTACTGCGTGGGCATGTGCGGAGACGGCGCC 2640
GAGCTGGTGTGCGAGCTACAGAAGCTTCAGTACTGCGTGGGCATGTGCGGAGACGGCGCC 2633
************************************************************

AATGACTGTGGGGCCCTGAAGGCGGCTGATGTCGGCATCTCGCTGTCCCAGGCAGAAGCC 2700
AATGACTGTGGGGCCCTGAAGGCGGCTGATGTCGGCATCTCGCTGTCCCAGGCAGAAGCC 2693
************************************************************

TCAGTGGTCTCACCCTTCACCTCGAGCATGGCCAGTATTGAGTGCGTGCCCATGGTCATC 2760
TCAGTGGTCTCACCCTTCACCTCGAGCATGGCCAGTATTGAGTGCGTGCCCATGGTCATC 2753
************************************************************

AGGGAGGGGCGCTGTTCCCTTGACACTTCGTTCAGCGTCTTCAAGTACATGGCTCTGTAC 2820
AGGGAGGGGCGCTGTTCCCTTGACACTTCATTCAGCGTCTTCAAGTACATGGCTCTGTAC 2813
****************************  *****************************

AGCCTGACCCAGTTCATCTCCGTCCTGATCCTCTACACGATCAACACCAACCTGGGTGAC 2880
AGCCTGACCCAGTTCATCTCCGTCCTGATCCTCTACACGATCAACACCAACCTGGGTGAC 2873
************************************************************

CTGCAGTTCCTGGCCATCGACCTGGTCATCACCACCACAGTGGCAGTGCTCATGAGCCGC 2940
CTGCAGTTCCTGGCCATCGACCTGGTCATCACCACCACAGTGGCAGTGCTCATGAGCCGC 2933
************************************************************

ACGGGGCCAGCGCTGGTCCTGGGACGGGTGCGGCCACCGGGGGCGCTGCTCAGCGTGCCC 3000
ACGGGGCCAGCGCTGGTCCTGGGACGGGTGCGGCCACCGGGGGCGCTGCTCAGCGTGCCC 2993
************************************************************

GTGCTCAGCAGCCTGCTGCTGCAGATGGTCCTGGTGACCGGCGTGCAGCTAGGGGGCTAC 3060
GTGCTCAGCAGCCTGCTGCTGCAGATGGTCCTGGTGACCGGCGTGCAGCTAGGGGGCTAC 3053
************************************************************

TTCCTGACCCTGGCCCAGCCATGGTTCGTGCCTCTGAACAGGACAGTGGCCGCACCAGAC 3120
TTCCTGACCCTGGCCCAGCCATGGTTCGTGCCTCTGAACAGGACAGTGGCCGCACCAGAC 3113
************************************************************

AACCTGCCCAACTACGAGAACACCGTGGTCTTCTCTCTGTCCAGCTTCCAGTACCTCATC 3180
AACCTGCCCAACTACGAGAACACCGTGGTCTTCTCTCTGTCCAGCTTCCAGTACCTCATC 3173
************************************************************

CTGGCTGCAGCCGTGTCCAAGGGGGCGCCCTTCCGCCGGCCGCTCTACACCAATGTGCCC 3240
CTGGCTGCAGCCGTGTCCAAGGGGGCGCCCTTCCGCCGGCCGCTCTACACCAATGTGCCC 3233
************************************************************

TTCCTGGTGGCCCTGGCGCTCCTGAGCTCCGTCCTGGTGGGCCTTGTCCTGGTCCCCGGC 3300
TTCCTGGTGGCCCTGGCGCTCCTGAGCTCCGTCCTGGTGGGCCTTGTCCTGGTCCCCGGC 3293
************************************************************
```

```
CTCCTGCAGGGGCCGCTGGCGCTGAGGAACATCACTGACACCGGCTTCAAGCTGCTGCTG 3360
CTCCTGCAGGGGCCGCTGGCGCTGAGGAACATCACTGACACCGGCTTCAAGCTGCTGCTG 3353
************************************************************

CTGGGTCTGGTCACCCTCAACTTCGTGGGGGCCTTCATGCTGGAGAGCGTGCTAGACCAG 3420
CTGGGTCTGGTCACCCTCAACTTCGTGGGGGCCTTCATGCTGGAGAGCGTGCTAGACCAG 3413
************************************************************

TGCCTCCCCGCCTGCCTGCGCCGCCTCCGGCCCAAGCGGGCCTCCAAGAAGCGCTTCAAG 3480
TGCCTCCCCGCCTGCCTGCGCCGCCTCCGGCCCAAGCGGGCCTCCAAGAAGCGCTTCAAG 3473
************************************************************

CAGCTGGAACGAGAGCTGGCCGAGCAGCCCTGGCCGCCGCTGCCCGCCGGCCCCCTGAGG 3540
CAGCTGGAACGAGAGCTGGCCGAGCAGCCCTGGCCGCCGCTGCCCGCCGGCCCCCTGAGG 3533
************************************************************

TAG---------------------------------------------------------- 3543
---TCTAGAGGGCCCTTCGAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCATACC 3590


------------------------ 3543
GGTCATCATCACCATCACCATTGA 3614
```

MS-ATP13A2 sequence:

```
ATP13A2_Isoform1
ATP13A2_MS_sequence

ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
ATGAGCGCAGACAGCAGCCCTCTCGTGGGCAGCACGCCCACCGGTTATGGGACCCTGACG 60
************************************************************

ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
ATAGGGACATCAATAGATCCCCTCAGCTCCTCAGTTTCATCCGTGAGGCTCAGCGGCTAC 120
************************************************************

TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
TGTGGCAGTCCATGGAGGGTCATCGGCTATCACGTCGTGGTCTGGATGATGGCTGGGATC 180
************************************************************

CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
CCTTTGCTGCTCTTCCGTTGGAAGCCCCTGTGGGGGGTGCGGCTGCGGCTCCGGCCCTGC 240
************************************************************

AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
AACCTGGCCCACGCCGAAACACTCGTTATCGAAATAAGAGACAAAGAGGATAGTTCCTGG 300
************************************************************

CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
CAGCTCTTCACTGTCCAGGTGCAGACTGAGGCCATCGGCGAGGGCAGCCTGGAGCCGTCC 360
************************************************************

CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
CCACAGTCCCAGGCAGAGGATGGCCGGAGCCAGGCGGCAGTTGGGGCGGTACCAGAGGGT 420
************************************************************

GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
GCCTGGAAGGATACGGCCCAGCTCCACAAGAGCGAGGAGGCGGTGAGTGTCGGACAGAAG 480
************************************************************
```

```
CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
CGGGTGCTGCGGTATTACCTCTTCCAGGGCCAGCGCTATATCTGGATCGAGACCCAGCAA 540
************************************************************

GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
GCCTTCTACCAGGTCAGCCTCCTGGACCATGGCCGCTCTTGTGACGACGTCCACCGCTCC 600
************************************************************

CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
CGCCATGGCCTCAGCCTCCAGGACCAAATGGTGAGGAAGGCCATTTACGGCCCCAACGTG 660
************************************************************

ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
ATCAGCATACCGGTCAAGTCCTACCCCCAGCTGCTGGTGGACGAGGCACTGAACCCCTAC 720
************************************************************

TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
TATGGGTTCCAGGCCTTCAGCATCGCGCTGTGGCTGGCTGACCACTACTACTGGTACGCC 780
************************************************************

CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
CTGTGCATCTTCCTCATTTCCTCCATCTCCATCTGCCTGTCGCTGTACAAGACCAGAAAG 840
************************************************************

CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
CAAAGCCAGACTCTAAGGGACATGGTCAAGTTGTCCATGCGGGTGTGCGTGTGCCGGCCA 900
************************************************************

GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
GGGGGAGAGGAAGAGTGGGTGGACTCCAGTGAGCTAGTGCCCGGAGACTGCCTGGTGCTG 960
************************************************************

CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
CCCCAGGAGGGTGGGCTGATGCCCTGTGATGCCGCCCTGGTGGCCGGCGAGTGCATGGTG 1020
************************************************************

AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTGCCGGAGGGG 1080
AATGAGAGCTCTCTGACAGGAGAGAGCATTCCAGTGCTGAAGACGGCACTTCCGGAGGGG 1080
************************************************** **********

CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
CTGGGGCCCTACTGTGCAGAGACACACCGGCGGCACACACTCTTCTGCGGGACCCTCATC 1140
************************************************************

TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
TTGCAGGCCCGGGCCTATGTGGGACCGCACGTCCTGGCAGTGGTGACCCGCACAGGGTTC 1200
************************************************************

TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
TGCACGGCAAAAGGGGGCCTGGTGAGCTCCATCTTGCACCCCCGGCCCATCAACTTCAAG 1260
************************************************************

TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
TTCTATAAACACAGCATGAAGTTTGTGGCTGCCCTCTCTGTCCTGGCTCTCCTCGGCACC 1320
************************************************************

ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
ATCTACAGCATCTTCATCCTCTACCGAAACCGGGTGCCTCTGAATGAGATTGTAATCCGG 1380
************************************************************
```

```
GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCCTGCTGCCATGACTGTGTGC 1440
GCTCTCGACCTGGTGACCGTGGTGGTGCCACCTGCCCTGCCTGCTGCCATGACTGTGTGC 1440
************************************************************

ACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTGCGC 1500
ACGCTCTACGCCCAGAGCCGACTGCGGAGACAGGGCATTTTCTGCATCCACCCACTGCGC 1500
************************************************************

ATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGCACCCTCACTGAG 1560
ATCAACCTGGGGGGCAAGCTGCAGCTGGTGTGTTTCGACAAGACGGGCACCCTCACTGAG 1560
************************************************************

GACGGCTTAGACGTGATGGGGGTGGTGCCCCTGAAGGGGCAGGCATTCCTGCCCCTGGTC 1620
GACGGCTTAGACGTGATGGGGGTGGTGCCCCTGAAGGGGCAGGCATTCCTGCCCCTGGTC 1620
************************************************************

CCAGAGCCTCGCCGCCTGCCTGTGGGGCCCCTGCTCCGAGCACTGGCCACCTGCCATGCC 1680
CCAGAGCCTCGCCGCCTGCCTGTGGGGCCCCTGCTCCGAGCACTGGCCACCTGCCATGCC 1680
************************************************************

CTCAGCCGGCTCCAGGACACCCCCGTGGGCGACCCCATGGACTTGAAGATGGTGGAGTCT 1740
CTCAGCCGGCTCCAGGACACCCCCGTGGGCGACCCCATGGACTTGAAGATGGTGGAGTCT 1740
************************************************************

ACTGGCTGGGTCCTGGAGGAAGAGCCGGCTGCAGACTCAGCATTTGGGACCCAGGTCTTG 1800
ACTGGCTGGGTCCTGGAGGAAGAGCCGGCTGCAGACTCAGCATTTGGGACCCAGGTCTTG 1800
************************************************************

GCAGTGATGAGACCCCCACTTTGGGAGCCCCAGCTGCAGGCAATGGAGGAGCCCCCGGTG 1860
GCAGTGATGAGACCTCCACTTTGGGAGCCCCAGCTGCAGGCAATGGAGGAGCCCCCGGTG 1860
**********  *******************************************

CCAGTCAGCGTCCTCCACCGCTTCCCCTTCTCTTCGGCTCTGCAGCGCATGAGTGTGGTG 1920
CCAGTCAGCGTCCTCCACCGCTTCCCCTTCTCTTCGGCTCTGCAGCGCATGAGTGTGGTG 1920
************************************************************

GTGGCGTGGCCAGGGGCCACTCAGCCCGAGGCCTACGTCAAAGGCTCCCCGGAGCTGGTG 1980
GTGGCGTGGCCAGGGGCCACTCAGCCCGAGGCCTACGTCAAAGGCTCCCCGGAGCTGGTG 1980
************************************************************

GCAGGGCTCTGCAACCCCGAGACAGTGCCCACCGACTTCGCCCAGATGCTGCAGAGCTAT 2040
GCAGGGCTCTGCAACCCCGAGACAGTGCCCACCGACTTCGCCCAGATGCTGCAGAGCTAT 2040
************************************************************

ACAGCTGCTGGCTACCGTGTCGTGGCCCTGGCCAGCAAGCCACTGCCCACTGTGCCCAGC 2100
ACAGCTGCTGGCTACCGTGTCGTGGCCCTGGCCAGCAAGCCACTGCCCACTGTGCCCAGC 2100
************************************************************

CTGGAGGCAGCCCAGCAACTGACGAGGGACACTGTGGAAGGAGACCTGAGCCTCCTGGGG 2160
CTGGAGGCAGCCCAGCAACTGACGAGGGACACTGTGGAAGGAGACCTGAGCCTCCTGGGG 2160
************************************************************

CTGCTGGTCATGAGGAACCTACTGAAGCCGCAGACAACGCCAGTTATCCAGGCTCTGCGA 2220
CTGCTGGTCATGAGGAACCTACTGAAGCCGCAGACAACGCCAGTTATCCAGGCTCTGCGA 2220
************************************************************

AGGACCCGCATCCGCGCCGTCATGGTGACAGGGGACAACCTGCAGACAGCGGTGACTGTG 2280
AGGACCCGCATCCGCGCCGTCATGGTGACAGGGGACAACCTGCAGACAGCGGTGACTGTG 2280
************************************************************
```

```
GCCCGGGGCTGTGGCATGGTGGCCCCCCAGGAGCATCTGATCATCGTCCACGCCACCCAC 2340
GCCCGGGGCTGTGGCATGGTGGCCCCCCAGGAGCATCTGATCATCGTCCACGCCACCCAC 2340
************************************************************

CCTGAGCGGGGTCAGCCTGCCTCTCTCGAGTTCCTGCCGATGGAGTCCCCCACAGCCGTG 2400
CCTGAGCGGGGTCAGCCTGCCTCTCTCGAGTTCCTGCCGATGGAGTCCCCCACAGCCGTG 2400
************************************************************

AATGGCGTTAAGGATCCTGACCAGGCTGCAAGCTACACCGTGGAGCCAGACCCCCGATCC 2460
AATGGCGTTAAGGATCCTGACCAGGCTGCAAGCTACACCGTGGAGCCAGACCCCCGATCC 2460
************************************************************

AGGCACCTGGCCCTCAGCGGGCCCACCTTTGGTATCATTGTGAAGCACTTCCCCAAGCTG 2520
AGGCACCTGGCCCTCAGCGGGCCCACCTTTGGTATCATTGTGAAGCACTTCCCCAAGCTG 2520
************************************************************

CTGCCCAAGGTCCTGGTCCAGGGCACTGTCTTTGCCCGCATGGCCCCTGAGCAGAAGACA 2580
CTGCCCAAGGTCCTGGTCCAGGGCACTGTCTTTGCCCGCATGGCCCCTGAGCAGAAGACA 2580
************************************************************

GAGCTGGTGTGCGAGCTACAGAAGCTTCAGTACTGCGTGGGCATGTGCGGAGACGGCGCC 2640
GAGCTGGTGTGCGAGCTACAGAAGCTTCAGTACTGCGTGGGCATGTGCGGAGACGGCGCC 2640
************************************************************

AATGACTGTGGGGCCCTGAAGGCGGCTGATGTCGGCATCTCGCTGTCCCAGGCAGAAGCC 2700
AATGACTGTGGGGCCCTGAAGGCGGCTGATGTCGGCATCTCGCTGTCCCAGGCAGAAGCC 2700
************************************************************

TCAGTGGTCTCACCCTTCACCTCGAGCATGGCCAGTATTGAGTGCGTGCCCATGGTCATC 2760
TCAGTGGTCTCACCCTTCACCTCGAGCATGGCCAGTATTGAGTGCGTGCCCATGGTCATC 2760
************************************************************

AGGGAGGGGCGCTGTTCCCTTGACACTTCGTTCAGCGTCTTCAAGTACATGGCTCTGTAC 2820
AGGGAGGGGCGCTGTTCCCTTGACACTTCATTCAGCGTCTTCAAGTACATGGCTCCGTAC 2820
***************************** ************************ ****

AGCCTGACCCAGTTCATCTCCGTCCTGATCCTCTACACGATCAACACCAACCTGGGTGAC 2880
AGCCTGACCCAGTTCATCTCCGTCCTGATCCTCTACACGATCAACACCAACCTGGGTGAC 2880
************************************************************

CTGCAGTTCCTGGCCATCGACCTGGTCATCACCACCACAGTGGCAGTGCTCATGAGCCGC 2940
CTGCAGTTCCTGGCCATCGACCTGGTCATCACCACCACAGTGGCAGTGCTCATGAGCCGC 2940
************************************************************

ACGGGGCCAGCGCTGGTCCTGGGACGGGTGCGGCCACCGGGGGCGCTGCTCAGCGTGCCC 3000
ACGGGGCCAGCGCTGGTCCTGGGACGGGTGCGGCCACCGGGGGCGCTGCTCAGCGTGCCC 3000
************************************************************

GTGCTCAGCAGCCTGCTGCTGCAGATGGTCCTGGTGACCGGCGTGCAGCTAGGGGGCTAC 3060
GTGCTCAGCAGCCTGCTGCTGCAGATGGTCCTGGTGACCGGCGTGCAGCTAGGGGGCTAC 3060
************************************************************

TTCCTGACCCTGGCCCAGCCATGGTTCGTGCCTCTGAACAGGACAGTGGCCGCACCAGAC 3120
TTCCTGACCCTGGCCCAGCCATGGTTCGTGCCTCTGAACAGGACAGTGGCCGCACCAGAC 3120
************************************************************

AACCTGCCCAACTACGAGAACACCGTGGTCTTCTCTCTGTCCAGCTTCCAGTACCTCATC 3180
AACCTGCCCAACTACGAGAACACCGTGGTCTTCTCTCTGTCCAGCTTCCAGTACCTCATC 3180
************************************************************
```

```
CTGGCTGCAGCCGTGTCCAAGGGGGCGCCCTTCCGCCGGCCGCTCTACACCAATGTGCCC 3240
CTGGCTGCAGCCGTGTCCAAGGGGGCGCCCTTCCGCCGGCCGCTCTACACCAATGTGCCC 3240
************************************************************

TTCCTGGTGGCCCTGGCGCTCCTGAGCTCCGTCCTGGTGGGCCTTGTCCTGGTCCCCGGC 3300
TTCCTGGTGGCCCTGGCGCTCCTGAGCTCCGTCCTGGTGGGCCTTGTCCTGGTCCCCGGC 3300
************************************************************

CTCCTGCAGGGGCCGCTGGCGCTGAGGAACATCACTGACACCGGCTTCAAGCTGCTGCTG 3360
CTCCTGCAGGGGCCGCTGGCGCTGAGGAACATCACTGACACCGGCTTCAAGCTGCTGCTG 3360
************************************************************

CTGGGTCTGGTCACCCTCAACTTCGTGGGGGCCTTCATGCTGGAGAGCGTGCTAGACCAG 3420
CTGGGTCTGGTCACCCTCAACTTCGTGGGGGCCTTCATGCTGGAGAGCGTGCTAGACCAG 3420
************************************************************

TGCCTCCCCGCCTGCCTGCGCCGCCTCCGGCCCAAGCGGGCCTCCAAGAAGCGCTTCAAG 3480
TGCCTCCCCGCCTGCCTGCGCCGCCTCCGGCCCAAGCGGGCCTCCAAGAAGCGCTTCAAG 3480
************************************************************

CAGCTGGAACGAGAGCTGGCCGAGCAGCCCTGGCCGCCGCTGCCCGCCGGCCCCCTGAGG 3540
CAGCTGGAACGAGAGCTGGCCGAGCAGCCCTGGCCGCCGCTGCCCGCCGGCCCCCTGAGG 3540
************************************************************

TAG------------------------------------------------------------ 3543
---TCTAGAGGGCCCTTCGAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCATACC 3597


------------------------ 3543
GGTCATCATCACCATCACCATTGA 3621
```

CURRICULUM VITAE

Name Surname : Ezgi ERTEREK

EDUCATION

- **B.Sc. :** 2020, Istanbul Technical University, Science and Lecture Faculty, Molecular Biology and Genetics Department

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

Poster presentation: "Investigation of the effect of ATP13A2 mutations on intracellular iron accumulation" at 21th national neuroscience kongre