Evolutionary Analysis of Makorin Ring Finger Protein 3 Reveals Positive Selection in Mammals

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ABSTRACT: Makorin ring finger proteins (MKRNs) are part the of ubiquitin-proteasome system; a complex system important for cell functions. Ubiquitin fate through proteolytic, non-proteolytic pathways varies, depending on covalent linkage between ubiquitin and protein substrates. Makorin ring finger protein 3 is an integral part of covalent linkage of ubiquitin to protein substrates. Similar to others imprinted genes, MKRN3 also evolve under positive selection; however, which codons are specifically selected in MKRN3 during evolution are needed to be explored. Different maximum-likelihood (ML) codon-based methodologies were used to ascertain positive selection signatures in 22 mammalian sequences of MKRN3 to probe an individual codon for positive selection signatures. By applying the HyPhy software package implemented in the Data Monkey Web Server and CODEML implemented in PAML, evolutionary analysis based on two Ml frameworks were conducted. The analysis was executed by comparing M1a against M2a, M7 against M8, and PAML models and *2ΔLnl* (*LRT*) was resulted by likelihood logs. M1a contributed ω1 (*dN/dS*) with *LRT* value (*ΔLnl*) 12.01, and positive selection was found in M2a with ω3 = 2.23603. To further improve selection test, M8 was compared to M7 with 2Δ*Lnl* (*LRT*) 30.17, and M8 showed positive selection with ω = 1.55759. The data were fit to M8 than M7, which suggests that M8 was the most significant model of selection. M8 was judged encouraging for this analysis and used to establish a positive selection of MKRN3 proteins. We found Gly312 as a positively selected amino acid in a zinc finger motif/Really Interesting New Gene (RING) finger motif; the former ones' region is involved in RNA binding and the later ones in ubiquitin ligase activity of the protein, vital for protein function. Selection analyses of MKRNs might advance the developments in unique approaches that could lead to genetic progress over the selection of superior individuals with the breeding values higher for certain traits as ancestries to get the next generation.

KEYWORDS: MKRN3, selection, evolution, puberty, maximum likelihood

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Introduction

Ribonucleoproteins encoded by the makorin ring finger protein (MKRN) gene family have distinctive organization comprising zinc finger motifs with various C3H zinc fingers, such as makorin-specific Cys-His organization and a ring zinc finger. The main features of MKRN3 include: really interesting new gene (RING) finger motif, zinc finger motifs followed by makorin zinc finger and a zinc finger motives.[1,2] Makorin ring finger protein 3 functions could be predicted via zinc finger motif in the zinc finger proteins. RNA-binding proteins and protein-protein interactions (PPI) are mediated by C3H zinc fingers and RING zinc fingers, respectively.[3-5] The RING zinc finger is central to E3 ligases enzyme that mediates the relocation of ubiquitin between target protein substrates and E2 ubiquitin-conjugating enzyme.[6] A decline is predicted in MKRN3 expression when there is an upsurge in gonadotropin-releasing hormone (GnRH) stimulatory factors or GnRH expression. Regulator of GnRH secretions including kisspeptin, neurokinin B, and dynorphin use arcuate nucleus as a gate way.[7] Puberty has resulted in a gain of excitatory inputs and loss in inhibitory inputs. Fluctuations in MKRN3 expression in the hypothalamic arcuate nucleus advise its significant role in the inhibition of GnRH discharge during the pre-pubertal dormant period. Makorin ring finger protein 3's possible action as an E3 ligase to prevent stimulatory effect, mutates to MKRN3's loss of function that causes premature

hypothalamic-pituitary-gonadal axis stimulation, phenotypically expressed as Central Precocious Puberty (CPP).[8]

The study of loss of function mutations in MKRN3 can help to diagnose CPP, as puberty initiation marks are not simply measurable especially in boys, leading to early diagnosis of early treatment. Furthermore, it will help in early diagnosis and guide genetic counseling of CPP in familial cases. It has not been confirmed whether the effect of the MKRN3 mutation is sexually dimorphic or not, but it is evident that these mutations initiate the puberty at an early age in both sexes.[9] There is no conflict about the MKRN3 importance in the hypothalamic-pituitary-gonadal axis, but how MKRN3 regulates GnRH secretion is not yet confirmed. The current studies are relating the contribution of MKRN3 genes with the age of menarche reinforce in pubertal timing. The exploration of a recognized inhibitory factor in hypothalamic-pituitary-gonadal axis led to stirring a new field in the neuroendocrine. Further investigations will illuminate the defined mechanism of action of GnRH secretion.[10] Positive Darwinian selection is a vital source of evolutionary research and a significant influence behind the species divergence. Darwinian selection has a crucial role in evolutionary biology and a core cause of species divergence. Positive selection detection techniques can be valuable implements for acquisition intuition into gene function.[11] Therefore, an extensive variety of approaches for detection of positively selected genes have been adapted, which make use of arrays of substitutions between species through phylogenetic methods and patterns of interspecies polymorphism which primarily rely on population genetic methods.[2]

Materials and Methods

Sequences analysis

In this analysis, we used MKRN3 gene coding nucleotides and amino acid sequences, collected from GenBank, Ensemble, and Uniprot database; www.ncbi.nlm.nih.gov/genbank, respectively. Clustal Omega employed in MEGA 6.0 was used to align the protein sequences. The accomplished protein sequences were affiliated through Clustal Omega built in MEGA 6.0 program,[12] and followed by manual adjustment.

Codon-based positive selection analysis

The HyPhy package and CODEML built in web server data monkey (http://www.datamonkey.org/)[13] and PAML version 4,[2] respectively, were used to compare diverse ω ratios (*dN/dS*) for determining positive selection among codons of mammalian MKRN3 by using maximum-likelihood approaches (MLAs). The analysis includes two bounds; the first bound is to catch the maximum-likelihood (ML) proportion for concluding positive selection with $\omega > 1$. The log for likelihood ($2\Delta l$) at d.f. = 2 is correlated with chi-square distribution. M7 postulates a β-distribution, if ω ratio falls between 0 and 1. A substitute, M8 comprises of two parameters (ω and β), and the sites under positive selection have ω values greater than one. The second key bound is to figure out positively selected amino acids if the presence is established by likelihood test. Different ω classes for posterior probabilities of each site were estimated subsidiary by using the Bayes theorem.[14] Probabilities $\omega > 1$ indicates that the amino acids are likely to be under selected. By using locations of positive-selected amino acids, crystal structure was draw with the help of Phyre and Swiss model-based programs.[2] Bioinformatics tools were used for the prediction of conserved amino acid localization in proteins; built on phylogenetic associations between sequences online (http://consurftest.tau.ac.il).[15] To validate the selection pressure on codon sites of aligned sequences of MKRN3, an online webserver was used (http://selecton.tau.ac.il/). The ML methods through Bayesian implication approach were used to measure the ω ratio among various codons from the aligned sequences made by selection.[16] Furthermore, different color scales were used on Selecton outcomes to describe numerous types in selection.

3D structure predictions and modeling

The sequence conservation of MKRN3 was explored by multiple sequence alignment (MSA) tools, Clustal Omega, and pictured by GenDoc. The resulted three-dimensional (3D) structure was enhanced by energy minimization variation in UCSF Chimera program. Primary sequences of MKRN3_Zinc finger domain were subjected to Swiss Model portal[17] to predict suitable structures. MolProbity server was used to do structural confirmation of predicted model.[18] Ramachandran Plot 2.0 tool was used to calculate Ramachandran values.[19] Visualization and geometry optimization of predicted structure was done in UCSF Chimera.[20] The Con Surf tool was applied to identify the evolutionary conserved amino acid positions in the protein based on phylogenetic relationship.[21] Positively selected positions, identified in more than one MLA were considered as more traditional approach used previously.[22]

String-based network analysis for PPI

String-based interaction network analysis (version 9.1, http://www.string-db.org/) for proteins was performed to document the MKRN3-interacted genes[23]; and the PPI encoded by the MKRN3 was pursued. An assembled score (>0.4) was considered as the cut-off standard. Bioinformatics database involves proteins communication intricate in several pathways. The middle nodes proteins were highly connected besides vital biological function and were recognized by assessing the between value and the number of line networks between proteins of each node. Cytoscape software was used to visualize the string bases constructed network (http://www.cytoscape.org/).[2]

Results

To identify the evolution pattern in MKRN3 gene in mammals, we retrieved MKRN3 gene available coding sequences in various mammalian species from public databanks. To recognize the sources for evolutionary changes in genes of domesticated and wild mammalian species, we performed phylogenetic analysis of aligned gene sequences by using MEGA. Maximum-likelihood and neighbor-joining (NJ) methods were used to generate Gene trees in MEGA 6.0 with the bootstrap value set to 1000. PAML CODEML program-based codon simulations were used to deduce ω values by MLAs for all mammalian MKRN3 codon sequences (Table 1). Analysis was executed by comparing M1a against M2a and M7 against M8 PAML models, and 2Δ*Lnl* (*LRT*) was resulted by likelihood logs. M1a contributed ω1 (*dN/dS*) with *LRT* value (Δ*Lnl)* 12.01, and positive selection was found in M2a with ω3 = 2.23603. To further improve selection test, M8 was compared with M7, with 2Δ*Lnl* (*LRT*) 30.17, and M8 showed positive selection with ω = 1.55759. The data were fit to M8 than M7, which suggests that M8 was the most significant model of selection. M8 was judged encouraging for this analysis and used to establish positive selection of MKRN3 proteins. The M8 represents 0.14% positively selected sites with ω = 1.55759, which is strong evidence of positive selection (Table 1). Makorin ring finger protein 3 codon sites were identified under positive selection through different likelihood methods (PAML, SLAC, IFEL, and FEL; Table 1).

Positive Selection in Amino Acids

Evolutionary conserved amino acid's position is mandatory for maintenance of structure and function in protein. Consequently, uncovering selected positions may instruct the selection fleets and spots the functionally important positions for MKRN3 protein. To perceive such positions, Bayes technique was used to determine the posterior likelihoods for each site. The site with posterior probabilities $\omega > 1$ are likely to be positively selected. Using case excess (BEB) analysis for MKRN3 amino acids corresponding to human protein sequence, eight sites were positively identified, but no site could be identified at 99% or 95% posterior probability (Table 2). Positive selection was confirmed through the selecton server which uses the Mechanistic Empirical Combination (MEC) model for the estimation of selection pressure for individual codons to avoid false-positive results of PAML. Several codons in MKRN3 have adaptive selection pressure (Table 2; Figure 1), recognized in positive selection.

We found Gly312 as a positively selected amino acid in a zinc finger motif or a RING finger motif (Figure 2); former ones' region is involved in RNA binding and later ones in the ubiquitin ligase activity of the protein, vital for protein function. Glycine also acts as a metabolic regulator and an antioxidant. Several proteins have highly conserved glycine because of its unique properties. Glycine is the smallest amino acid with no side chains besides important for proteins that require flexibility, in hinge regions, or for ion gates that must open and closes under varying circumstances. Really interesting new gene finger proteins are categorized as part of the main class of E3s; proteins that expedite ubiquitination, posttranscriptional protein adaptations, or if they have other distinct functions ought to be apparent. Mammalian genome has hundreds of RING finger proteins genes. The proficiency of these proteins to interact with E2s and to potentially mediate ubiquitination has been inferred from in vitro studies. It needs to establish bonafide E3s among these both for heterologous substrates and targets for ubiquitination. The number of potential E3s is fostered by the conjunctional association of specific RING finger proteins with other proteins that provide docking sites for substrates.

3D Structure Modeling and Analysis of MKRN3

Three-dimensional structure of MKRN3 zinc finger domain was estimated and confirmed through MolProbity, ERRAT, and Verify3D server to evaluate structure quality, that is, Ramachandran outliers, Ramachandran plot, favored rotamers, and poor rotamers. Root mean square deviation (RMSD) value 0.156 Å was estimated by superimposing the template and predicted using UCSF Chimera software. The results of constructed 3D structure showed that it contained two α-helices represented with different colors and an additional α-helix which may be involved in release of sex pheromones and binding of ligands (Figure 3).

PPI Network

By exploring MKRN3-encoded protein in the STRING databank, several proteins were found to be linked with MKRN3 through protein interaction network. The PPI linkage had 11 nodes (denote MKRN3 encoded proteins) and 42 edges (line networks between nodes; Figure 4).

In the PPI network, MKRN3 is networked with the other vital genes which are co-expressed. We found 11 genes: GABRB3, SNRPN, ATP10A, HERC2, NDN, SNURF, NPAP1, MAGEL2, OCA2, and UBE3A (Figure 3). Among these MAGEL2, SNRPN, HERC2, and UBE3A are important genes that are involved in reproduction through biological pathways and are up regulated with MKRN3 (Figure 3). The biological pathways of all the interacted proteins are involved in different reproductive processes, such as MAGEL2 increases ubiquitin ligase activity of RING-type zinc finger-containing E3 ubiquitin-protein ligase, cytokine receptor binding, and UBE3A is involved Ubiquitin protein ligase E3A; E3 ubiquitin-protein ligase which accepts ubiquitin from an E2 ubiquitin-conjugating enzyme and others proteins are linked together in different biological pathways.

Discussion

In humans, puberty is initiated with recurrence of the pulsatile discharge of the GnRH from hypothalamic pituitary neurons.

Table 1. PAML site models of positive selection-derived log likelihood values and test.

GENE MKRN3	N, 22	LC, 810	S, 9.8848	MODEL, M1A	PARAMETER ESTIMATES, P1 = 0.48153 P2 = 0.51847	2ΔL M3 VS M0	2ΔL M2 VS M1	2ΔL M8 VS M7	POSITIVELY SELECTED SITES			
									PAML	SLAC	IFEL	FEL
				M2a	ω*1* = 0.16566; ω*2* = 1.00000	806.3	12.01	30.17	301 G,302 V,303 S,306 G,312 G,326 H,346 L,364 R	237,295, 307,345,357	44,171, 184,189,	44,171,184, 189,199,415, 451,460, 474
					P1 = 0.46918; p2 = 0.47309; p3 = 0.05773							
				M3	ω*1* = 0.16843; ω*2* = 1.00000; ω*3* = 2.23603							
					P = 1 0.21535; p2 = 0.47500; p3 = 0.30965							
				M7	ω*1* = 0.03316; ω*2* = 0.40678; ω*3* = 1.23988							
				M8	p = 0.51857; q = 0.49088							
					p0 = 0.85820; p = 0.63098; q = 0.82606							
					p1 = 0.14896; ω = 1.55759							

Abbreviations: *n*, number of sequences; *Lc*, codons after alignment gaps are removed; *S*, tree length, measured as the number of nucleotide substitutions per codon; p1, positive selected sites in proportion; p0, sites under selective constraint; p and q, distribution of beta parameters.
Sites under positive selection recognized under M8 are listed conferring to the human nucleotide sequence.

Table 2. Positively selected sites based on Bayes empirical Bayes analysis under different PAML site models.

GENE	MODEL	CODONS	AMINO ACIDS	POSTERIOR PROBABILITY	POST MEAN ± SE FOR ω
MKRN3	M8: selection, beta + ω	301	G	0.911	1.448 ± 0.184
		302	V	0.901	1.442 ± 0.194
		303	S	0.829	1.393 ± 0.247
		306	G	0.684	1.295 ± 0.311
		312	G	0.787	1.367 ± 0.275
		326	H	0.761	1.349 ± 0.278
		346	L	0.655	1.275 ± 0.319
		364	R	0.628	1.248 ± 0.340

Figure 1. Selection densities among MKRN3 gene structures based on mechanistic empirical combination (MEC) model of selection online tool. Different color intensities represent selection density; positive selection (yellow and brown); neutral selection (gray and white), and negative selection (purple).

Humans with reproductive ailments and animal models have been used to study GnRH physiology and ontogeny pathways. Makorin ring finger protein 3 derived only from RNA transcribed from the paternally inherited copy of the gene due to maternal imprinting which is involved in cell signaling and ubiquitination is encoded by MKRN3 gene located on the chromosome 15.[10] Reproduction-related genes evolve rapidly through positive selection, lined in branch length and evidenced under positive selection,[24] we observed positive selection in MKRN3. Both the pro and mature region of MKRN3 were subjected to evaluation and found positive selection in various codons for all branches of the phylogenetic tree in mammals (Figure 2 and Table 1).

Estimation for constraints in adoptive selection was done by measuring positive selection estimates using MEC model. Phylogenetic analysis reveals the ML phylogenetic relationship of mammalian MKRN3 and suggests significant discrepancy and a rapid evolution compared to other genes. Empirical Bayes-based selection analysis suggests positive selection in numerous amino acid sites for MKRN3 and spots positive-selection signals at various codon sites of MKRN3 (Table 2; Figure 1). The amino acid replacements might be a result of distinct variation from their common ancestors which favor previous submissions,[25] as orthologs diverge from their most modern common ancestor, their different evolutionary routes direct to deviation in discerning constraints on homologous positions. Our analysis of MKRN provides new insights into a positive selection that might be involved in the selection of some biological pathways in recent evolutionary periods.[26] Therefore, the identification of the selection in mammalian genome undertakes a vital research platform for modern evolutionary research. Our findings are in agreements with previous studies in which mutations were identified that changes the amino acid sites in MKRN3 protein and change the function of protein. In this study, we found the amino acid substitutions under positive Darwinian selection by the influence of natural selection pressure. We detected zinc finger motif or RING finger motif which includes a positive-selected amino acid Gly312. (Figure 2) and have a predicted role for protein activities including RNA binding and ubiquitin ligase, respectively, indispensable for protein function.

In addition to metabolic regulator and antioxidant; glycine has flexibility to adopt wide range of main-chain dihedral angles to represent either L or D amino acids.[27] Several proteins comprise of conserved glycine residues suggesting its role in flexibility and mobility. Literature reported that substitution of conserved glycines in the enzyme acylphosphatase results an increased tendency to aggregate, and this may be an important concerns for protection from the amyloid formation associated to many neurological diseases.[28] A number of dimerization-activated proteins suggest that glycine has central role in dimerization. The terminal residue of certain peptides has highly conserved glycine that plays a vital role by supporting binding to the plasma membrane via myristoylation.[28] Currently, in human with CPP, 12 different mutations of MKRN3 have been reported. It is anticipated that normally branch length and prompt gene evolution are linked to positive selection, and the reproduction linked genes evolve promptly and ascertained positive selection regularly.[24] It has been reported that ω ratios (dN/dS) might increase because of GC-based gene conversion, predominantly in primates and exhibit false-positive selection of amino acids in branch site model.[29] Our findings revealed positive-selection sites that were located in zinc finger motif which are involved in ubiquitin ligase activity and important protein function. The missense mutations (Gly-312-Asp-, Cys-340-Gly, Arg-365-Ser, Phe-417-Ile, and His-420-Gln) were positioned within a RING finger and zinc finger motives or, ligase activity of the protein and regions involved in RNA and ubiquitin attachment, respectively.[9] Bayes Empirical Bayes-based selection analyses revealed positive selection of various amino acid sites

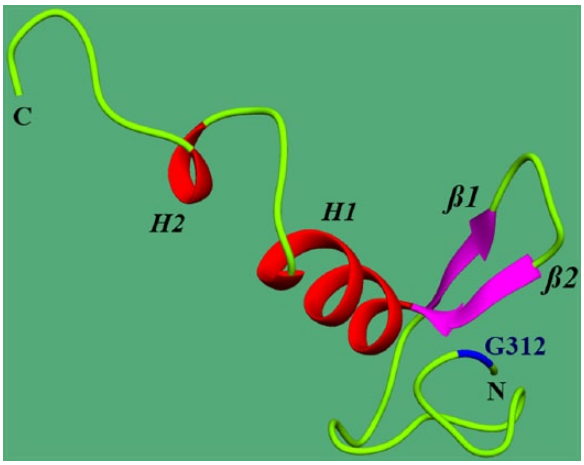

Figure 2. Positive-selected amino acids locality in MKRN3_zinc finger domain genes.
Taking human MKRN3_zinc finger domain crystal structure in account, crystal structure was mapped with positive -selected site by Phyre tool (http://www.sbg.bio.ic.ac.uk/phyre2/html). Altogether deposits for selection area recognized in ring finger domain that is important in ubiquitination pathways.

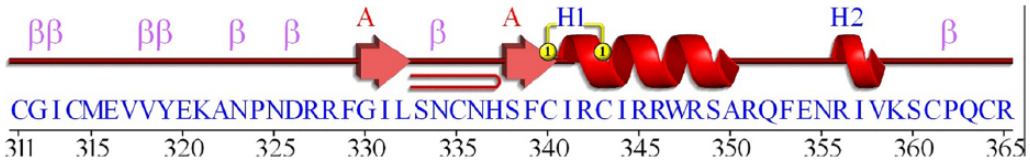

Figure 3. Secondary structures of makorin ring finger protein 3 (MKRN3).
The conserved residues are denoted by capital letters, and α-helices are represented as color coded in the tertiary structure.

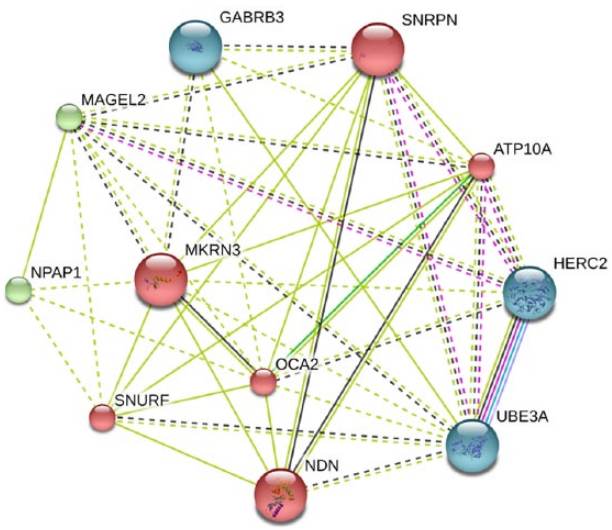

Figure 4. String-based protein-protein interaction (PPI) established by database for MKRN3 genes.
Down-regulated genes (gray circles) and up-regulated genes (red circles). Line thickness (interaction strength), negative and positive correlation coefficients are represented by dash and solid edge, respectively. Posttranscriptional modifications in proteins or splice isoforms are denoted by network nodes, and a single node represents all the proteins produced by a single-protein coding gene locus.

in MKRN3 gene. We observed 258S, 270A, 272G, 284T, 285C, and 304L codon sites have positive-selection signals at (Figure 2). The positively selected amino acid sites such as alanine, leucine, arginine, cysteine, and lysine are important for signaling involved in ubiquitin ligase activity and important protein function such as ligase activity of the protein and regions to be involved in RNA binding. Really interesting new gene finger proteins are the members of the largest class of E3s to date that intervenes ubiquitination and other protein modifications or if they have other distinct functions should soon become apparent. Mammalian genomes comprise hundreds of RING finger proteins. Concluding from in vitro studies, we conclude that many of these have the capacity to interact with E2s and to potentially mediate ubiquitination. It remains to be uncovered how many of these will be bonafide E3s for heterologous substrates, targets for ubiquitination, or both. The number of potential E3s is further enhanced by the combinatorial association of certain RING finger proteins with other proteins that provide docking sites for substrates. Our study revealed positive selection at MKRN3 with $\omega > 1$(Table 1). This suggests rapid evolution of non-synonymous (dN) than synonymous positions in purifying or balancing selection of fresh variants that showed high allelic polymorphism[2,21,30] that could transform structural confirmation of protein by incorporating any alteration, thus impairing the signaling pathways.[22]

Conclusions

Numerous amino acid sites experiencing positive selection have a defined role in protein and subsequently for precocious puberty in women. This study presented comprehensive analyses to define MKRN3 genetic implication. Selection analyses of makorin ring finger proteins might advance the developments in unique approaches that could lead to genetic progress over the selection of superior individuals with the breeding values higher for certain traits as ancestries to get the next generation.

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Author Contributions

MJA and HIA wrote the original draft of manuscript. MJA, HIA, MMA, SM, GH, and RHM helped in data analysis. AE and FU helped in English editing and gave the critical review of the manuscript. AL and LY supervised the study.

Data Availability

Data will be openly available for all readers.

Supplemental Material

In this analysis, we used MKRN3 gene coding nucleotides and amino acid sequences, retrieved from GenBank, Ensemble, and Uniprot online; www.ncbi.nlm.nih.gov/genbank, respectively (See Table S1 in the Supplementary Material for Gene Accession Number).

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