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CLINICAL RESEARCH / KLİNİK ARAŞTIRMA

The Investigation of the Effects of a Novel Missense Mutation in *Katanin-like 2 (KATNAL2)* Gene on Microtubule-related Proteins in Patient Fibroblasts Using a Proteomic Approach

Katanin-like 2 (KATNAL2) Genindeki Yeni bir Yanlış Anlam Mutasyonunun Hasta Fibroblastlarındaki Mikrotübül İlişkili Proteinler Üzerine Etkilerinin Proteomik Bir Yaklaşım ile İncelenmesi

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Abstract

Objective: The proteome is all the proteins produced or modified by an organism or system. Proteomics, which is an important component of functional genomics, describes the detection and characterization of a complete set of proteins present in a cell, organ, or organism at a given time. The *katanin-like 2 (KATNAL2)* gene encodes a protein with a microtubule-severing function, based on studies in cell cultures and lower organisms. However, there are no functional studies investigating the function of this gene in humans, yet. In this study, we aimed to perform proteomic analysis to investigate the effects of the variant in the *KATNAL2* gene on the binding of various proteins to microtubules in the fibroblasts of a patient with epilepsy, autistic symptoms and intellectual disability with a homozygous pathogenic variant in the *KATNAL2* gene.

Methods: Fibroblasts obtained from the patient by skin biopsy were grown in cell culture and protein isolation was performed from them. Following pelleting of microtubules and associated proteins, proteomic analysis was performed using liquid chromatography followed by tandem mass spectrometry.

Results: Quantitative data analysis was performed using the SAINTexpress tool on 5 different fractions cut from the gel, identifying 1246 proteins, 38 of which differentially bound to microtubules between patient and control cells. The data obtained from the proteomic and the bioinformatic analyzes indicate that because of the mutation in the *KATNAL2* gene in the patient, the extracellular matrix proteins such as collagens that interact with the cytoskeleton are organized differently from those in normal cells.

Conclusion: Here, we leveraged the power of proteomics to identify the altered interactome of the microtubules in skin fibroblasts with a missense mutation in *KATNAL2*. Our results point to altered extracellular matrix-cell interactions in *KATNAL2*-mutant fibroblasts.

Keywords: Childhood, intellectual disability, proteomics, KATNAL2, microtubule-related proteins

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Öz

Amaç: Proteom, bir organizma veya sistem tarafından üretilen veya modifiye edilen proteinlerin tamamıdır. Fonksiyonel genomiğin önemli bir bileşeni olan proteomik ise, belirli bir zamanda bir hücrede, organda veya organizmada bulunan proteinlerin tespiti ve karakterizasyonunu tanımlar. *KATNAL2* geninin, hücre kültürü ve düşük organizmalarda yapılan çalışmalara göre mikrotübül kesici işlevi olan bir protein kodladığı bilinmektedir; ancak insanlarda bu proteinin işlevini araştıran fonksiyonel çalışmalar henüz yoktur. Bu çalışmada, *KATNAL2* geninde patojenik bir varyantı homozigot olarak taşıdığı saptanan, epilepsi, otistik belirtiler ve zeka geriliği olan bir hastanın fibroblastlarından, söz konusu gendeki varyantın çeşitli proteinlerin mikrotübüllere bağlanması üzerindeki etkilerini araştırmak üzere proteomik analiz yapmayı amaçladık.

Yöntem: Mikrotübüller ve bağlı proteinlerin çöktürülmesi sonrası sıvı kromatografisi, ardından tandem kütle spektrometrisi yöntemi kullanılarak proteomik analiz gerçekleştirildi.

Bulgular: Jelden kesilen 5 farklı fraksiyon üzerinde SAINTexpress aracı kullanılarak nicel veri analizi yapıldı ve 38'i hasta ve kontrol hücreleri arasında mikrotübüllere farklı miktarda bağlanan 1246 protein belirlendi. Proteomik ve biyoinformatik analizlerden elde edilen veriler, *KATNAL2* genindeki mutasyon sonucunda hücre iskeleti ile etkileşime giren kollajen gibi hücre dışı matriks proteinlerinin, normal hücrelerdekinden farklı şekilde organize edildiğini gösterdi.

Sonuç: Bu çalışmada, *KATNAL2* geninde bir yanlış anlam mutasyonu taşıyan hastanın fibroblastlarında mikrotübül ilişkili proteinlerin interaktomundaki değişimleri tanımlamak üzere proteomik tekniklerin gücünden yararlandık. Sonuçlarımız, *KATNAL2* mutant fibroblastlarda değişen ekstrasellüler matriks ve hücreler arası interaksiyonun etkilendiğine işaret etmektedir.

Anahtar Kelimeler: Çocukluk, zihinsel yetersizlik, proteomik, *KATNAL2*, mikrotübül ilişkili proteinler

Introduction

Biological studies today rely on combining the results from methods that analyze DNA, mRNA, noncoding RNAs, proteins with their interactions and the nucleotide modifications that form the epigenome into datasets representing a diverse array of "omics" data. "Multiomics" is a biological analysis approach that integrates diverse types of omics data in which the data sets are multiple "omes", such as the genome, exome, transcriptome, epigenome, proteome, metabolome, etc. Combining these "omes" provides an opportunity to analyze the complexity of biological data more comprehensively to find novel associations between genotypes and phenotypes, reveal relevant biomarkers, better understand the underlying etiology and the pathways involved[1,2]. After genomics and transcriptomics, proteomics is the next step in the study of biological systems. Proteomics is the study of the set of proteins produced by an organism, tissue, or cell, with the aim of understanding the behavior of these proteins in varying environments and conditions. Generally, proteomics studies provide data on three properties of proteins in a sample: Location, abundance and turnover, and post-translational modifications of the proteins. Specifically, proteomics is used to investigate when and where proteins are expressed and the rates of protein production, degradation, and steady-state abundance, how proteins are modified, the movement of proteins between subcellular compartments, the involvement of proteins in metabolic pathways and how proteins interact. Depending on the purpose of the experimental study, researchers may be directly interested in all or some part of these data or use them to extract additional information[3,4].

In terms of proteomic analysis methods, several high-throughput technologies have been developed to investigate proteomes. The most commonly used proteomic analysis methods are mass spectrometry (MS)-based techniques such as tandem-MS and gel-based techniques. Through these high-throughput technologies, huge amounts of data are generated. Databases that record and store such data allow the researcher to make connections between their results and the existing knowledge[5].

In this study, we performed proteomic analysis in the fibroblasts of a patient with epilepsy, autistic symptoms and mental retardation, in whom a pathogenic variant was detected in the *KATNAL2* gene, whose function is not fully known in humans. The KATNAL2 protein is a member of the katanin family. Katanins are a major class of microtubule severing enzymes. Katanins cut long microtubules to form shorter microtubule fragments, which can be easily transported to the ends of axons by motor proteins for axon elongation[6,7]. Studies in cultured cells and lower organisms have demonstrated their roles in neuron development, cell migration, formation of axons and dendritic extensions in neurons, and formation of spindles during cell division and locomotory organelle formation. Katanins are composed of a catalytic and smaller p60 subunit (A subunit, which contains the AAA ATPase domain), and a regulatory and larger p80 subunit (B subunit). The human genome encodes two alternatively spliced isoforms of the canonical p60 A

subunit (*KATNA1* chromosomal locus 6q25.1, protein IDs NP_001191005.1 and NP_008975.1) and two additional p60-like proteins (*KATNAL1* chromosomal locus 13q12.3, protein ID NP_115492.1; *KATNAL2* chromosomal locus 18q21.1, protein ID NP_112593.2). Similarly, in addition to the canonical p80 B subunit (*KATNB1* chromosomal locus 16q21, protein ID NP_005877.2), the human genome encodes an additional p80-like protein (*KATNBL1* chromosomal locus 15q14, protein ID NP_078989.1)[8]. The *KATNAL2* gene is expressed at a high level in the central nervous system in humans. It has been identified as a risk gene for autism spectrum disorder (ASD) in a few reports. In a study by Yuen et al.[9] where whole-genome sequencing (WGS) of quartet families with ASD was performed, in a family, a child was found to have inherited a non-sense mutation in the ASD-risk gene, *KATNAL2* from his mother.

Neale et al.[10] reported 3 loss-of-function mutations in the *KATNAL2* gene among 935 cases with ASD and they defined *KATNAL2* as a genuine autism risk factor. O'Roak et al.[11] reported whole-exome sequencing (WES) for parent-child trios exhibiting sporadic ASDs, including 189 new trios and 20 that were previously reported. Additionally, O'Roak et al.[12] sequenced the exomes of 50 unaffected sibs corresponding to 31 of the new and 19 of the previously reported trios, for 677 individual exomes from 209 families. In proband exomes, the authors identified a splice site mutation in the *KATNAL2* gene. In another study, Sanders et al.[13] combined all de novo events in their sample with those identified in the study by O'Roak et al.[12] and observed from 414 probands that the *KATNAL2* gene carried 2 highly disruptive mutations.

Although the basic function of the KATNAL2 protein is widely known from the studies in cell cultures and lower organisms, no functional studies have been reported in humans. Here, we aimed to identify the altered microtubule-binding proteins via liquid chromatography-tandem MS (LC-MS/MS) proteomic analysis in the fibroblasts of a patient with epilepsy, mental retardation and autistic behavior, who had a homozygous disease-causing change in *KATNAL2* (p.Ser392Pro).

Materials and Methods

Case Presentation

An 11-year-old boy was admitted with the complaints of intellectual disability, autistic behavior, and epileptic seizures. There was a first cousin marriage between his parents and the patient had a healthy brother aged 6-year-old. The patient was born at term without any complications. From early infancy, psychomotor retardation and hypotonia were noticed in the patient. In terms of neuromotor developmental skills, he was holding his head at age 3, he could sit without support at age 6, and he could walk without support at the age of 9. He could produce vowel sounds at the age of 8, however, he could not still produce the voice of babbling, and he still could not make meaningful words and sentences. The patient could never achieve stool or urinary continence. His epileptic seizures started at 6 months of age. Initially, prolonged hypomotor seizures during the awake period were seen. Later, he demonstrated myoclonic seizures with head drops. Phenobarbital, valproate and clonazepam treatments were ineffective for seizure treatment. After the addition of levetiracetam to the treatment, his seizures stopped by 2 years of age.

In the first neurologic examination, it was noted that his social communication was very limited. Eye contact was diminished. The patient could only understand and follow one or two commands given by his father, such as bringing the car key. The patient did not respond when his name was called. He had unintentional stereotypes such as shaking of the head and body. The patient's dysmorphic features were prominent with the marked nose bridge, deep set eyes, folded palpebral fissures, exotropia, plump lips, macroglossia, mild retrognathia, wide and asymmetric nipples, kyphosis, bilateral dystrophic nails, fingertips, narrow and sharp fingers, and hyper-extensible joints. The remaining examination findings were normal.

Laboratory tests including complete blood count, liver and renal function tests, creatine kinase, thyroid tests, lactate, pyruvate, organic acids, tandem MS, very long chain fatty acids levels in serum, metabolic tests in cerebrospinal fluid and karyotype analysis were normal. There were no abnormal finding in his electrocardiography, echocardiography, electroencephalography and abdominal ultrasonography. Cranial magnetic resonance imaging was also normal. Since the patient could not be diagnosed with clinical findings and laboratory tests, WES was performed in the patient and his family. WES and ensuing bioinformatic analysis revealed a novel homozygous variant in the *KATNAL2* gene (NM_031303.3):c.1174T>C (p.Ser392Pro), that was confirmed by Sanger sequencing (Figure 1 A and B). The variant is not observed in Genome Aggregation Database and is classified as variant of uncertain significance/likely pathogenic according to the American College of Medical Genetics classification.

Skin Biopsy and Dermal Fibroblast Culture

The study protocol was approved by the Local Ethics Committee (5097-GOA) (decision no: 2019/29-36). Written informed consent was obtained from the parents of the patient. Normal human skin fibroblasts were cultured from skin biopsies obtained from two healthy controls.

The patient fibroblasts used in our study were obtained from skin punch biopsy. Skin biopsy was taken using a sterile 3 mm punch biopsy apparatus after disinfecting and anesthetizing the skin part from the inner area of the left forearm of the patient using a standard procedure[14].

Proteomic Analysis of the Fibroblasts

To understand the effects of the variant in the *KATNAL2* gene in the binding of various proteins to microtubules, proteomic analysis was performed, as described previously, using LC method followed by tandem mass spectrometry (LC-MS/MS)[15].

Samples obtained after microtubule precipitation in age-sex-matched control and patient fibroblasts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Statistical Analysis

Quantitative LC-MS/MS data analysis was performed using the SAINTexpress tool on 5 different fractions cut from the gel[16]. To understand the relationships between the microtubule-binding proteins and to understand how they are related to each other, gene ontology (GO) term enrichment/overrepresentation analyses of interaction network was performed using the STRING database and the *Enrichr* tool[17,18].

There were no study limitations during our research.

Results

Coomassie staining and fractionation of samples obtained from patient and control fibroblasts after microtubule precipitation on SDS-PAGE gel are shown in Figure 2. Quantitative LC-MS/MS data analysis on 5 different fractions cut from the gel identified that, among 1246 microtubule-binding proteins 38 were differentially bound to microtubules between patient and control cells (SAINTscore=1, Bayesian false discovery rate <0.05) (Table 1). Mapping of relationships between 38 proteins that bind to microtubules differentially between patient and control fibroblasts using the STRING database and the enrichment/overrepresentation analyses of GO terms related to biological process, molecular function and cellular components using the *Enrichr* tool are shown in Figures 3A-D. The data obtained from the proteomic and the bioinformatic analyzes indicate that because of the mutation in the *KATNAL2* gene, the extracellular matrix proteins such as collagens that interact with the cytoskeleton are organized differently from those in normal cells. Strikingly, all the 38 proteins were relatively enriched in *KATNAL2*-patient fibroblasts compared with healthy controls. While our results point to clear differences between extracellular matrix-cellular interactions, the exact molecular mechanisms of the defect due to mutant *KATNAL2* can only be understood through in-depth functional studies of the interactions of the *KATNAL2* protein with the most significantly altered proteins.

Discussion

Biological systems are subject to various perturbations including cell cycle, cellular differentiation, carcinogenesis and environmental factors. Transcriptional and translational responses to these perturbations result in functional changes to the proteome implicated in response to the stimuli. Therefore, describing and quantifying proteome-wide changes in protein abundance is crucial for understanding biological processes more holistically, at the level of the entire system[19]. In this way, proteomics can be seen as complementary to genomics, transcriptomics, epigenomics, metabolomics, and other -omics approaches in integrative analyses attempting to define biological phenotypes more comprehensively. Advances in quantitative proteomics would clearly enable a more in-depth analysis of cellular systems.

In proteomics, there are some commonly used methods to study proteins. Generally, proteins can be detected using antibodies or MS. If a complex biological sample is being analyzed, either a very specific antibody must be used, or because there are too many analyzes to be performed in the sample, biochemical separation must be used for accurate detection and quantification before the detection step[20,21]. Therefore, we used the well-established method of microtubule-pelleting to avoid the enormous noise that would be caused by the total cellular proteome. As KATNAL2's function is tightly linked to the regulation of microtubules and related structures, this approach allowed the identification of altered proteins and pathways, successfully.

Here we leveraged the power of proteomics to identify the altered interactome of the microtubules in skin fibroblasts with a missense mutation in *KATNAL2*. Our results point to

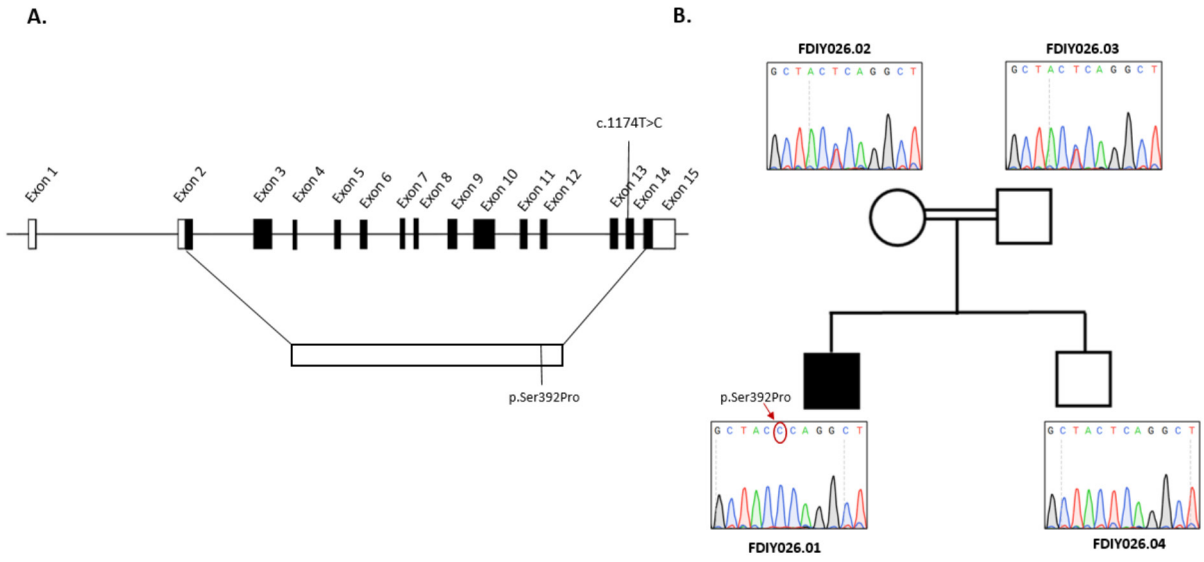


Figure 1. A) Schematic representation of the *KATNAL2* gene and **B)** Variant segregation

KATNAL2: Katanin-like 2

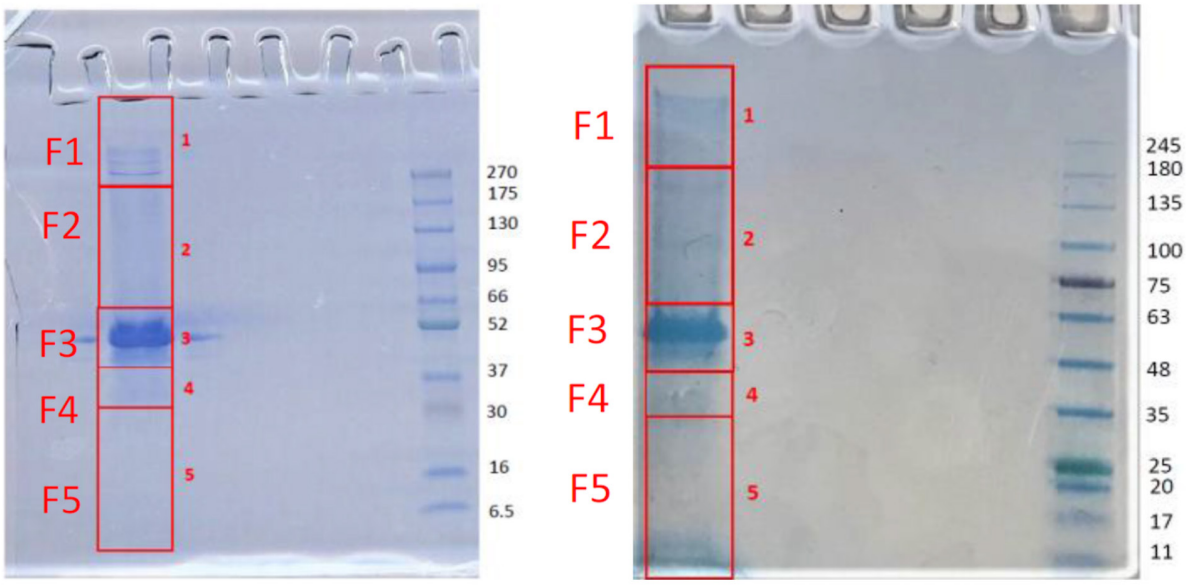


Figure 2. Coomasie staining and fractionation of samples obtained from patient (left) and control (right) fibroblasts after microtubule precipitation on SDS-PAGE gel

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

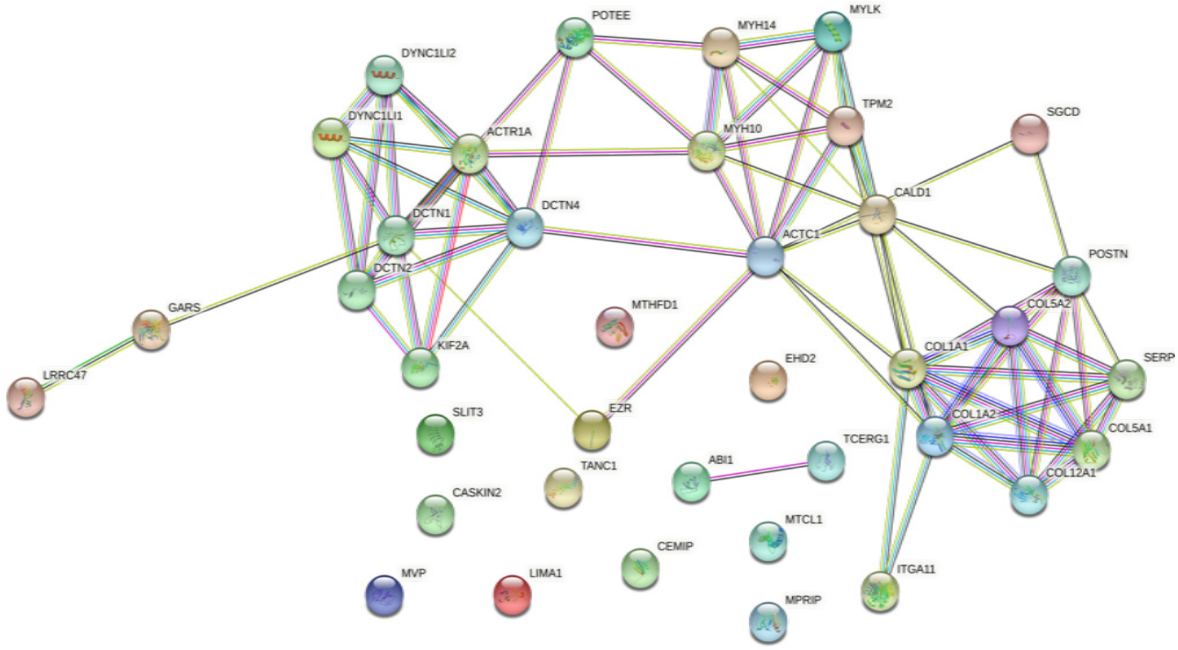
altered extracellular matrix-cell interactions in *KATNAL2*-mutant fibroblasts. To our knowledge, this is the first time such a link has been established, at least in human cells.

Our observation that all differentially bound proteins were higher in the patient pellets, and none were depleted, implies that the mutant cell microtubules tend to form stronger interactions with the binding proteins in the presence of mutant *KATNAL2*. One could speculate that the suboptimal activity of mutant *KATNAL2* alters the microtubule dynamics in a way that

Table 1. Proteins that bind to microtubules differentially in fibroblasts with the homozygous *KATNAL2* variant

	Prey	Prey gene	Spec	Spec sum	Avg spec	Saint score	Log odds score	Fold change	BFDR
0	O14776	TCERG1	3|3	6	3	1	5.39	30	0
1	Q8WXE0	CASKIN2	3|3	6	3	1	5.39	30	0
2	Q9UHB6	LIMA1	9|10	19	9.5	1	5.62	6.33	0
3	Q8IZP0	ABI1	3|6	9	4.5	1	5.39	45	0
4	O75094	SLIT3	9|7	16	8	1	12.06	80	0
5	Q9UJW0	DCTN4	5|4	9	4.5	1	7.13	45	0
6	P11586	MTHFD1	12|34	46	23	1	4.77	7.67	0
7	Q9NZN4	EHD2	16|14	30	15	1	6.95	5	0
8	Q15063	POSTN	3|3	6	3	1	5.39	30	0
9	P05997	COL5A2	22|20	42	21	1	31.75	210	0
10	Q9UKX5	ITGA11	3|9	12	6	1	5.39	60	0
11	O00139	KIF2A	13|13	26	13	1	8.67	6.5	0
12	Q15746	MYLK	3|4	7	3.5	1	5.39	35	0
13	Q8WUJ3	CEMIP	16|21	37	18.5	1	17.06	18.5	0
14	Q7Z406	MYH14	5|4	9	4.5	1	7.13	45	0
15	O43237	DYNC1LI2	7|11	18	9	1	4.93	9	0
16	P35580	MYH10	4|6	10	5	1	7.13	50	0
17	P61163	ACTR1A	6|11	17	8.5	1	10.45	85	0
18	Q8N1G4	LRRC47	3|345	348	174	1	5.39	1740	0
19	Q13561	DCTN2	14|35	49	24.5	1	9.87	12.25	0
20	P02452	COL1A1	22|16	38	19	1	14.45	12.67	0
21	P08123	COL1A2	18|22	40	20	1	28.79	200	0
22	P41250	GARS	3|3	6	3	1	5.39	30	0
23	Q14203	DCTN1	38|68	106	53	1	23.58	8.15	0
24	P68032	ACTC1	132|103	235	117.5	1	5.66	11.75	0
25	Q9Y4B5	MTCL1	7|8	15	7.5	1	12.06	75	0
26	P15311	EZR	4|5	9	4.5	1	7.13	45	0
27	P07951	TPM2	24|66	90	45	1	37.62	450	0
28	Q92629	SGCD	9|18	27	13.5	1	15.2	135	0
29	Q9Y6G9	DYNC1LI1	6|11	17	8.5	1	6.12	17	0
30	Q6S8J3	POTEE	23|251	274	137	1	36.15	1370	0
31	Q9C0D5	TANC1	5|6	11	5.5	1	8.81	55	0
32	P20908	COL5A1	22|3	25	12.5	1	5.39	125	0
33	Q14764	MVP	41|34	75	37.5	1	18.03	5.36	0
34	Q05682	CALD1	59|59	118	59	1	21.38	3.69	0
35	Q6WCQ1	MPRIP	3|3	6	3	1	5.39	30	0
36	Q99715	COL12A1	33|14	47	23.5	1	22.82	235	0
37	P50454	SERPINH1	27|87	114	57	1	17.05	12.67	0

BFDR: Bayesian false discovery rate, KATNAL2: Katanin-like 2


POTEE
MYH14
MYLK
DYNC1LI2
DYNC1LI1
ACTR1A
MYH10
TPM2
SGCD
CALD1
DCTN1
DCTN4
ACTC1
DCTN2
POSTN
GARS
MTHFD1
COL5A2
KIF2A
COL1A1
SERP
LRRC47
EHD2
SLIT3
EZR
COL1A2
COL5A1
TCERG1
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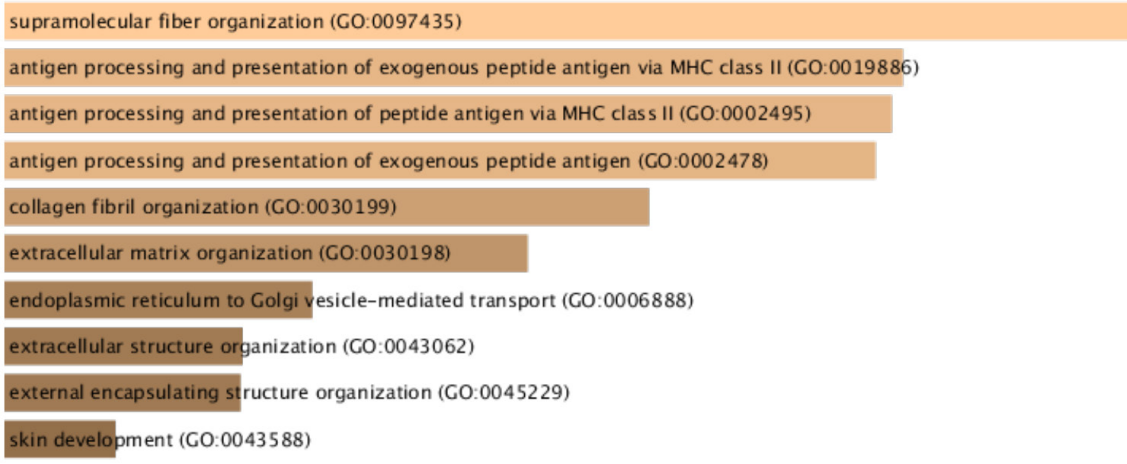
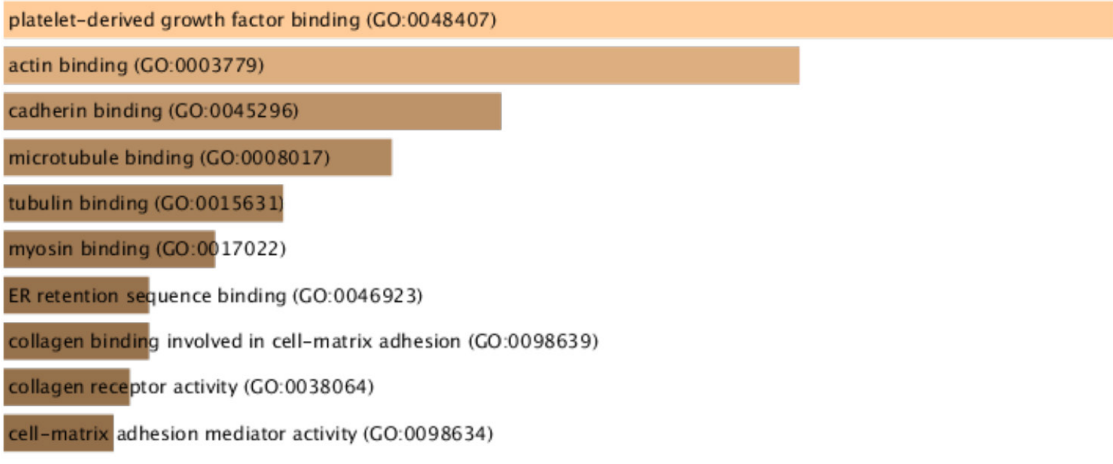
TANC1
COL12A1
CASKIN2
MTCL1
CEMIP
ITGA11
MVP
LIMA1
MPRIP

A)


supramolecular fiber organization (GO:0097435)
antigen processing and presentation of exogenous peptide antigen via MHC class II (GO:0019886)
antigen processing and presentation of peptide antigen via MHC class II (GO:0002495)
antigen processing and presentation of exogenous peptide antigen (GO:0002478)
collagen fibril organization (GO:0030199)
extracellular matrix organization (GO:0030198)
endoplasmic reticulum to Golgi vesicle-mediated transport (GO:0006888)
extracellular structure organization (GO:0043062)
external encapsulating structure organization (GO:0045229)
skin development (GO:0043588)

B)


platelet-derived growth factor binding (GO:0048407)
actin binding (GO:0003779)
cadherin binding (GO:0045296)
microtubule binding (GO:0008017)
tubulin binding (GO:0015631)
myosin binding (GO:0017022)
ER retention sequence binding (GO:0046923)
collagen binding involved in cell-matrix adhesion (GO:0098639)
collagen receptor activity (GO:0038064)
cell-matrix adhesion mediator activity (GO:0098634)

C)

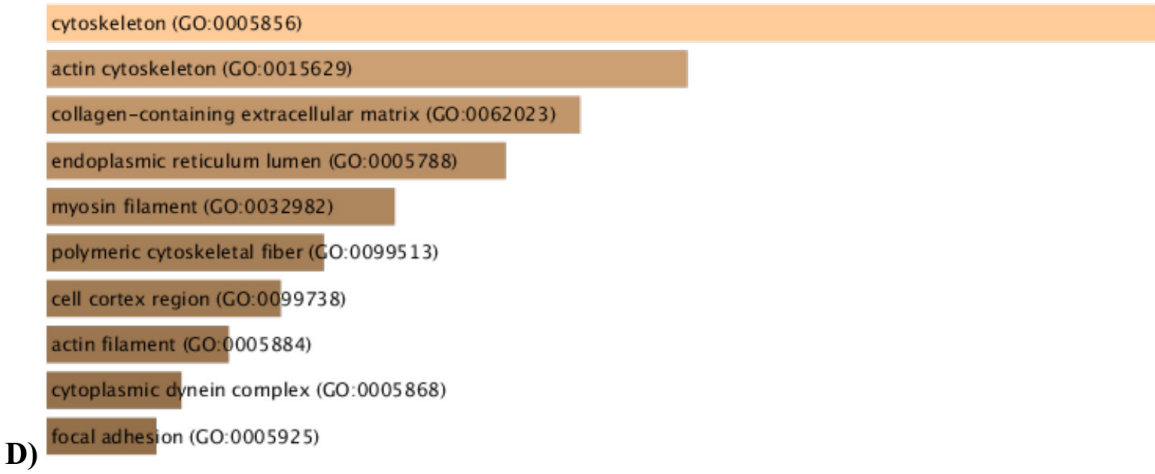


Figure 3. A) Mapping of relationships between 38 proteins that bind to microtubules differentially between patient and control fibroblasts using STRING database. **B-D)** Enrichment analyses of GO terms related to biological process, molecular function and cellular component, using *Enrichr* tool

GO: Gene ontology

more readily allows microtubule-protein interactions. Alternatively, altered cytoskeletal organization might lead to changes in cell's biophysical properties, which would indirectly cause alterations in cell-cell and cell-extracellular matrix interactions. Our preliminary data suggest no change in cellular shape or an overt change in cellular dynamics (data not shown), however, we cannot rule out altered cytoskeletal organization.

Conclusion

We have demonstrated here, for the first time, altered interactions between extracellular matrix and cells in *KATNAL2*-mutant fibroblasts using the proteomics method. Further studies will be required to pinpoint the exact molecular alterations leading to the severe neurological disease observed in *KATNAL2* patients. One possible candidate could be POTEE (POTE Ankyrin domain family, member E) protein, due to its hub-gene role among the 38 proteins and high-level of upregulation (1370-fold).

Ethics

Ethics Committee Approval: This study was approved by the Dokuz Eylül University Ethics Committee (decision no: 2019/29-36, date: 02.12.2019).

Informed Consent: Written informed consent was obtained from the parents of the patient.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.S.H., A.Y., Concept: A.S.H., A.Y., E.S., Y.O., Design: A.S.H., A.Y., E.S., Y.O., Data Collection or Processing: A.S.H., E.S., Y.O., Analysis or Interpretation: A.S.H., E.S., Y.O., Literature Search: A.S.H., Y.O., Writing: A.S.H., Y.O.

Conflict of Interest: No conflict of interest was declared by the authors.

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