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Araştırma Makalesi

Assessing the Functional Properties of the *TMCO1* Sequence Variants by Using *In Silico* Analyses

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ABSTRACT

Transmembrane and Coiled-Coil Domains 1 (TMCO1) protein is encoded by *TMCO1* gene consists of 7 exons. Previous studies have identified multiple *TMCO1* variants in patients with cerebro-facio-thoracic dysplasia (CFTD) and *TMCO1* locus was also shown to be associated with primary open angle glaucoma (POAG). However, there are limited number of research exist reporting associations of the *TMCO1* gene sequence variants and majority of the findings affirm the pathogenicity of the nonsense and frameshift *TMCO1* variants and their associations with clinical phenotypes. Thus functional properties of the single nucleotide variants causing amino acid changes in the TMCO1 are yet to be comprehensively elucidated. In this study, we evaluated the effects of amino acid substitutions on protein structure, identified their putative roles in post-translational modifications (PTM) and in regulatory mechanism for TMCO1 protein. We classified 41 missense variants as pathogenic based on combined scores of common in silico tools (SIFT, MutationTaster2, Polyphen2). Of these 41 variants, four (p.K211Q, p.K105E, p.S235F, p.K237R) were identified to be located in PTMs and regulatory protein binding sites; thus they were proposed to be putative functional variants. Moreover, rs1387528611 (p.Lys128Gln) had also strong evidence (RegulomeDB score=2b) for its possible regulatory function. The results of our in silico analyses highlight the functional importance of the missense *TMCO1* variants that may contribute to the *TMCO1*-associated disease phenotypes and further in vivo evaluation yet to be needed to uncover their role in human diseases.

Keywords: *TMCO1, Cerebro-facio-thoracic dysplasia, RegulomeDB, SNV, post-translational modifications, in silico analyses*

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TMCO1 Gen Sekans Varyanlatlarının Fonksiyonel Özelliklerinin *In Silico* Analizlerlerle Değerlendirilmesi

ÖZET

Transmembran and Coiled-Coil Domains 1 (TMCO1) proteini, *TMCO1* geni tarafından kodlanır ve 7 ekzondan oluşur. Önceki çalışmalar serebrofasiotorasik displazili (SFTD) hastalarda çok sayıda *TMCO1* varyantı tanımlamış ve *TMCO1* lokusunun primer açık açılı glokom hastalığı ile (PAAG) ilişkili olduğunu göstermiştir. Bununla birlikte *TMCO1* gen sekansı varyantlarının ilişkilerini bildiren sınırlı sayıda araştırma vardır ve elde edilen bulguların çoğu anlamsız mutasyonlar ve çerçeve kayması mutasyonlarının *TMCO1* varyantlarının patojenliğini ve klinik fenotiplerle ilişkilerini belirtmektedir. Bu nedenle, TMCO1'de aminoasit değişikliklerine neden olan tek nükleotid varyantlarının fonksiyonel özellikleri henüz tam olarak açıklanamamıştır. Bu çalışmada aminoasit değişikliklerinin protein yapısı üzerindeki etkilerini, post-translasyon modifikasyonlardaki (PTM) ve TMCO1 proteini için düzenleyici mekanizmadaki olası rollerini belirledik. Yaygın olarak kullanılan in silico araçları (SIFT, MutationTaster2, Polyphen2) ile yaptığımız analizin değerlendirmesine göre 41 adet yanlış anlamlı mutasyon barındıran varyantı patojenik olarak sınıflandırdık. Bu 41 varyanttan dördü (p.K211Q, p.K105E, p.S235F, p.K237R) PTM ve düzenleyici protein bağlama bölgelerinde yer almaktadır, bu nedenle bu varyantların fonksiyon üzerinde etkili olduğunu düşündük. Bununla birlikte, rs1387528611 (s.Lys128Gln) varyantının (RegulomeDB skoru= 2b) düzenleyici varyant olabileceğine dair güçlü biyolojik kanıtlar olduğunu saptadık. In silico analizlerimizin sonuçları, *TMCO1* ile ilişkili hastalık fenotiplerine katkıda bulunabilecek yanlış anlamlı *TMCO1* varyantların fonksiyonel önemini ve insan hastalıklarındaki rollerini ortaya çıkarmak için in vivo değerlendirmenin işlevsel önemini vurgulamaktadır.

Anahtar Kelimeler: *TMCO1, Serebrofasiyotorasik displazi, RegulomeDB, SNV, Post-translasyonel modifikasyonlar, in silico analiz*

I. INTRODUCTION

Transmembrane and Coiled-Coil Domains 1 (TMCO1) encodes a 239 amino acid transmembrane protein which belongs to DUF841 superfamily in eukaryotes [1]. The protein was first identified by Iwamuro (1999) in an in vitro translation study conducted in rabbit reticulocyte lysates [2]. In this study, the protein was shown to be located in endoplasmic reticulum (ER) and golgi in COS 7 cells. The highest expression level of *TMCO1* was found in thymus, prostate, testis and small intestine whereas the lowest expression level was detected in brain, placenta, lung and kidney. In a subsequent study of porcine cells, TMCO1 was sublocalized in mitochondria in PK-15 cells [1].

A genome-wide homozygosity mapping study localized the disease location on chromosome 1q23.3-q24.1 in an Amish family with 11 affected individuals with autosomal recessive inheritance. Following candidate gene sequencing a 2bp homozygous deletion (c.139_140delAG) was identified in all of the patients resulting in frameshift mutation and truncated TMCO1 protein (p.Ser47Ter) [3]. The patients had craniofacial dysmorphism, skeletal anomalies and mental retardation. The craniofacial dysmorphism was presented with brachycephaly, flat face, low hairline, low-set ears, high arched palate, and cleft lip and palate while the skeletal anomalies of the patients were pectus excavatum, club feet in early infancy, scoliosis, and long, hyperextensible fingers in puberty. Following these findings, four more studies reported three homozygous nonsense (p.Arg87Ter, p.Arg114Ter,

p.Ser98Ter) and a splice-site mutation (c.323+3G>C) in the *TMCO1* gene in (CFTD, MIM#213980) in different cases of non-Amish patients with CFTD who had similar clinical features [4-8]. In 2011, a genome wide association study (GWAS) study also reported two susceptibility loci -one was near *TMCO1* gene (rs4656461)- were associated with primary open angle glaucoma (POAG) [9]. POAG is the most common type of glaucoma which is characterized by apoptosis and death of retinal ganglion cells resulting in visual loss. The GWAS locus, rs4656461, were shown to be associated with POAG in Australians, Pakistanis, Europeans and non-Hispanic Whites, yet no association was detected in African Americans and Chinese individuals.[10-16]. Further studies that focus on another variant (rs7555523) at *TMCO1* locus showed association with POAG and Intraocular Pressure (IOP) in Chinese and European Cohorts, respectively [16, 17]. However, lack of association of this variant with POAG was observed in Saudi Cohort [18]. Subsequent studies aiming to determine disease associated variants near or at *TMCO1* results identification of associations of rs7518099 and rs4657473 with IOP in Caucasian and POAG in African Americans, respectively. However, lack of associations of rs10800149 and rs7518099 with POAG were shown in African and Afro-Americans [12, 14, 15].

Recent studies started to enlighten the molecular function of the TMCO1 as a ER transmembrane protein in Ca2+ channel in response to excess Ca2+ load in vitro. Homotetramerization of TMCO1 is observed if the ER Ca2+ capacity is overloaded. Moreover, TMCO1 knockout mice showed similar phenotype to human CFTD as expected since TMCO1 is highly conserved among many species [19]. Interestingly female Tmco1-/- mice exhibited subfertility with reduced follicle development and declined in ovarian follicles [20].

To date, the *TMCO1* sequence variants have been shown to be associated with Glaucoma and linked to CFTD in a limited number of studies [3-5, 8, 21-23]. Variants affecting the structure of the TMCO1 protein particularly frameshift and nonsense variants were observed in critical phenotypes. Thus, more studies are needed to elucidate the roles of *TMCO1* genetic variation on human disease and identify the functional properties of its sequence variants. In this study, we evaluated the functional importance of *TMCO1* variants using in silico tools since their clinical interpretations and pathogenicity assessments are crucial to understand their contribution to the *TMCO1*-associated clinical phenotypes.

II. METHODS

A. RETRIEVING SINGLE NUCLEOTIDE VARIANTS DATABASE

Data of single nucleotide variants (SNVs) corresponding to the human *TMCO1* gene were acquisted from Variation Viewer https://www.ncbi.nlm.nih.gov/variation/view by selecting the NCBI Reference Sequence as GRCh38.p12, NC_000001.11, NM_019026, (ENST00000392129.10). We filtered the variants included in the dbSNP database and only missense and nonsense SNVs were analyzed in the current study. Bioinformatic tools and databases used to assess functional importance of the filtered variants were depicted in the workflow (Figure 1).

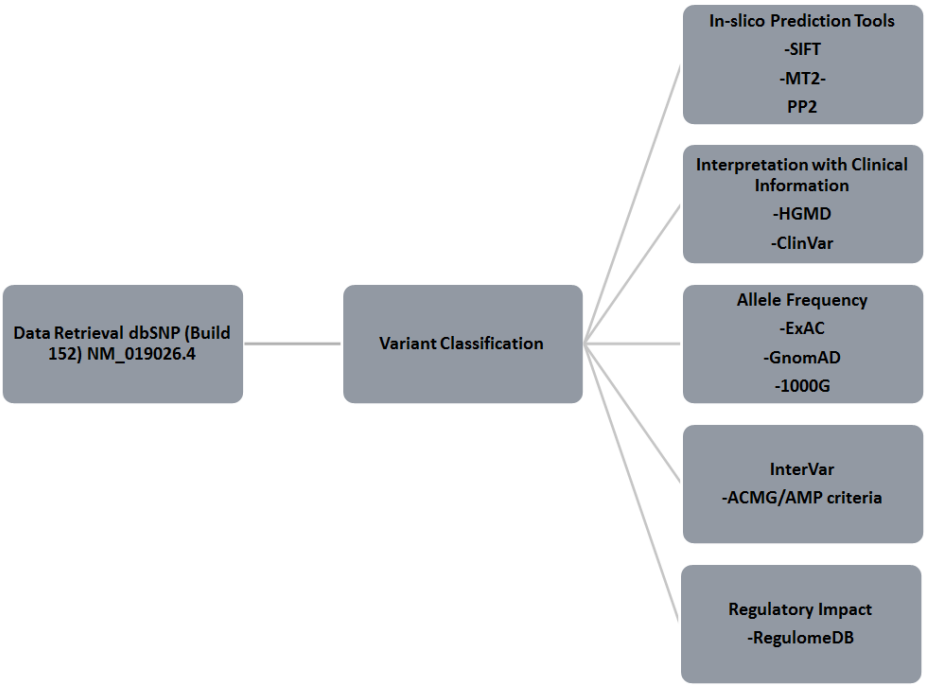

Figure 1. The workflow of the *TMCO1* variant classification.

B. EVALUATION OF PATHOGENICITY OF MISSENSE TMCO1 VARIANTS

Pathogenicity interpretation of a sequence variant requires meticulous assessments considering different aspects including the observed allele frequency of the variant in healthy individuals and in clinical phenotypes. Two bioinformatic tools and public databases were used to evaluate sequence variants by incorporating genetic data with clinical information: ClinVar https://www.ncbi.nlm.nih.gov/clinvar/ and HGMD (Human Gene Mutation Database) Professional 2018.3 (http://www.hgmd.cf.ac.uk/). We also used InterVar (http://quanli.tk/wInterVar/) which interprets the clinical significance of the sequence variants by using criterias of the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP) [24].

Population allele frequencies of the *TMCO1* variants were extracted from three resources aggregating data of large-scale sequencing projects: 1) The Exome Aggregation Consortium (ExAC) Browser, 2) 1000 Genome Data Browser and 3) Genome Aggregation Database (GnomAD). We used different in silico predictive tools which have specific computational algorithms incorporated to assess the tolerance and pathogenicity of the *TMCO1* variants. Predictors used in this study were Sorting Intolerant From Tolerant (SIFT), Mutation Taster 2 (MT2) and Polymorphism Phenotyping v.2 (Polyphen-2) for evaluating the functional consequences of all missense variants. SIFT predicts functional importance of the amino acid substitutions on protein based on the evolutionary sequence homology and interprets variants as deleterious or tolerated [25] (http://sift.jcvi.org). Structural, sequence and phylogenetic annotations were used in Polyphen-2 (PP2) to characterize the pathogenicity of the substitutions on protein level and predictions were classified as benign, possibly damaging, or probably damaging [26] (http://genetics.bwh.harvard.edu/pph2). PP2 requires specific protein identifier for the interested variant to be analyzed and Q9UM00 was retrieved from UniProtKB database for analyzing *TMCO1* sequence variants in PP2. SIFT and PP2 predictions score only

missense variants however MT2 can also handle indels. MT2 (http://www.mutationtaster.org) classifies variants as disease causing or polymorphism [27]. We included all missense variants for classifying their effects on TMCO1 and combined the scores of three commonly used algorithms (SIFT, MT2 and PP2) particularly for evaluating missense variants' pathogenicity. Combined in silico score (CIS) were assigned as pathogenic or benign when the results of the three algorithms were same in terms of prediction. Variants with inconsistent results attained from the algorithms were scored as VUS (Variant of Uncertain Significance).

C. REGULATORY FUNCTIONS OF THE TMCO1 VARIANTS USING REGULOMEDB

TMCO1 variants that may have potential regulatory functions were explored using RegulomeDB database integrating data from experiments and computational predictions [28]. RegulomeDB assigns scores from 1 to 6 for SNVs based on their possible regulatory impacts; lower scores suggest strong evidence that variant reside in the regulatory regions. In this study, we prioritized the variants with a RegulomeDB score <3 and considered them as regulatory SNV of the *TMCO1* gene. Scoring scheme for RegulomeDB database is listed in Table 1.

Table 1. *The representations of the RegulomeDB scores*

RegulomeDB Score	Biological Evidence
1a	eQTL + TF binding + matched TF motif + matched DNase Footprint + DNase peak
1b	eQTL + TF binding + any motif + DNase Footprint + DNase peak
1c	eQTL + TF binding + matched TF motif + DNase peak
1d	eQTL + TF binding + any motif + DNase peak
1e	eQTL + TF binding + matched TF motif
1f	eQTL + TF binding / DNase peak
2a	TF binding + matched TF motif + matched DNase Footprint + DNase peak
2b	TF binding + any motif + DNase Footprint + DNase peak
2c	TF binding + matched TF motif + DNase peak
3a	TF binding + any motif + DNase peak
3b	TF binding + matched TF motif
4	TF binding + DNase peak
5	TF binding or DNase peak
6	other

D. ANALYSES OF PHOSPHORYLATION-RELATED TMCO1 VARIANTS

We also explored the post-translational modification (PTM) sites of TMCO1 protein and identified the reported non-synonymous (nsSNVs) exist in these regions. PhosphoSitePlus (PSP) database (https://www.phosphosite.org) which contains experimental data for PTMs including phosphorylation, acetylation, ubiquitylation and methylation was used to define PTM sites in the TMCO1 protein. Over 95% of the PTM sites defined in PSP are from mass spectrometry (MS) experiments [29].

III. RESULTS

As a result, 155 nsSNVs were downloaded from the Variation Viewer (dbSNP build 152) of which 142 were missense and 13 were nonsense variants (Table 2). The distributions of variants according to the exon position was not uniform. The majority of the nsSNVs (46.45 %) were located in the Exon 1 followed by Exon 6 and Exon 7. The number of nsSNVs located in the Exon 2, 3, 4 and 5 were comparable to each other (Figure 2).

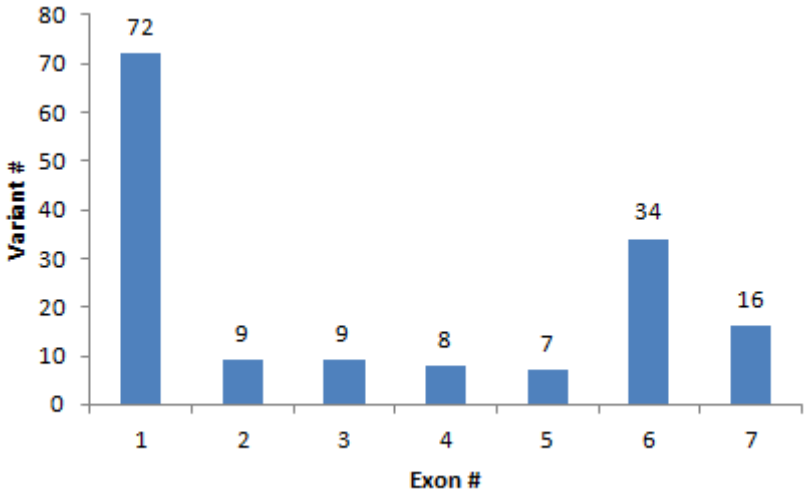

Figure 2. *Distributions of nsSNVs across exons of the TMCO1 gene.*

The classification of all variants according to the ACMG/AMP criteria using InterVar database resulted 17 pathogenic/likely pathogenic and 138 VUS; of 142 missense variants 6 were classified as pathogenic/likely pathogenic and 135 were VUS variants. ClinVar is a public tool to combine the variant information with the clinical findings. *TMCO1* gene variants were searched in the ClinVar database and only one missense variant (rs1332024737) and 4 nonsense variants were identified as VUS and pathogenic, respectively; no clinical data is available for the remaining variants (Table 2). HGMD is another public database to link between gene mutations and disease phenotypes worldwide. Only 2 variants (rs765824628, rs201213306) are present in HGMD Professional database and all were classified as DM (Disease Mutation) for CFTD.

Prediction of the consequence of the missense mutations on TMCO1 protein was achieved using three different *in silico* tools: SIFT, PP2, and MT2. Submitting the 155 variants to SIFT tool resulted in 95 damaging and 48 tolerated variants. No information was obtained for 12 nonsense variants. According to the PP2 prediction, 14 variants were classified as damaging, 20 as possibly damaging, 41 as probably damaging, 29 as benign and no data was available for the 51 variants. Mutation Taster is the third prediction tool and 101 variant were classified as disease causing, while 54 were predicted as polymorphism. The results of our *in silico* prediction classification combining the results of three predictors (SIFT, PP2, MT2) are as follows; 98 variants were categorized as pathogenic (%25), 57 were VUS and none of them were categorized as benign variant.

Table 2. *Results of analyses of TMCO1 nsSNVs using in-slico tools and bioinformatic sources.*

RefSNP ID	Molecular Consequences	A.a. Change	InterVar	In-Slico*	SIFT	PP2	RegulomeDB	MT2	1000G	ExAC	GnomAD	ClinVar	HGMD
rs896888634	MS	p.Cys68Trp	VUS	P	D	PD	4	DC	NA	NA	NA	NA	NA
rs1448690745	MS	p.Asp58Ala	VUS	P	D	PD	4	DC	NA	NA	3.98E-06	NA	NA
rs1332024737	MS	p.Leu60Pro	VUS	P	D	PD	NA	DC	NA	NA	3.98E-06	VUS	NA
rs757084156	MS	p.Thr69Pro	VUS	P	D	PD	4	DC	NA	8.25E-06	1.77E-05	NA	NA
rs1053090890	MS	p.Thr77Ile	VUS	P	D	PD	5	DC	NA	NA	NA	NA	NA
rs573501926	MS	p.Leu80Arg	VUS	P	D	PD	5	DC	2.00E-04	8.25E-06	3.98E-06	NA	NA
rs368923697	MS	p.Arg83Lys	P	P	D	PD	5	DC	NA	NA	NA	NA	NA
rs1256737415	MS	p.Lys105Glu	VUS	P	D	PSD	5	DC	NA	NA	3.98E-06	NA	NA
rs761130784	MS	p.Glu106Gly	VUS	P	D	PD	5	DC	NA	8.31E-06	1.77E-05	NA	NA
rs1364557967	MS	p.Glu121Lys	VUS	P	D	PD	5	DC	NA	NA	3.99E-06	NA	NA

rs1387528611	MS	p.Lys128Gln	VUS	P	D	PD	2b	DC	NA	NA	3.98E-06	NA	NA
rs1390115490	MS	p.Lys128Asn	VUS	P	D	PD	3a	DC	NA	NA	3.98E-06	NA	NA
rs1253586909	MS	p.Leu134Pro	VUS	P	D	PD	4	DC	NA	NA	NA	NA	NA
rs763875549	MS	p.Arg138Gln	VUS	P	D	PSD	NA	DC	NA	8.39E-06	7.96E-06	NA	NA
rs760232273	MS	p.Met139Val	VUS	P	D	PD	NA	DC	NA	8.39E-06	3.98E-06	NA	NA
rs771825350	MS	p.Ile145Ser	VUS	P	D	B	6	DC	NA	8.34E-06	3.98E-06	NA	NA
rs759265609	MS	p.Phe160Leu	VUS	P	D	PD	NA	DC	NA	1.00E-05	NA	NA	NA
rs774273682	MS	p.Asp161Tyr	VUS	P	D	PD	NA	DC	NA	1.00E-05	NA	NA	NA
rs773388746	MS	p.Val164Gly	VUS	P	D	PD	NA	DC	NA	4.00E-05	NA	NA	NA
rs1349622376	MS	p.Val165Met	VUS	P	D	PD	NA	DC	NA	NA	NA	NA	NA
rs1319496801	MS	p.Ala166Ser	VUS	P	D	PD	NA	DC	NA	NA	3.56E-06	NA	NA
rs11557504	MS	p.Leu168Ile	VUS	P	D	PD	NA	DC	NA	NA	NA	NA	NA
rs748433524	MS	p.Phe170Cys	VUS	P	D	PD	NA	DC	NA	8.25E-06	3.98E-06	NA	NA
rs776944318	MS	p.Pro172Arg	VUS	P	D	PD	NA	DC	NA	8.25E-06	3.98E-06	NA	NA
rs780554889	MS	p.Leu179Pro	VUS	P	D	PD	NA	DC	NA	8.25E-06	1.19E-05	NA	NA
rs1207775141	MS	p.Ser180Cys	VUS	P	D	PD	NA	DC	NA	NA	NA	NA	NA
rs746331589	MS	p.Thr190Ala	VUS	P	D	PSD	NA	DC	NA	6.59E-05	2.78E-05	NA	NA
rs777377528	MS	p.Asp191Gly	VUS	P	D	PD	NA	DC	NA	1.65E-05	1.19E-05	NA	NA
rs1293672835	MS	p.Phe196Ile	VUS	P	D	PD	6	DC	NA	NA	3.98E-06	NA	NA
rs767085714	MS	p.Leu197Pro	VUS	P	D	PD	6	DC	NA	8.24E-06	3.98E-06	NA	NA
rs751410355	MS	p.Tyr198Cys	VUS	P	D	PD	6	DC	NA	8.24E-06	3.19E-05	NA	NA
rs1390745807	MS	p.Cys201Tyr	VUS	P	D	PD	NA	DC	NA	NA	NA	NA	NA
rs373042992	MS	p.Gln207His	VUS	P	D	PD	6	DC	NA	8.24E-06	3.98E-06	NA	NA
rs1398266846	MS	p.Lys211Gln	VUS	P	D	PD	6	DC	NA	NA	3.99E-06	NA	NA
rs915211345	MS	p.Gly214Ser	VUS	P	D	PD	NA	DC	NA	NA	NA	NA	NA
rs1302569629	MS	p.Pro217Ser	VUS	P	D	PSD	NA	DC	NA	NA	NA	NA	NA
rs1425428199	MS	p.Gly227Glu	VUS	P	D	PSD	6	DC	NA	NA	3.98E-06	NA	NA
rs1392427324	MS	p.Gly230Arg	VUS	P	D	PD	NA	DC	NA	NA	NA	NA	NA
rs1459734421	MS	p.Ser235Phe	VUS	P	D	PSD	6	DC	NA	NA	3.98E-06	NA	NA
rs768112478	MS	p.Lys237Arg	VUS	P	D	PD	6	DC	NA	8.25E-06	3.98E-06	NA	NA
rs779514586	MS	p.Phe238Leu	VUS	P	D	PSD	6	DC	NA	1.00E-05	3.98E-06	NA	NA

***TMCO1* variants designated as VUS by combined in-slico scores**

RefSNP ID	Molecular Conseq.	A.a. Change	InterVar	İn-Slico	SIFT	PP2	RegulomeDB	MT2	1000G	ExAC	GnomAD	ClinVar	HGMD
rs747266207	MS	p.Met1Val	LP	VUS	T	NA	4	P	NA	NA	**7.33E-06**	NA	NA
rs1045406608	MS	p.Met1Arg	LP	P	D	NA	4	P	NA	NA	NA	NA	NA
rs745928907	MS	p.Pro2Thr	VUS	P	D	NA	2a	P	NA	NA	NA	NA	NA
rs1161605092	MS	p.Arg5Trp	VUS	P	D	NA	2a	P	NA	NA	6.79E-06	NA	NA
rs1476414936	MS	p.Arg5Pro	VUS	VUS	T	NA	2a	P	NA	NA	6.73E-06	NA	NA
rs1374771274	NS	p.Cys7Ter	VUS	VUS	T	D	2a	DC	NA	NA	1.31E-05	NA	NA
rs775810253	MS	p.Asp8Asn	VUS	P	D	NA	2a	P	NA	5.10E-05	6.52E-06	NA	NA
rs772583336	MS	p.Asp8Glu	VUS	P	D	NA	2b	P	NA	NA	NA	NA	NA
rs949839058	MS	p.Leu9Phe	VUS	P	D	NA	4	P	NA	NA	1.28E-05	NA	NA
rs1176744246	MS	p.Arg10Gln	VUS	P	D	NA	4	P	NA	NA	NA	NA	NA
rs1288692026	MS	p.Ala11Val	VUS	P	D	NA	4	P	NA	NA	6.18E-06	NA	NA
rs1340894520	MS	p.Val12Leu	VUS	P	D	NA	4	P	NA	NA	NA	NA	NA
rs937760628	MS	p.Val12Ala	VUS	P	D	NA	4	P	NA	NA	NA	NA	NA
rs780791020	MS	p.Arg13Gly	VUS	P	D	NA	4	P	NA	NA	1.36E-04	NA	NA
rs754441437	MS	p.Arg13Ser	VUS	P	D	NA	4	P	NA	7.11E-05	3.53E-05	NA	NA
rs1335826460	MS	p.Val14Ile	VUS	P	D	NA	4	P	NA	NA	NA	NA	NA
rs373051778	MS	p.Leu16Pro	VUS	P	D	NA	4	P	NA	NA	1.66E-05	NA	NA
rs1432218976	NS	p.Leu17Ter	P	P	NA	D	4	DC	NA	NA	3.19E-05	NA	NA
rs765958391	MS	p.Leu17Phe	VUS	P	D	NA	4	P	NA	2.74E-05	5.43E-06	NA	NA
rs1411818361	MS	p.Leu18Phe	VUS	P	D	NA	4	P	NA	NA	5.39E-06	NA	NA
rs1264007339	MS	p.Leu18His	VUS	P	D	NA	3a	P	NA	NA	NA	NA	NA
rs758191413	MS	p.Gly19Cys	VUS	P	D	NA	3a	P	NA	NA	5.30E-06	NA	NA
rs145109620	MS	p.Gly20Asp	VUS	P	D	NA	3a	P	2.00E-04	NA	NA	NA	NA
rs764920804	MS	p.Gly21Arg	VUS	P	D	NA	3a	P	NA	2.22E-05	5.04E-06	NA	NA
rs1388231675	MS	p.Gly21Asp	VUS	P	D	NA	3a	P	NA	NA	NA	NA	NA
rs75840847	NS	p.Gly22Ter	P	P	NA	D	1f	DC	4.00E-02	4.21E-05	8.63E-06	NA	NA
rs764292839	MS	p.Gly22Val	VUS	P	D	NA	4	P	NA	2.09E-05	9.96E-06	NA	NA
rs760783250	MS	p.Val23Asp	VUS	P	D	NA	4	P	NA	1.88E-05	8.32E-06	NA	NA

rs1175720485	MS	p.Tyr24His	VUS	P	D	NA	4	P	NA	NA	3.19E-05	NA	NA
rs772491686	NS	p.Tyr24Ter	VUS	VUS	T	D	4	P	NA	1.77E-05	8.15E-06	NA	NA
rs746077564	MS	p.Gly25Arg	VUS	P	D	NA	4	P	NA	1.76E-05	4.67E-06	NA	NA
rs1340749721	MS	p.Ser26Gly	VUS	P	D	NA	4	P	NA	NA	4.49E-06	NA	NA
rs566687101	MS	p.Ser26Arg	VUS	P	D	NA	2b	P	2.00E-04	1.39E-03	7.61E-04	NA	NA
rs1235539060	MS	p.Arg27His	VUS	P	D	NA	2b	P	NA	NA	NA	NA	NA
rs1205580878	MS	p.Phe28Leu	VUS	P	D	NA	2b	P	NA	NA	3.19E-05	NA	NA
rs771167297	MS	p.Arg29Cys	VUS	P	D	NA	2b	P	NA	2.65E-05	2.97E-05	NA	NA
rs778089145	MS	p.Arg29Leu	VUS	P	D	NA	2b	P	NA	2.59E-05	1.12E-05	NA	NA
rs62622803	MS	p.Phe30Leu	VUS	P	D	NA	2b	P	6.00E-03	1.49E-03	1.12E-03	NA	NA
rs1336516207	MS	p.Thr31Ala	VUS	P	D	NA	2b	P	NA	NA	4.13E-06	NA	NA
rs746449477	MS	p.Thr31Ser	VUS	P	D	NA	2b	P	NA	1.16E-05	8.23E-06	NA	NA
rs796123797	MS	p.Phe32Val	VUS	P	D	NA	2b	P	NA	NA	7.29E-06	NA	NA
rs779533546	MS	p.Pro33Leu	VUS	VUS	T	NA	4	P	NA	1.07E-05	4.07E-06	NA	NA
rs988874481	MS	p.Gly34Val	VUS	P	D	NA	4	P	NA	NA	8.11E-06	NA	NA
rs750014669	MS	p.Cys35Gly	VUS	VUS	T	NA	4	P	NA	2.08E-05	2.02E-05	NA	NA
rs765112121	MS	p.Cys35Tyr	VUS	VUS	T	NA	4	P	NA	5.08E-05	3.58E-05	NA	NA
rs377564729	MS	p.Arg36Lys	VUS	P	D	NA	4	P	NA	9.91E-06	8.05E-06	NA	NA
rs200952577	MS	p.Ala37Gly	VUS	P	D	NA	4	P	2.00E-04	2.89E-05	1.43E-05	NA	NA
rs1223127433	MS	p.Ser39Thr	VUS	P	D	NA	3a	P	NA	NA	NA	NA	NA
rs775773638	MS	p.Ser39Phe	VUS	P	D	NA	3a	P	NA	3.00E-05	3.55E-05	NA	NA
rs906155551	MS	p.Trp41Gly	VUS	P	D	NA	3a	P	NA	NA	NA	NA	NA
rs1412053241	MS	p.Arg42Trp	VUS	P	D	NA	3a	P	NA	NA	NA	NA	NA
rs774391939	MS	p.Val43Leu	VUS	P	D	NA	3a	P	NA	1.74E-05	7.98E-06	NA	NA
rs771345698	MS	p.Val43Ala	VUS	P	D	NA	3a	P	NA	8.69E-06	8.00E-06	NA	NA
rs763169187	MS	p.Arg44Lys	VUS	VUS	T	NA	4	P	NA	8.61E-06	NA	NA	NA
rs773712450	MS	p.Val45Gly	VUS	P	D	NA	4	P	NA	5.13E-05	2.79E-05	NA	NA
rs1225818105	MS	p.Glu51Lys	VUS	VUS	D	B	4	P	NA	NA	3.98E-06	NA	NA
rs770351223	MS	p.Met52Val	LP	VUS	D	B	4	DC	NA	2.00E-05	1.41E-05	NA	NA
rs1287927441	MS	p.Met55Leu	VUS	VUS	T	B	4	DC	NA	NA	7.96E-06	NA	NA
rs1226699617	MS	p.Met55Ile	VUS	VUS	T	PSD	4	DC	NA	NA	3.98E-06	NA	NA
rs548152171	MS	p.Ala57Pro	VUS	VUS	T	B	4	DC	2.00E-04	1.66E-05	7.96E-06	NA	NA
rs771532906	MS	p.Thr59Ser	VUS	VUS	T	B	4	DC	NA	8.27E-06	NA	NA	NA
rs745557634	MS	p.Leu60Phe	VUS	VUS	T	B	4	DC	NA	2.00E-05	3.98E-06	NA	NA
rs778373100	MS	p.Val63Leu	VUS	VUS	T	B	3a	DC	NA	8.26E-06	3.98E-06	NA	NA
rs1412319588	MS	p.Val67Leu	VUS	VUS	T	B	4	DC	NA	NA	3.98E-06	NA	NA
rs753646054	MS	p.Leu71Val	VUS	VUS	T	PSD	4	DC	NA	1.00E-05	7.95E-06	NA	NA
rs1187974061	MS	p.Leu72Val	VUS	VUS	T	PSD	4	DC	NA	NA	3.98E-06	NA	NA
rs929157192	MS	p.Gly75Ser	VUS	VUS	T	PD	2b	DC	NA	NA	NA	NA	NA
rs773786877	NS	p.Trp78Ter	P	P	NA	D	5	DC	NA	1.65E-05	7.96E-06	NA	NA
rs201984392	NS	p.Trp78Ter	P	P	NA	D	5	DC	NA	8.25E-06	3.98E-06	NA	NA
rs1410430424	MS	p.Asp85Glu	VUS	VUS	T	B	5	DC	NA	NA	3.98E-06	NA	NA
rs1478544261	MS	p.Lys86Glu	VUS	VUS	T	B	5	DC	NA	NA	NA	NA	NA
rs772945927	MS	p.Lys86Asn	VUS	VUS	T	B	5	DC	NA	1.65E-05	1.41E-05	NA	NA
rs1482755273	MS	p.Arg89Lys	VUS	VUS	T	B	5	DC	NA	NA	3.98E-06	NA	NA
rs1482168240	MS	p.Thr109Ala	VUS	VUS	T	B	5	DC	NA	NA	3.98E-06	NA	NA
rs776021960	MS	p.Gly113Val	VUS	VUS	D	B	5	DC	NA	8.30E-06	7.97E-06	NA	NA
rs765824628	NS	p.Arg114Ter	P	P	NA	D	5	DC	NA	5.81E-05	2.83E-05	P	DM
rs1285976846	MS	p.Arg114Gln	VUS	VUS	T	B	5	DC	NA	NA	3.98E-06	NA	NA
rs1246344989	MS	p.Lys117Gln	VUS	VUS	T	PSD	5	DC	NA	NA	3.98E-06	NA	NA
rs1341633252	MS	p.Lys119Glu	VUS	VUS	D	PSD	5	DC	NA	NA	3.98E-06	NA	NA
rs1049109060	MS	p.Asn130Ser	VUS	VUS	T	B	3a	DC	NA	NA	1.59E-05	NA	NA
rs1483437354	MS	p.Asn131Ser	VUS	VUS	T	PSD	3a	DC	NA	NA	3.98E-06	NA	NA
rs143695489	MS	p.Leu134Val	VUS	VUS	T	PD	4	DC	NA	8.30E-06	3.98E-06	NA	NA
rs1448621317	MS	p.Met136Thr	P	VUS	T	B	4	DC	NA	NA	3.98E-06	NA	NA
rs1178647962	MS	p.Met136Ile	P	VUS	T	B	4	DC	NA	NA	NA	NA	NA
rs201213306	NS	p.Arg138Ter	P	P	NA	D	NA	DC	NA	4.20E-05	2.39E-05	P	DM
rs201283060	MS	p.Cys148Ser	VUS	VUS	T	B	6	DC	NA	NA	3.98E-06	NA	NA
rs1366520384	MS	p.Gly154Arg	VUS	VUS	T	PSD	NA	DC	NA	NA	3.19E-05	NA	NA
rs774226905	MS	p.Asn157Ser	VUS	VUS	T	PSD	NA	DC	NA	1.67E-05	1.59E-05	NA	NA
rs767326894	MS	p.Ile159Met	VUS	VUS	T	PD	NA	DC	NA	4.00E-04	1.97E-05	NA	NA

rs766367718	MS	p.Asp161Val	VUS	VUS	T	PD	NA	DC	NA	5.00E-05	NA	NA	NA
rs1286184183	MS	p.Arg163Gly	VUS	VUS	T	PSD	NA	DC	NA	NA	NA	NA	NA
rs769729611	MS	p.Ala166Gly	VUS	VUS	T	PD	NA	DC	NA	8.26E-06	4.00E-06	NA	NA
rs1553249737	NS	p.Tyr175Ter	P	P	NA	D	NA	DC	NA	NA	NA	P	NA
rs769148322	MS	p.Ile176Phe	VUS	VUS	T	B	NA	DC	NA	8.25E-06	3.98E-06	NA	NA
rs1199319494	NS	p.Gln177Ter	P	P	NA	D	NA	DC	NA	NA	NA	NA	NA
rs1247427997	NS	p.Arg182Ter	P	P	NA	D	NA	DC	NA	NA	3.98E-06	P	NA
rs1451007549	MS	p.Thr189Ala	VUS	VUS	T	B	6	DC	NA	NA	3.98E-06	NA	NA
rs758994650	MS	p.Thr189Ile	VUS	VUS	T	B	6	DC	NA	2.00E-05	3.19E-05	NA	NA
rs755533576	MS	p.Cys192Ser	VUS	VUS	D	B	NA	DC	NA	8.24E-06	3.98E-06	NA	NA
rs752363853	MS	p.Ile195Val	VUS	VUS	T	PSD	6	DC	NA	8.24E-06	3.98E-06	NA	NA
rs11557505	MS	p.Leu197Val	VUS	VUS	T	PD	6	DC	NA	NA	NA	NA	NA
rs1430886686	MS	p.Leu200Ile	VUS	VUS	T	B	NA	DC	NA	NA	3.98E-06	NA	NA
rs1431597019	MS	p.Met203Val	VUS	VUS	T	PSD	NA	DC	NA	NA	NA	NA	NA
rs1306085130	MS	p.Ser204Leu	VUS	VUS	T	PD	NA	DC	NA	NA	NA	NA	NA
rs1387136549	MS	p.Ile205Thr	VUS	VUS	T	PD	NA	DC	NA	NA	NA	NA	NA
rs765379963	NS	p.Arg206Ter	P	P	NA	D	6	DC	NA	8.24E-06	1.41E-05	NA	NA
rs201904119	NS	p.Arg219Ter	P	P	NA	D	NA	DC	NA	2.47E-05	1.59E-05	NA	NA
rs1371162932	MS	p.Arg219Gln	VUS	VUS	T	PSD	NA	DC	NA	NA	3.19E-05	NA	NA
rs150796970	MS	p.Ala221Ser	VUS	VUS	T	PSD	NA	DC	2.00E-04	4.53E-04	7.96E-06	NA	NA
rs759757338	MS	p.Thr222Ile	VUS	VUS	D	B	NA	DC	NA	8.24E-06	1.00E-05	NA	NA
rs771396376	MS	p.Gly227Arg	VUS	VUS	D	B	6	DC	NA	8.24E-06	3.19E-05	NA	NA
rs749769560	MS	p.Pro232Ser	VUS	VUS	D	B	NA	DC	NA	1.40E-04	6.72E-05	NA	NA
rs112969816	MS	p.Ser235Pro	VUS	VUS	D	B	6	DC	NA	NA	NA	NA	NA
rs1168267361	stop lost	p.Ter240Leuext*12	VUS	P	NA	D	NA	P	NA	NA	NA	NA	NA

**Combined scores of common in silico tools (SIFT, MutationTaster2, Polyphen2), VUS; Variant of uncertain significance, DC; disease causing, PSD; possibly damaging, PD; probably damaging, P; pathogenic, NA; not available, MS; Missense, NS; Nonsense,*

We further analyzed the regulatory features of the all variants retrieved from dbSNP database and 18 nsSNVs were found to have strong evidence (RegulomeDB score <3) for being resided in regulatory regions. The nonsense *TMCO1*/rs75840847 variant (p.Gly22Ter) had the lowest RegulomeDB score of 1f. It was shown to reside binding site of 80 proteins such as CTFC, HNF1A, HNF4A, GABPA, GABPB1, POLR2A, RAD21, etc. The minor allele frequency of the rs75840847 in the public databases is <0.04 (Table 2). Of the 41 pathogenic missense variants classified based on the CIS classification, only *TMCO1*/rs1387528611 (p.Lys128Gln) had the RegulomeDB score =2b indicating this variant may affect the binding of different transcription factors including ATF2, TBL1XR1, MEF2A, MEF2C, and RUNX3. This variant were found in populations covered in GnomAD with a minor allele frequency of 3.98E-06.

Searching PTMs sites in the *TMCO1* using PhosphoSitePlus database revealed 15 sites of which 9 had biological evidence for ubiquitylation and the remaining were site for phosphorylation (Figure 3). We also identified 6 amino acid substitutions occur in these PTM sites and of which 4 [p.Lys211Gln (rs1398266846), p.Lys105Glu (rs1256737415), p.Ser235Phe (rs1459734421), p.Lys237Arg (rs768112478)] were categorized as pathogenic based on CIS classification (Table 3).

Table 3. *Phosphorylation-related variants identified in the TMCO1 gene*

PTM Sites	Flanking Sequence	PTMs	Phosphorylation-related variant	RegulomeDB Scores
K91	TDKYKRLKAEVEKQS	Ubiquitylation	NA	-
K96	RLKAEVEKQSKKLEK	Ubiquitylation	NA	-
K104	QSKKLEKKKETITES	Ubiquitylation	NA	-
K105	SKKLEKKKETITESA	Ubiquitylation	p.Lys105Glu	5
T107	KLEKKKETITESAGR	Phosphorylation	NA	-

S111	KKETITESAGRQQKK	Phosphorylation	NA	-
K117	ESAGRQQKKKIERQE	Ubiquitylation	p.Lys117Gln	5
K167	FDGRVVAKLPFTPLS	Ubiquitylation	NA	-
T171	VVAKLPFTPLSYIQG	Phosphorylation	NA	-
K211	SIRQNIQKILGLAPS	Ubiquitylation	p.Lys211Gln	6
T222	LAPSRAATKQAGGFL	Phosphorylation	p.Thr222Ile	NA
K223	APSRAATKQAGGFLG	Ubiquitylation	NA	-
S235	FLGPPPPSGKFS	Phosphorylation	p.Ser235Phe	6
K237	GPPPPSGKFS	Ubiquitylation	p.Lys237Arg	6
S239	PPPSGKFS	Phosphorylation	NA	-

PTM: Post-translational modifications

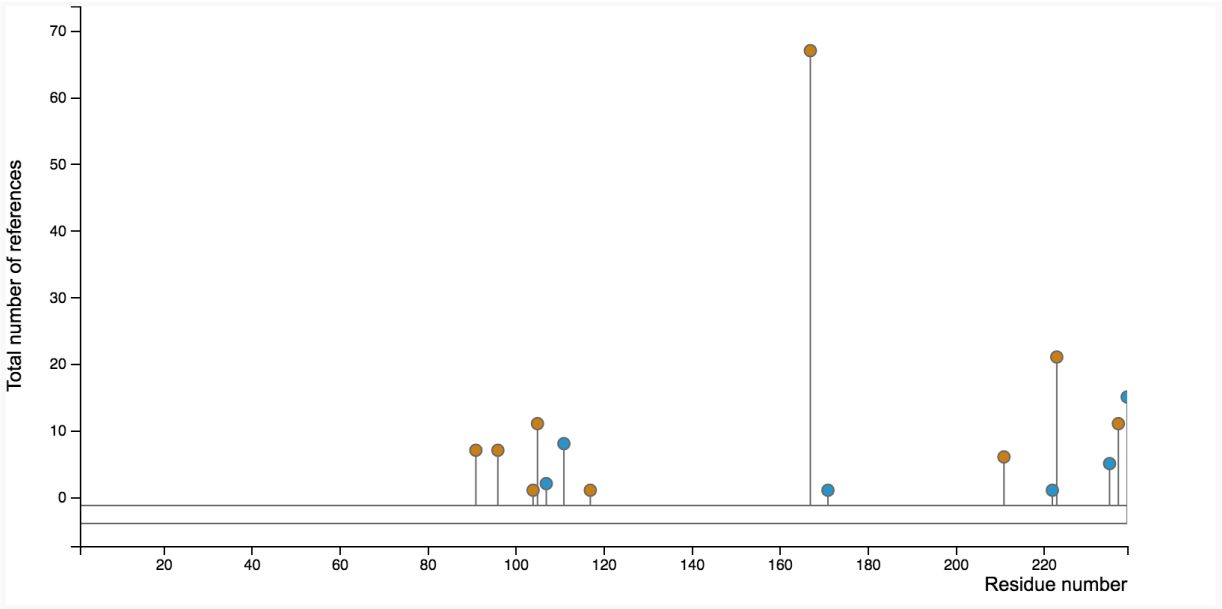

Figure 3. *Post-transcriptional modification (PTM) sites of the TMCO1 gene. The number of the papers define the specific modification sites in their methods is shown on the Y-axis. Blue dots indicate phosphorylation sites; orange dots indicate ubiquitylation sites.*

IV. DISCUSSION

Membrane proteins comprise almost 30% of total proteins and have key roles in human metabolism and diseases [30]. TMCO protein have 7 members in humans; TMCO1 is one of the paralogs of this subfamily and "Calcium load-activated calcium channel" (UniProt KB: Q9UM00 for human) is the recommended name since its function was clarified by Wang et al. 2016 [19]. The protein has a coiled-coil domain (aa# 84-140) and three transmembrane domains; (aa# 12-30), (aa# 61-81), and (aa# 142-160) (Interpro: https://www.ebi.ac.uk/interpro/protein/Q9UM00)

In vitro and knockout-mouse studies showed that overfilling of ER by calcium is prevented through TMCO1 protein. Thus, pathogenic variants in the *TMCO1* gene resulting dysfunctional protein were known to cause ER Ca2+ overload and observed in individuals with CFTD. To the best of our

knowledge, 5 different mutations has been linked to CFTD until today. One was a nonsense variant (rs201213306 (c.412C>T, p.Arg138Ter) located in exon 5 (Table 2). This variant was named as c.259C>T (p.Arg87Ter) in previous reports due to different usage of accession IDs [4,5]. Except this variant, remaining four variants were either a deletion, frameshift or a splice donor mutation resulting in truncated TMCO1 protein in patients with CFTD [3,6,7,8]. The linkage studies showed us that only the variants that cause a premature stop codon in the TMCO1 protein causes severe CFTD phenotype. Recently, *TMCO1* gene was also proposed to be associated with primary open angle glaucoma (POAG) in a GWAS which causes a highly different phenotype than CFTD. Their results exhibited associations of rs4656461, located downstream of TMCO1, and rs4977756 at CDKN2B-AS1 (Cyclin Dependent Kinase Inhibitor 2B- antisense RNA) with POAG in Australian population [9]. These information suggests that variants in *TMCO1* gene may contribute to the risk of variable phenotypes.

Up to date, none of the *TMCO1* missense variants have been reported to be associated with obvious clinical phenotype. In this study, we investigated the putative effects of missense *TMCO1* variants on protein function, gene regulation and post-translational mechanisms to prioritize possible functional and regulatory missense *TMCO1* variants. In recent years, several prediction tools were developed for pathogenicity assessment of variants located in the coding regions of the genome [31,32]. It is highly important to evaluate their predicted results meticulously since they use distinct scoring algorithms and attained results are not always concordant [33,34]. All of the predictors have some limitations yet some are superior in certain aspects to others [35,36]. In the current study, we mainly aimed to reveal functional potentials of the *TMCO1* missense variants therefore SIFT, MT2, and PP2 which are the most commonly used predictors for amino acid substitutions, were used in the in silico analysis. In order to strength our analyses and classify variants with strong evidence, we combined the scores of the three algorithms and compared our combined scores with ACMG variant interpretations retrieved from InterVar. Of the 141 missense variants, only 90 variants had predictions from all three database and predictions of the 51 variants were based on the two databases due to missing data in databases. We found 41 variants predicted to have significance impact on protein structure of which were classified as VUS based on ACMG criterias and 11 were not found in the databases of large-scale sequencing projects (1000G, GnoMAD, ExAC). The allele frequencies of these variants in 1000G, GnoMAD and ExAC were ranged from 2.10E-4 to 3.56E-06. This information highlighted the rare occurrence of the variants in the screened populations (European, Asian, American, African and Ashkenazi Jewish) and supported their potential functional relevance.

We also searched the ClinVar database for all nsSNVs to retrieve the clinical information attributed to the variants however only 6 variants (rs765824628, rs201213306, rs1553249737, rs1247427997, rs765379963, rs1332024737) were found to be submitted to the ClinVar and had clinical information. Five were the well-known pathogenic *TMCO1* variants which were also classified as pathogenic based on in silico scores and rs765379963 (p.Leu60Pro) were classified as VUS in the ClinVar.

Accumulating evidence sheds light on the impacts of PTM-associated SNVs (SNVs located in the PTM sites) on human diseases, particularly cancer [37,16]. Thus, our analyses implied multiple putative PTM-associated SNVs residing in PTM sites of the *TMCO1* gene which may be associated with human diseases. However, we also identified SNVs which may have dual role in amino acid and regulatory coding as may be part of codons called “duons” by Stergachis et al. [38]. Duons were known to have dual functions since they have also transcription factor binding sites aside from coding [39,40]. *TMCO1*/rs1387528611 (p.Lys128Gln) which was predicted to impact protein function may act as duon sequence variant since it was shown to be potential site for transcription factor binding (RegulomeDB score=2b).

In conclusion, we hereby comprehensively investigated the potential pathogenic properties of the *TMCO1* coding variants resulting amino acid substitutions as well as assessed their putative roles in gene regulation and PTMs by in silico analyses. Our results highlight the functional potentials of coding variants in *TMCO1* which was known to cause severe phenotypes by mostly the impact of frameshift and nonsense variants. Future studies unraveling the roles of postulated functional variants and expanding this knowledge may have substantial impact to elaborately understand the pathogenetics of *TMCO1*-associated diseases.

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