

Prediction of deleterious nsSNPs of mouse DROSHA gene targeting miR-16 and miR-21 maturation in vascular smooth muscle cells: Integrated computational and experimental study

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Abstract

Background

MicroRNAs (miRNAs) are a class of small endogenous RNAs to regulate cell function and fate. Drosha, the nuclease to process pri-miRNAs to pre-miRNAs, is a key molecule to the biogenesis of miRNAs. The critical mutations in the DROSHA sequence to affect miRNA maturation remain largely unknown. Thus, we used *in silico* algorithms, protein-protein docking (PPD), molecular dynamics (MD) simulation, and experimental studies to determine and characterize the role of single nucleotide polymorphism of (SNPs) of the Drosha gene in the mouse vascular smooth muscle cells (VSMCs).

Methods

Different *in silico* algorithms, including SIFT, PROVEAN, I-Mutant 2.0, SNAP and PANTHER were used to predict the mutations on Drosha. Then, we characterized the binding efficiency of mutated DROSHA with Dgcr8 using PPD and MD simulations. Further, we mutated that potential site on Drosha to mouse vascular smooth muscle cells (VSMCs) and determined its cellular role in miR-16 and miR-21 maturation.

Results

All the selected algorithms indicated that the deleterious nonsynonymous SNPs in site 401 (E401Q) on DROSHA are a potential mutation region. The results of binding affinity, RMSD, RMSF, and radiation of gyrus indicated expressed that the changes in a glutamic acid to glutamine of Drosha may alter miRNA maturation through Dgcr8. After the over-expression of mutant Drosha in the mouse VSMCs, the expressions of the anti-tumor miR-16 and the oncogenic miR-21, the two most highly expressed miRNAs in VSMC, were significantly down-regulated. It is also noted that the expression of miR-21 had a greater decrease than that of miR-16 by the mutation, suggesting the E401Q might be helpful to attenuate the oncogenic miR-21 production.

Conclusions

Our data, for the first time, disclose the role of the E401Q on DROSHA in mouse VSMCs.

1. Introduction

microRNAs (miRNAs), the non-coding RNAs with the short length of ~ 22 nucleotides [1], have the partially complementary sequences (seed regions) with the 3' UTR of their target mRNAs [2], which lead to the degradation of the mRNAs post-transcriptionally, and the inhibition of protein translation [3]. miRNAs have been identified to play key roles in modulating essential cellular processes and pathogenesis via downregulating their target genes [4]. The original sequences to generate mature miRNAs are in the regions of genomes within cell nucleus, including the introns, the non-coding exons, and the loci between genes. Initially, the miRNA hosting sequences are transcribed to pri-miRNAs by polymerase II, followed by being processed to pre-miRNAs through the activity of DROSHA [5]. The RNase III domain of DROSHA cleaves the double-strand stem of pri-miRNA, the structure that is recognized and combined by DGCR8 (the protein in complex with Drosha to form the microprocessor) [6]. The pre-miRNAs are then exported to the cytoplasm, and further processed by Dicer to produce the mature miRNAs. According to their regulatory functions, miRNAs can be divided into anti-tumor miRNAs (e.g., miR-16) or onco-miRNAs (e.g., miR-21). miR-16 and miR-21 have been well investigated in various cells [7, 8]. Particularly, they are both highly expressed in VSMCs, ranking top 2 among other major VSMC marker miRNAs [9], suggestive of their vital roles in the regulation of the functions of vascular smooth muscle cell (VSMC).

VSMC is the main cell type in the blood vessel wall, controlling the vascular movement [10]. Therefore, VSMC malfunction is closely relevant to vascular diseases, such as hypertension, atherosclerosis, restenosis, and vascular calcification [11–13]. As stated above, DROSHA, the miRNA biogenesis enzyme, is pivotal to the canonical miRNA maturation pathway. Previous work proved that the Drosha deletion in mouse VSMCs gives rise to the embryonic lethality at E14.5, and the reduced VSMC proliferation and differentiation. In addition, the loss of Drosha alters the miRNA expression profile in mouse VSMC, resulting in the downregulation of a series of highly expressed VSMC miRNAs [14]. The data indicate that Drosha is important to VSMC growth and function via affecting miRNA expressions. Furthermore, the similar phenomenon was documented when DGCR8 or Dicer was disrupted in mouse VSMCs [15, 16]. The work on zebrafish also indicates that Drosha is important to the vascular development and the maintenance of vascular integrity [17]. The aberrant expression of miRNAs causes vascular diseases, and the miRNA expression is largely dependent on DROSHA behavior as described. Although the structure of human DROSHA was characterized [18], the precise sites critical to miRNA maturation is still rarely understood. Here, by using a series of computational methodologies, we found that the site 401 on DROSHA sequence may be highly related to miRNA maturation. Further, we over-expressed the mutant Drosha sequence that the site 401 was mutated from glutamic acid to glutamine (E401Q) for the mouse VSMCs with wild-type Drosha knockdown, and thereby compared the expressive changes of miR-16 and miR-21. Our work will cast light on disclosing the in-depth mechanism for DROSHA cleavage in the process of miRNA biogenesis and may provide potential target for ameliorating the related diseases.

2. Materials and Methods

2.1. Chemicals

2.1. Data collection

Mus musculus Drosha genome information was collected from the National Center for Biotechnology Information (NCBI; Gene ID: 14000) and ENSEMBL (Accession No: ENSMUSG00000022191). In addition, Drosha (accession number: Q5HZJ0) and Dgcr8 (accession number: Q9EQM6) protein FASTA sequences from the mouse were collected from the Uniprot database. The single nucleotide polymorphism (SNP) information ID and mRNA accession number (NM) of the Drosha gene were obtained from the mouse SNP information database (www.informatics.jax.org/snp) for further computational analysis [19].

2.2 Prediction of disease-related SNP

SNPs in the Drosha coding region can disrupt protein structure. In this study, we used the Sorting Intolerant from Tolerant (SIFT) [20] and PANTHER [21] tools to predict disease-associated SNPs in the Drosha coding region. SIFT is a program that predicts SNPs based on amino acid sequence homology and physicochemical properties determined by the substituted amino acids. A cutoff score ≥ 0.05 indicates that the amino acid substitution is deleterious and a score $\leq$ indicates resistance. Screening for unacceptable polymorphisms (SNAP) was used to predict the functional effects of nonsynonymous SNPs based on neural network algorithms. A cut-off scores above 50 gave a strong signal of effect, while a cut-off scores below - 50 gave a strong signal of neutrality [24]. PANTHER Position-Specific Evolutionary Conservation (PSEP) measures the length of time (in millions of years) that a position is conserved in the present-day protein by tracing it back to its immediate reconstructed ancestor. The longer a position is held, the more likely it will be counterproductive. Injury thresholds were (time > 450 my) and benign (time < 200 my) [25].

Prediction of protein stability

I-Mutant 2.0, a support vector machine (SVM) network-based tool, is used for the prediction stability of proteins during single-point mutations from structure or sequences. The well-trained datasets of this tool were retrieved from Thermodynamic Database for Protein and Mutant (ProTherm) database with its corresponding free energy change values (Capriotti et al., 2005). The dynamics and flexibility of protein structure is also an essential factor in its function. In I-Mutant 2.0, DDG > 0 means that the mutated protein has high stability and vice versa [22]. Protein variation effect analyzer (PROVEAN) v1.1 tool was used to predict whether a protein sequence variation affects protein function. A delta alignment algorithm based averaged within and across clusters to generate the final score. The pre-defined threshold was (<-2.5) "deleterious" and threshold (>-2.5) for neutral" effect [23]. PredyFlexy server (https://www.dsimb.inserm.fr/dsimb_tools/predyflexy/index.html) was applied to predict the flexibility of the structures, by using the X-ray B-factors and molecular dynamics-based root-mean-square-fluctuations (RMSF) descriptors (de Brevern et al., 2012). Based on the confidence index (CI) value and flexibility class (0 denotes rigid; 1 denotes intermediate; 2 denotes flexible), the detailed flexibility of the structures was examined.

Prediction of intrinsically disordered residues

Certain regions of proteins, which are not likely to construct a defined three-dimensional (3D) structure, are called Intrinsically disordered regions (IDRs) (Kulkarni and Uversky, 2019). The naturally occurred IDRs of w-Drosha and m-Drosha sequences were predicted by using a meta-predictor known as PONDER-FIT (<http://original.disprot.org/pondr-fit.php>), based on the consensus artificial neural network (ANN) method (Xue et al., 2010).

Physicochemical parameter analysis

Using the ExPASy ProtParam web-interface the different physiochemical properties including the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, anticipated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) was calculated for the w-Drosha and m-Drosha sequences.

2.3 Homology modeling

Three dimensional structures of the mouse Drosha is not available in protein database. The protein sequences of w-Drosha and m-Drosha were submitted to I-TASSER modeling server [26]. It generated multiple models based on the HHSEARCH and **SP3** algorithms using 5ZAK as template was considered for further analysis. w-Drosha and m-Drosha sequences share significant sequence identity with 5ZAK. The modeled structure quality was examined by using the PROCHECK and PROSA programs [27, 28] and verified structures were used for protein-protein docking and molecular dynamics (MD) simulations.

Protein-Protein docking of w-Drosha and m-Drosha with Dgcr8

Using High Ambiguity Driven Protein-Protein Docking (HADDOCK) v 2.4 (Van Zundert et al., 2016) modeled structures of w-Drosha and m-Drosha proteins were docked into the binding motif of Dgcr8 to assess miRNA biogenesis. Here, a three-step(s) HADDOCK protocol was performed for docking calculations. In the first stage, rigid body docking (it0) generated 1000 structures, and the best 200 structures based on intermolecular energies were transferred to the second stage. A semi-flexible refinement (it1). In the third step, MD refinement is performed using a well-defined solvent (water), then the model is clustered according to contact network similarity at the interface or backbone interface-ligand RMSD similarity, which were scored accordingly (Vangone et al., 2017; Kurkcuoglu et al., 2018; Van Zundert et al., 2016; Kurkcuoglu et al., 2020). The HADDOCK scoring function contains a linearly weighted combination of various energy terms. In addition, we used LIGPLOT + v.2.2.4 to visualize 2D interactions between wild-type and mutant forms of the Drosha and Dgcr8 complex.

Calculation of binding free energies and dissociation constants

Using the PROtein BinDing Energy (PRODIGY) tool to calculate the binding affinities and dissociation constants of the Dgcr8-Drosha complex based on intermolecular contacts.

2.4 MD simulations

A protein-protein complex with a more negative HADDOCK score was used to prepare the input files for the MD simulations. The CHARMM-GUI Web Interface-Solution Builder plugin was used to generate solvated complexes with TIP3P water molecules (Jo et al., 2014). The desired residue ID numbers for the Dgcr8 and Drosha proteins from the complex were selected. A rectangular water box with an edge spacing of 10.0 Å and containing 0.15 M potassium chloride (KCl) ions was added by Monte Carlo ion placement. After applying periodic boundary conditions, PME-FTT (Particle-mesh Ewald) mesh information is generated, and the system is locally minimized to remove unwanted contacts. Furthermore, we implemented CHARMM36-m force field-assisted hydrogen mass redistribution to improve protein-protein simulation conformational probes (Sajadi and Rowley, 2018). Constant volume and temperature (NVT) and constant pressure and temperature (NPT) ensembles were used to generate equilibration and production input files for MD simulations at 303.15K. In addition, MD simulations of solvated protein-peptide complexes were performed using the 100 ns gmx module of the GROMACS 5.1.2 package (Abraham et al., 2015; Abraham et al., 2016). Energy minimization was performed by a 5000-step steepest descent algorithm, resulting in a system free of cubic collisions and anomalous geometries. The system was then equilibrated under an isothermal-isochoric ensemble (NVT/NPT) at 300 temperatures with a step length of ps (0.002) using the Leep-Frog algorithm. Finally, fabrication was performed at desired distances with a Coulomb cutoff (1.0 Å) and a short-range van der Waals cutoff (1.0 Å) in 50,000 steps. The complex was restarted from the last frame of the production run to achieve the target 100 ns simulation and generate corresponding trajectories representing the atomic motions of the system. After successfully completing a 100 ns simulation, the position of the α carbon (C α), the root mean square deviation (RMSD), the root mean square variation (RMSF), the radius of gyration (Rg), and the accessible surface area of the solvent Dgcr8 (SASA) and the protein complexes were calculated.

2.5 Cell culture

The C57BL/6 strain immortalized mouse VSMC cells were utilized in the tests. They were grown in the minimal Eagle's medium (MEM) (Gibco), which contains 10% fetal bovine serum (FBS), at 37°C with 5% ambient carbon dioxide. If sub-cultured, 0.25% trypsin was used to separate the cells. The cells were placed in full MEM with 10% dimethyl sulfoxide and frozen in liquid nitrogen to preserve the cell line (DMSO). The previously frozen cells were defrosted in a 37°C water bath, centrifuged for 3 minutes at 1000 rpm to remove the DMSO-containing MEM, and then resuspended in the new MEM for further culture. Except for employing Dulbecco's modified Eagle's medium, HEK293V cell culture for lentivirus production was similar to that of VSMCs.

2.6 Knockdown of Drosha in mouse VSMCs

To lower the wild-type Drosha expression in mouse VSMCs, the lentiviral vector (pLVshRNA-Puro) harboring the targeting sequence (GCAGGAATGACTGAATGAACC) was used to knock down Drosha by interfering with its mRNA5'UTR. The vector without insertion was used as the control. The HEK293V cells of 90–95% confluent was then transfected by the plasmid and two helper plasmids (pH1 and pH2) to package lentiviruses. The cell culture media were replaced freshly 24 h post-transfection and collected after the other 24 h. The lentiviruses were obtained by using the Lentivirus Purification kit (P2010, inovogen). The VSMCs of approximately 80% confluent were transduced by the lentiviruses with polybrene (6 $\mu\text{g}/\mu\text{L}$) and cultured for 48 h (renewing the media once 24 h after transduction). The VSMCs were screened by the puromycin at the concentration of 3 $\mu\text{g}/\text{mL}$. To test the RNAi efficiency, the quantitative real-time RT-PCR was applied to compare the Drosha expressions in control and Drosha knocked down VSMCs.

2.7 Overexpression of mutant Drosha for the mouse VSMCs with wild-type Drosha knockdown

To determine the role of E401Q on DROSHA in miRNA maturation in VSMCs, the mutant Drosha was delivered to the VSMCs with wild-type Drosha knockdown. Comparatively, the Drosha sequence without mutation was also re-expressed in the knocked down VSMCs. The mutant or wild-type Drosha sequence was cloned to the lentiviral vector (pLV-Bsd), followed by lentivirus production and transduction to VSMCs with native Drosha knockdown, the similar methods as described in 2.6 except for the screen by blasticidin at the concentration of 10 $\mu\text{g}/\text{mL}$. The expression of mutant or wild-type Drosha mRNA in VSMCs was determined by real-time RT-PCR.

2.8 Real-time RT-PCR to quantify Drosha mRNA

The total RNA of VSMCs were isolated using TRIZOL method, and reverse transcribed according to the instruction of HiScriptReverse Transcriptase Kit (Vazyme). The primers for the amplification of wild-type Drosha or mutant Drosha were

F: GTCGTACCAACCCAGGACGA and

R: GCGCTCGGAGGCTCATATTGATA, and the primers for GAPDH as the internal reference were

F: AGGTTGTCTCTCGGACTTCA and

R: GTCCAGGGTGGGCTTCTTACT.

The PCR system of 20 μL volume contained the 1 μL cDNA template, 0.8 μL + 0.8 μL primers, 10 μL 2 $\times$ SYBR Green Mix, and 7.4 μL ddH₂O. In the reaction, cDNA was pre-denatured at 95°C for 2 min, prior to the 95°C for 5 sec and the 60°C for 30 sec with 40 cycles. The melting curve was performed from

65°C to 95°C, with the increase of every 0.5°C/5 sec. The expression of wild-type or mutant Drosha mRNA, normalized to that of the GAPDH, was quantified as $2^{-\Delta\Delta Ct}$.

2.9 Real-time RT-PCR to quantify mature miR-16 and miR-21

To compare the expression of miR-16 or miR-21, the two important miRNAs with the opposite functions, altered by Drosha mutation, the real-time RT-PCR was performed to quantify mature miR-16-5p and miR-21-5p in VMSCs of different genotypes. The VSMC RNA was extracted by using the Direct-zol RNA MiniPrep Kit (Zymo Research), and reverse transcribed via a 10 μ L system containing 1 μ L 10 $\times$ Reaction buffer *E. coli* poly(A) polymerase, 1 μ L ATP (1mM), 1 μ L RT primer (10 μ M), 1 μ L dNTP mix (1mM each), 0.5 μ L M-MuLV reverse transcriptase, 0.2 μ L *E. coli* poly (A) polymerase, 100 ng RNA sample, and RNase-free water added to the total volume of 10 μ L. The reaction was at 42°C for 1 hour and 95°C for 5 min. The primers to amplify miR-16 were F: GGGGTagcagcacgta and R: CAGGTCCAGTTTTTTTTTTTTTcg, while miR-21 were F: GGGGtagcttatcagactg and R: CAGGTCCAGTTTTTTTTTTTTTtca. mU6-F and -R, the primers for internal reference amplification, were ctgcgttcggcagcacata and tgcgtgtcatccttgcgca, respectively. The 20 μ L PCR system was composed of 1 μ L cDNA template, 0.4 μ L + 0.4 μ L primers, 10 μ L 2 $\times$ SYBR Green Mix, and 8.2 μ L ddH₂O. The PCR began with the pre-denaturation at 95°C for 3 min, followed by the 31 cycles including 95°C for 30 s, and 55°C for 10 s. The reaction was completed after the last step of 72°C for 10 min. The melting curves and the quantification of miR-16 or miR-21 expression were performed the same as those in 2.8.

2.10 Statistical analysis

From three independent experiments, data were shown as mean $\pm$ standard deviation, and the difference was calculated by the student's *t*-test that *p* value less than 0.05 was considered significant.

3. Results

3.1 Calculation of deleterious non-synonymous SNPs using SIFT and PANTHER program

Predicted and calculated SNP data on the Drosha gene was retrieved from mouse SNP information database for this study. In total 35 SNPs of Drosha gene, 13 SNPs was found to be protein coding non-synonymous, 22 SNPs (rs38201751; rs37196657; rs38935668; rs46453469; rs32044887; rs31637395; rs32449566; rs37100157; rs32274343; rs37574965; rs36834569; rs31634346; rs37065962; rs31895334; rs32477308; rs31807236; rs4827900; rs31833279; rs13470544; rs38018886; rs37316206; rs37176538; rs36530504) were non-protein coding occurred in mRNA 5'UTR regions. The coding non-synonymous regions were selected for further analysis. SIFT tool is employed to identify the conservation level of the particular position of the DROSHA through sequence homology method. From 13 nsSNPs were showed score ranging from 0.05 to 1.0 respectively which are illustrated in Table 1. PANTHER is a hidden Markov model (HMM) based tool was employed to validate the DROSHA function upon single point mutation and it is able to classify proteins by function, thus adding another layer of complexity to refine SNP prediction. The distribution of predicted nsSNPs in DROSHA gene was shown in Fig. 1. Out of 13 nsSNPs, only one nsSNPs that demonstrated subPSEC score less than - 4.6 was selected as deleterious and the amino acid substitution position at E401Q showed good correlations with SIFT, PROVEAN, I-Mutant 2.0 and SNAP. Therefore, the SNP ID: rs212801796 was selected for modeling analysis.

Table 1
List of nsSNPs that were analyzed by SIFT, PROVEAN, I-Mutant 2.0, SNAP and PANTHER.

SNP ID	Allele	AA Change	SIFT		PANTHER		PROVEAN		I-Mutant		SNAP	
			Score	Prediction	subPSEC	Prediction	Score	Prediction	DDG	Stability	Score	Effect
rs3681267	C/T	P211G	0.41	Tolerated	-0.88	Begign	-0.47	Neutral	-2.11	Decrease	13	Effect
rs32031213	C/T	S149Q	1	Tolerated	-0.49	Damaging	0.53	Neutral	-0.16	Increase	-37	Neutral
rs3681822	C/T	P191T	0.37	Tolerated	-0.88	Begign	0.61	Neutral	-1.13	Decrease	-23	Neutral
rs31704434	A/G	P113R	0.52	Tolerated	-1.67	Damaging	0.94	Neutral	-0.18	Increase	53	Effect
rs38678852	A/G	S87V	0.35	Tolerated	-0.88	Begign	-1.05	Neutral	0.04	Increase	8	Effect
rs48279007	G	R15P	1	Tolerated	-1.67	Damaging	0.28	Neutral	-0.47	Decrease	73	Effect
rs31936126	C/T	P320S	0.85	Tolerated	-0.46	Begign	0.12	Neutral	-1.75	Decrease	-5	Neutral
rs228110127	A/C	N382T	0.11	Tolerated	-0.88	Begign	-1.7	Neutral	0.45	Decrease	5	Effect
rs219409109	G/C	E388D	0.34	Tolerated	-0.88	Begign	-0.89	Neutral	-0.59	Decrease	-55	Neutral
rs228498905	T/C	V394A	0.79	Tolerated	-0.13	Begign	-0.36	Neutral	-0.37	Decrease	-59	Neutral
rs212801796	G/C	E401Q	0.05	Deleterious	-1.67	Damaging	-0.47	Neutral	-0.35	Decrease	64	Effect
rs231394879	G/T	E403D	0.26	Tolerated	-0.13	Begign	-0.71	Neutral	-0.47	Decrease	-76	Neutral
rs38201751	C/G	R15P	1	Tolerated	-1.67	Damaging	0.28	Neutral	-0.47	Decrease	73	Effect

3.2 Homology modeling and quality assessment of the model

There were 19% percentages of sequence identity observed between the target and template of DROSHA. Hence, we prefer to follow threading based approach for model native and mutant TYR structures. 3-D models of native and mutant (E401Q) DROSHA structures (Fig. 2) based on the following PDB ID: 5zakA used as template. The root mean square deviation (RMSD) value of the modeled structures were calculated by structural superposition with templates structures on chimera and found to be 0.68 Å. This value indicates a high structural similarity in the 3D structures. The low RMSD values of the homology model were evidence for a model of acceptable quality. Ramachandran plot indicates that good stereo chemical quality of modeled proteins. In native structure of DROSHA showed 72.8% of residues are in most favored regions, 18.3% residues are in additional allowed regions, and 6.1% residues are in generously allowed regions. In the mutant E401Q structure of DROSHA showed 65.2% of residues in most favored region, 21.18% of residues in additional allowed region and 8.7% of residues are in generously allowed region. Overall G-factors (acceptable between 0 and 0.5) of DROSHA structures predicted by PROCHECK is acceptable range 0.3 for both native and mutant type. In addition, some physiochemical properties of native and mutant structures of DROSHA were summarized in Table 2.

Table 2
Physiochemical properties of native and mutant structures of DROSHA.

Protein	Total Assessable Area (Å ²)	Free energy of folding (kcal/mol)	No. of H bonds
DROSHA	48941.2	-1215.89	905
DROSHA ^{E401Q}	51528.6	-1206.93	814

3.3 Molecular dynamics simulation for stability analysis

The RMSD describes the preliminary analysis of backbone atoms of the native and mutant structure of DROSHA is calculated against the simulation time between 0 and 20000 ps. RMSD is a key parameter to estimate the equilibration of MD trajectories. In the native structure of DROSHA we can observed that the average RMSD trajectory value ranging between 0.12 nm and 0.85 nm. We observed the deviation of the native structure throughout the simulation time. After 4000 ps increase in the RMSD trajectory value was about 0.60 nm, also after 5900 ps the RMSD value seems to be dropped to 0.57 nm. The gradual increase observed at 7500 ps and dropped at 14500 ps. Then, the slight increase observed until the end of the simulation period. In the mutant structure of DROSHA the minimum RMSD trajectory value ranges between 0.14 nm and 0.80 nm. The mutant structure indicates the constant variation till the end of the simulation. We have observed changes in 3900 ps, 10500 ps and 15000 ps. The high RMSD value indicates lower stability of the protein (Fig. 3). From the observation of the RMSD results mutant structure of DROSHA is more stable than native structure.

3.4 Molecular dynamics simulation for flexibility analysis

The root mean square fluctuations (RMSF) plot describes that flexibility of backbone atoms of the native and mutant structure of DROSHA. The high RMSF value indicates more flexible movements whereas low RMSF value indicates restricted movements during simulation in relation to its average position. The RMSF ranges 4 nm and 28 nm in the native type. The high level of fluctuation observed at the residue position 15, 87, 113, 149, 191, 236, 320, 382, 388, 394, 402, 407 and 463. The highest number of fluctuation noticed at the position of 463 with the fluctuation is about 28 nm. The RMSF ranges 4 nm and 16 nm in the mutant type. The high level of fluctuation observed at the residue position 18, 93, 121, 156, 198, 257, 323, 389, 394, 401, 403 and 476. The highest number of fluctuation noticed at the position of 476 with the fluctuation is about 16 nm. From the RMSF results the native structure causes more fluctuation than mutant structure. The terminal residues of both the protein structures show high fluctuations (Fig. 4). It indicates the mutant structure of DROSHA is more stable than native type. Thus, the result demonstrated that E401Q mutation causes overall stability of the protein structure when compared to the native type protein structure.

3.5 The expressions of miR-16 and miR-21 were altered in the mouse VSMCs due to the E401Q on DROSHA

To disclose the consequence of Drosha E401Q mutation for miRNA maturation in VSMCs, the expression of miR-16 or miR-21 was compared between mouse VSMCs with and without Drosha mutation. In the Drosha knockdown VSMCs, the expression of Drosha mRNA was significantly reduced compared to that of the controls. Subsequently, the knocked down VSMCs were transduced by lentiviruses to over-express wild-type and mutant Drosha, respectively. Real time RT-PCR analysis showed that the mRNA level of wild-type or mutant Drosha was significantly increased thereof. Noticeably, the expression of mutant Drosha mRNA was higher than that of the re-expressed wild-type (Fig. 5). Further, the expressions of miR-16 and miR-21 were compared between the VSMCs with different genotypes. The two miRNAs were both significantly decreased in the Drosha knockdown VSMCs, as well as significantly re-increased after the recuperation of Drosha as predicted. Interestingly, with the mutation of Drosha, both miR-16 and miR-21 were significantly down-regulated, suggesting that glutamic acid the site 401 of Drosha is pivotal to the maturation of miRNA. However, the expression of miR-21 was reduced by Drosha mutation with the ratio of 1.79, which was greater than 1.28 of miR-16 (Fig. 6A&B), indicating that E401Q on Drosha may be useful to attenuate the regulatory effect of onco-miRNAs in VSMCs such as miR-21. In the real time RT-PCR analysis, the melting curves for wild-type/mutant Drosha, miR-16, miR-21, GAPDH, and U6 were shown in **Supplemental Fig. 1**, indicative of the specificity of the amplified products.

4. Discussion

Drosha, the endonuclease responsible for the key step of miRNA maturation, has been shown to be the oncogene in some cancer types, such as lung and cervical cancers. In the non-small-cell lung cancer and the cervical squamous cell carcinoma (SCC), Drosha is found to be highly expressed. Knockdown

of Drosha in the cervical SCC can reduce the cell motility/invasiveness, as well as change the global expression of miRNAs [31, 32]. In the Wilms tumor, a kidney cancer frequently affecting children, the occurrence of mutation from glutamic acid to lysine at site 1147 (E1147K) of Drosha is more than 60%, which impairs the miRNA biogenesis [33]. As previously denoted, Drosha is required for VSMC survival [14], and dysregulation of Drosha could also contribute to vascular diseases [34]. Therefore, Drosha may be a potential molecular target for disease treatment in various cells. Studies reported that the naturally occurring mutations (P32L, P100L, K226E and R279L) of Drosha are associated with hereditary hemorrhagic telangiectasia. Over-expression of P100L or R279L in zebrafish gives rise to the abnormalities of angiogenesis and vascular permeability [17]. These investigations point to the close relationships between certain Drosha mutations and diseases in the nature. In the present work, we found E401Q mutation for Drosha may affect miRNA maturation through calculation. Accordingly, we compared the expressions of miR-16 and miR-21, two well-investigated miRNAs with opposite functions, between wild type Drosha and muDrosha in mouse VSMCs, to testify the E401Q effect on miRNA biogenesis *in vitro*.

miR-16 is a key member of miR-15/107 family (miR-103a, miR-107, miR-15a, miR-15b, miR-16, miR-195, miR-424, miR-497, miR-503, and miR-646), a much focused miRNA group, as all members bearing the sequence AGCAGC near the 5' end of mature miRNAs [35, 36]. The accumulated evidences yet proved the tumor suppressive role of miR-16 for it targets multiple cancer-related genes. For instance, miR-16 is able to down-regulate the antiapoptotic B cell lymphoma 2 protein, Sal-like protein 4, and wild-type p53-induced phosphatase 1, therefore inhibits the chronic lymphocytic leukemia, gastric and lung cancers through reducing the tumor cell proliferation and enhancing the apoptosis [37–39]. In VSMCs, knockdown of miR-16 promotes angiotensin II (AngII) -induced cell proliferation through activating ERK1/2 and p38 pathways, indicating miR-16's capability to inhibit VSMC growth mediated by AngII. As is well-documented, VSMC proliferating ability enhanced by AngII is closely linked to the vascular diseases like hypertension, restenosis and atherosclerosis [9]. Therefore, miR-16 seems to be a “good” miRNA in VSMC.

On the contrary, miR-21 has been characterized as an oncogenic miRNA by targeting a series of tumor inhibitor proteins, such as phosphatase and tensin homolog, programmed cell death 4, and leucine zipper transcription factor-like 1, thereby promoting tumor growth, metastasis and invasion in manifold cancers including ovarian, renal and breast cancers [40–42]. miR-21 is also proven to stimulate VSMC proliferation in the injured artery by down-regulating c-Ski in rat [43], and to enhance the proliferation and migration in human airway smooth muscle cells, which may worsen the symptom of asthma [44]. High level of miR-21 involves in inducing contractile gene expression in VSMCs [45]. We previously demonstrated that miR-21 is associated with primary dysmenorrhea, a disease resulted from abnormal uterine smooth muscle cell contraction [46, 47]. These studies altogether revealed that miR-21 may play the opposite roles as miR-16, not only in cancers but in VSMC modulation.

In our data, the expressions of both miR-16 and miR-21 in mouse VSMCs were significantly down-regulated when Drosha was knocked down, indicating that the maturation of the two important miRNAs was inhibited with the decrease of Drosha. Further, we over-expressed the wild type Drosha and muDrosha for the VSMCs with Drosha knockdown, respectively. muDrosha expression was higher than the wild type one. However, the expression of miR-16 or miR-21 was significantly reduced in the muDrosha-expressing VSMCs, which reflects the fact that E401Q attenuates the productions of miR-16 and miR-21. This is in agreement with the knowledge that the major miRNA expressions are dependent on Drosha level. Interestingly, our data show that, with the mutation of E401Q, the expression of miR-21 falls more quickly than miR-16 (the decreasing ratios for miR-21 and miR-16 were 1.79 fold vs 1.28 fold). The evidence suggests that Drosha E401Q may exert greater impact on the expression of miR-21 than that of miR-16 in VSMCs. Hence, the present study might cast light on attenuating the effect of “deleterious” miRNA through re-designing the sequence of Drosha. This work, for the first time, predicted the potential site mutation on Drosha may influence miRNA maturation by virtue of computational strategies, and tested its effect on two key miRNA expressive alterations in mouse VSMCs. However, Future works are needed to testify more miRNAs regulated by the mutation, and to characterize the VSMC phenotypic variation thereof.

Conclusions

We theoretically calculated that the mutation of E401Q on Drosha may affect miRNA maturation. With such mutation in mouse VSMCs, we found that the levels of both miR-16 and miR-21, two key miRNAs highly expressed in VSMCs, were down-regulated. However, miR-21 is more likely to be suppressed by Drosha E401Q. In all, the data provide insight into modulating the VSMC functions via engineering the Drosha sequence to change the expressions of different miRNAs.

Declarations

Conflict of interest

All authors declare that they have no conflict of interest.

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Author Contributions

Dong-Qing Wei, Gurudeeban Selvaraj, and Pei Fan conceived and designed the calculations and experiments. Gurudeeban Selvaraj, Satyavani Kaliyamurthi, and Gilles H. Peslherbe performed the computational works. Pei Fan, Lu Zhang, and Rui-Fang Li performed the experiments and data analysis. Dong-Qing Wei, Gurudeeban Selvaraj, and Pei Fan wrote the manuscript. Dong-Qing Wei, Gilles Peslherbe, and Rui-Fang Li provided expert comments. All authors approved the manuscript.

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Figures

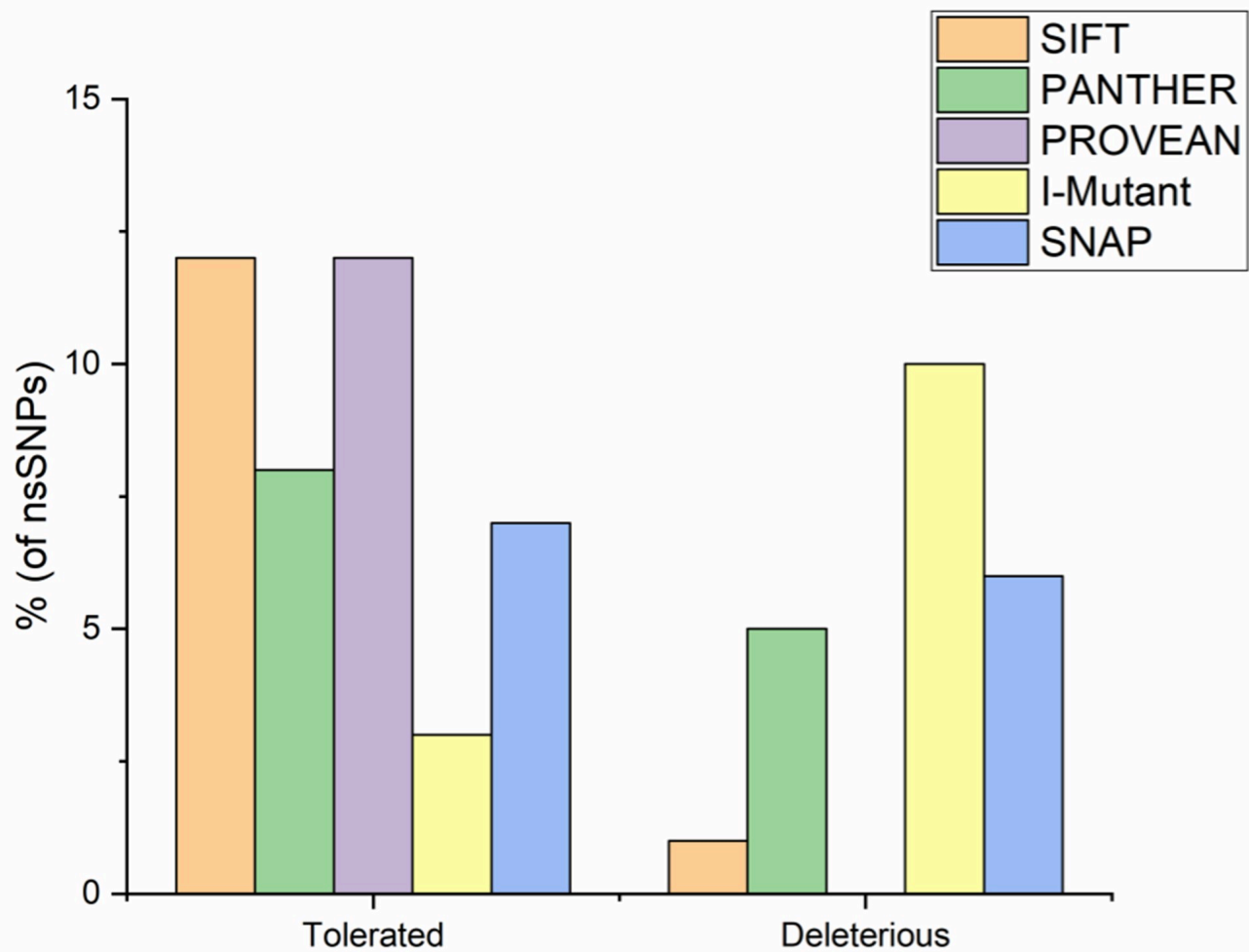


Figure 1

Bar diagram of distribution of predicted nsSNPs in Drosha gene analyzed by SIFT, PROVEAN, I-Mutant 2.0, SNAP and PANTHER. Red color bar indicates percentage of nsSNPs found to be deleterious by SIFT and PANTHER, decrease stability by I-Mutant 2.0 and damaging by SNAP. Blue color bar indicates percentage of nsSNPs tolerated by SIFT and PANTHER, increase the stability by I-Mutant 2.0 and neutral by SNAP.

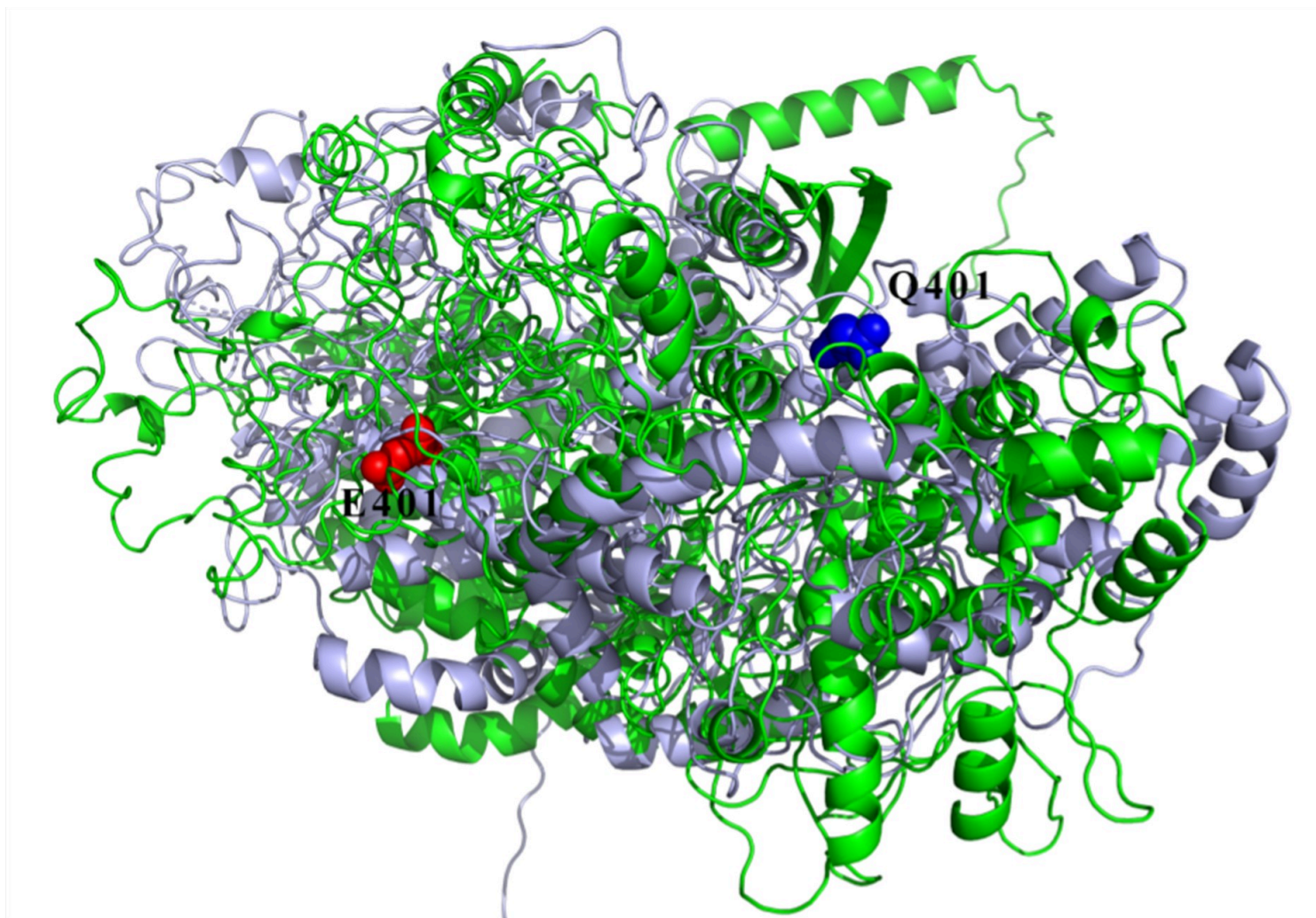


Figure 2

Superimposition of native and mutant (E401Q) modeled structures of DROSHA.

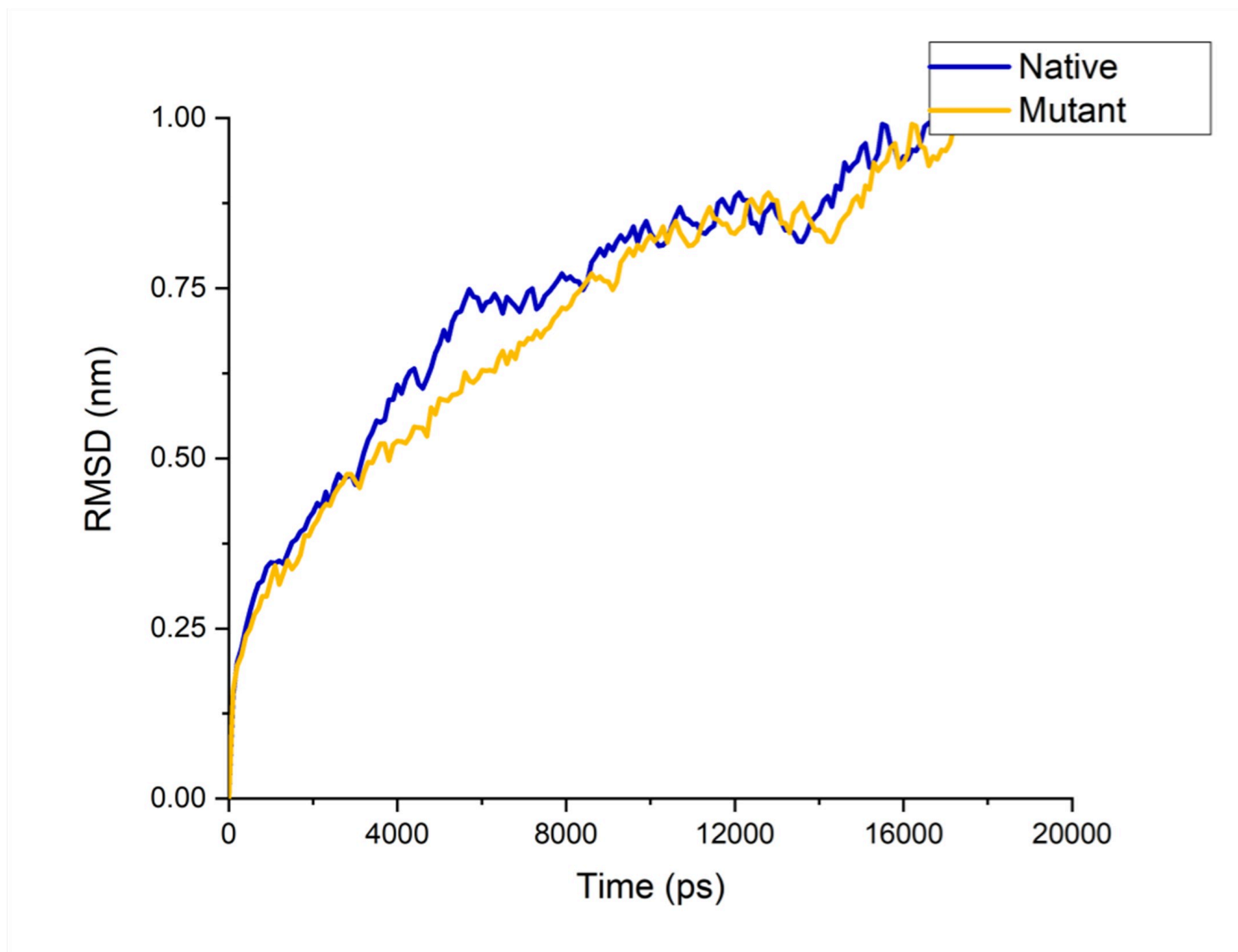


Figure 3

Backbone RMSD profile of native (blue) and mutant (orange) structure of DROSHA at 300K

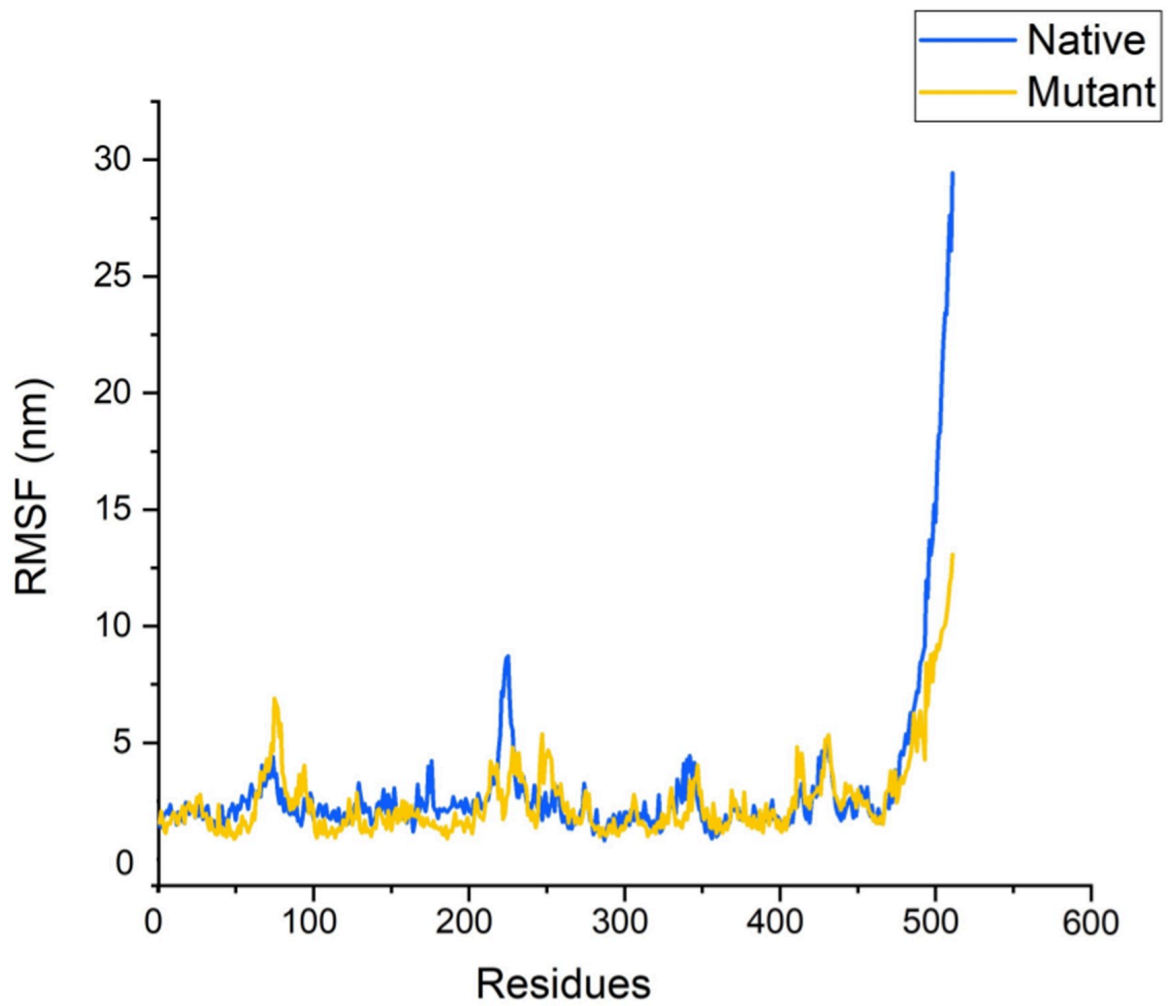


Figure 4

Backbone RMSF profile of native (blue) and mutant (orange) structure of DROSHA at 300K

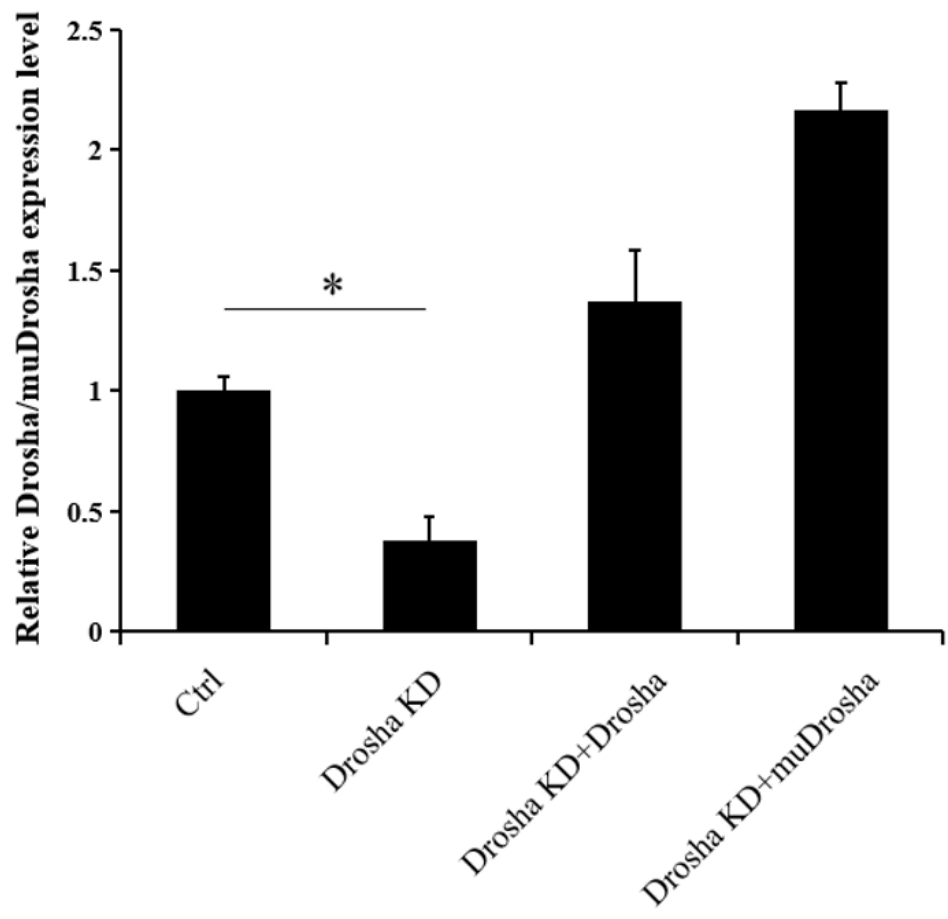


Figure 5

Wild-type or mutant Drosha is delivered to the mouse VSMCs with Drosha knockdown. The comparison of wild-type or mutant Drosha expression in mouse VSMCs between control (Ctrl), Drosha knockdown (Drosha KD), Drosha knockdown re-expressed with wild-type Drosha (Drosha KD + Drosha), and Drosha knockdown expressed with mutant Drosha (Drosha KD + muDrosha) groups, in which * means $p < 0.05$ that the difference is significant.

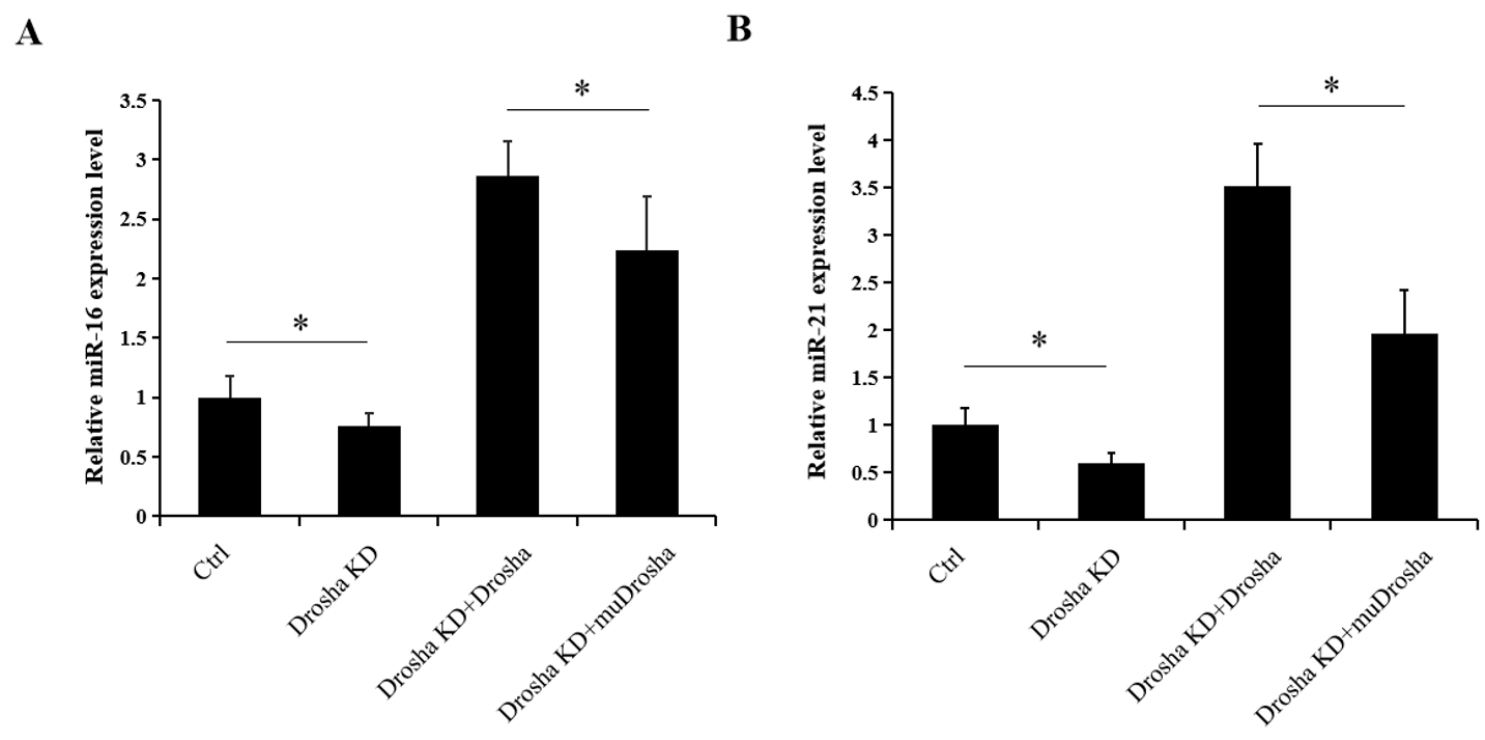


Figure 6

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The expressions of miR-16 and miR-21 are altered by E401Q on Drosha in the mouse VSMCs. **A&B** are the expressive comparisons of miR-16 and miR-21, respectively, between the four different groups (* $p < 0.05$).