LETTER TO THE EDITOR

Mutations induce conformational changes in folliculin C-terminal domain: possible cause of loss of guanine exchange factor activity and Birt-Hogg-Dubé syndrome

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Introduction

Birt–Hogg–Dubé (BHD) syndrome, an inherited kidney cancer syndrome, is characterized by cutaneous fibro folliculomas, pulmonary cysts, and an increased risk for the development of renal cell carcinoma (Vocke et al., 2005). The folliculin protein is known as kidney tumor suppressor (Hasumi et al., 2015). Germline mutations of the folliculin gene have been found to be responsible for BHD syndrome.

Nookala et al. (2012) have determined, at 2 Å resolution, the three-dimensional (3D) structure of the C-terminal domain of folliculin, called folliculin-CT. The fold of folliculin-CT is dominated by an α–β architecture with a core β-sheet and helices packed on one side followed by an all-helical region. They also demonstrated that folliculin-CT is expressed differentially in normal cells and is distantly related to “differentially expressed in normal cells and neoplasia” (DENN) domain proteins, a family of Rab guanine nucleotide exchange factors (GEFs). Biochemical analysis has also shown that folliculin has GEF activity. A database of folliculin mutations reported 70 mutations, of which most lead to the BHD phenotype (Lim et al., 2010). The tumor suppressor folliculin is a GEF for Rab35 and folliculin’s GEF activity toward its GTPase might be essential for important cellular processes. The mutations in Flcn lead to a loss of GEF activity which triggers cancer progression (Nookala et al., 2012). Folliculin (Flcn) interacts with Folliculin-interacting proteins 1 and 2 (FNIP1 and FNIP2).

In a recent study, authors demonstrated that heterozygous Fnip1/homozygous Fnip2 double-knockout mice developed kidney cancer at 24 mo of age, analogous to the heterozygous Flcn-knockout mouse model. These findings support the concept that interaction with Fnip1 and Fnip2 is essential for the tumor-suppressive function of Flcn, and kidney tumorigenesis in human BHD syndrome may be triggered by loss of interactions among Flcn, Fnip1, and Fnip2 (Hasumi et al., 2015).

It is surprisingly interesting that such a large number of mutations encompassing a single folliculin gene lead to single disease phenotype. Most of the mutations identified are frameshift which lead to a change in amino acid residue at a specific position in the folliculin (Lim et al., 2010).

Molecular dynamics (MD) simulations have become routine recently to understand structural changes that follow molecular interactions (Gadhe, Balupuri, & Cho, 2015; Punkvang, Hannongbua, Saparpakorn, & Pungpo, 2015; Wang, Yang, Shi, & Le, 2015). We have selected three folliculin-CT mutations, i.e. Ser386Asp, Leu418Trp, and His429Pro, to study the effect of these mutations on the conformation and role in loss of function. To find out the protein conformation which is associated with disease phenotype, we performed MD simulation on mutated protein variants. The molecular insight into the folliculin dynamics in the presence and absence of mutations may provide valuable information regarding the interactions essential for normal functioning of cellular process and the molecular basis of BHD syndrome.

Materials and methods

MD simulation

Classical MD simulation was performed for folliculin wild and mutant forms using the Gromacs package (Berendsen, van der Spoel, & van Drunen, 1995). The only crystal structure of human folliculin available is PDB ID 3V42 (Nookala et al., 2012), which has several missing residues. So, a model was generated using Swiss Model server (Schwede, Kopp, Guex, & Peitsch, 2003) by taking 3V42 as template. All the mutant forms were...
generated using Discovery studio (Accelrys Software Inc., 2011). The Gromos43a1 force field (van Gunsteren et al., 1996) was used to generate the topologies for proteins and for the simulation. Proteins were solvated in a cubic box under the periodic boundary conditions using a distance of 1.5 nm from the protein to the surface of the box. The simple point charge (SPT) water model was used in the present study. To neutralize the system, counter ions were added accordingly. Each system was subjected to energy minimization using the steepest descent integrator without constraints for 10,000 steps. Following the minimization step, system (folliculin, solvent, and ions) was equilibrated under NVT (number of atoms, volume, temperature) (canonical ensemble) and NPT (number of atoms, pressure, temperature) (isothermal–isobaric ensemble) conditions for 300 ps at 300 K after applying position restraints to the protein. Finally, a 100 ns production run was performed. The root mean square deviation (RMSD) and root mean square fluctuation (RMSF) calculations were done by least square fit. All visualizations were performed using Chimera (Pettersen et al., 2004).

**Principal component analysis**

Principal component analysis (PCA) was performed to obtain dominant and collective modes of the protein from the overall dynamics of the MD trajectory. PCA is based on the construction of a mass-weighted covariance matrix of the atom displacement. This covariance matrix is diagonalized to extract a set of eigenvectors and eigenvalues that reflect concerted motion of the molecule (Amadei, Linssen, & Berendsen, 1994). Eigenvectors represent the direction of motion, whereas the corresponding eigenvalues represent the amplitudes in those directions. The Gromacs in-built tool, gmx covar, was used to yield the eigenvalues and eigenvectors by calculating and diagonalizing the covariance matrix.

**Results and discussion**

**MD simulation in water**

RMSD trajectory of folliculin (wild and mutants) with respect to the initial conformation was calculated as a function of time to assess the conformational stability of the protein during the simulations. RMSD profile was found to be always less than 0.5 nm for all the variants of folliculin, indicating the stability of the system during 100 ns simulation (Figure 1(A)). However, all the mutant forms showed higher RMSD as compared to the wild folliculin. Total energy profiles (Figure 1(B)) showed a remarkable difference in the total energy. All the mutant forms accounted for much lower energy (higher negative energy)
as compared to wild form which indicated that mutant forms are more stable as compared to wild. The radius of gyration ($R_g$) of an object describes its dimensions, calculated as the root mean square distance between its center of gravity and its ends. In analyzing proteins, the radius of gyration is indicative of the level of compaction in the structure. All mutant forms showed decrease in the $R_g$ which clearly indicates their folding during MD simulation (Figure 2(A)). All mutations studied lead to a more compact structure of folliculin. Mutants Ser386Asp and Leu418Trp are found more compact. Intra-molecular hydrogen bonding was found similar in all the studied forms of folliculin (Figure 2(B)).

Further, the RMSF profile was recorded for each form of folliculin which reveals that dDENN domain residues in all mutant forms showed much higher fluctuation as compared to wild folliculin. The Leu418Trp form showed highest fluctuation in dDENN domain (Figure 3). These results collectively indicate that mutation induced conformational changes in dDENN domain of folliculin.

**Conformational changes**

Zhang, Iyer, He, and Aravind (2012) described the folliculin-CT domain and mentioned the last three helices

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**Figure 2.** (A) Radius of gyration and (B) Intra-molecular hydrogen bonding during the 100 ns molecular dynamics simulation (Blue – wild, Red – Ser386Asp, Green – Leu418Trp, and Purple – His429Pro).

**Figure 3.** RMSF profile showing the fluctuation in the folliculin residues (Blue – wild, Red – Ser386Asp, Green – Leu418Trp, and Purple – His429Pro). Circles are highlighting the d-DENN domain.
helices 8, 9, and 10) as d-DENN domain and rest of the part as core DENN domain. There are very limited studies regarding the folliculin DENN domain and its function; however, folliculin-CT has structural and functional similarities with the DENN1B as reported by Nookala et al. (2012).

To understand how folliculin interacts with the Rab35, it becomes essential to understand the interaction between DENN1B and Rab35. The Rab35-binding surface of the DENN1B domain is formed primarily by helices Helix 7, Helix 13, and Helix 14 in strand Sheet 7, and the loops Helix 5–Sheet 6, Helix 6–Sheet 7, Helix 7–Sheet 8, Helix 8–Helix 9, and Helix 13–Helix 14 (Wu et al., 2011). Helices 9–13 of DENN1B are structurally similar to the helices 8 and 9 of the folliculin, and Helix 14 of DENN1B shares position and structure with Helix 10 of folliculin. The C-terminal lobe residues primarily contribute to Rab35-binding surface. Rab35 binds the DENN1B domain via switches I and II. Switch I and II surround the nucleotide-binding pocket. A conformational change in switch I upon binding to the DENN domain lowers the affinity of Rab35 for GDP, facilitating GDP dissociation and allowing GTP to bind (Wu et al., 2011). Residues 33–45 and 64–77 in Rab35 constitute the switch regions I and II, respectively. I224 K/I226D/L233D/Y236K and M241R/P242R/Q359A mutations in DENND1B surface affect the binding of switches I and II and result in no observable complex of any mutant form with the Rab.

Figure 4. Comparison of average structure of all folliculin forms with initial structure (A) Wild, (B) Ser386Asp, (C) Leu418Trp, and (D) His429Pro (in all parts, peach-colored structure is the initial structure).
The GEF activity of the mutants for Rab35 was also not found significant as compared to the wild-type DENN1B. Interaction with both switch regions is important for binding as well as for GEF activity as revealed by results (Wu et al., 2011). In vitro biochemical analysis reveals that folliculin-CT has GEF activity toward Rab35 similar to the full-length folliculin (Nookala et al., 2012).

Comparison of the initial modeled structure with average structure of wild and mutant folliculin revealed that wild form showed a very close similarity with initial structure, while all mutant forms showed remarkable difference in organization, especially in helices 8, 9, and 10 (Figure 4). This structural variation occurred due to mutation that might lead to the loss of interaction with partner proteins such as Fnip1/2.

In a recent study, authors demonstrated that heterozygous Fnip1/homozygous Fnip2 double-knockout mice developed kidney cancer at 24 mo of age, analogous to the heterozygous Fcn-knockout mouse model. These findings support the concept that interaction of folliculin with Fnip1/Fnip2 is essential for the tumor-suppressive function of Fcn and kidney tumorigenesis in human BHD syndrome may be triggered by loss of interactions among Fcn, Fnip1, and Fnip2 (Hasumi et al., 2015).

These two results concluded that folliculin GEF activity may or may not require Fnip1/Fnip2, while folliculin–Fnip1/2 interaction is essential for tumor-suppressor activity. The mutation-induced structural changes in folliculin may disrupt the proper interaction with Gab35 and also with Fnip1/2. The residues of Helix 14 of DENN1B were reported to interact with switch II region and the mutation in Helix 14 (Q359A) significantly inhibits GEF activity (Wu et al., 2011). The switch undergoes conformational change under the influence of DENN1B. The studies available suggest that Rab GEFs remodel the nucleotide-binding site for Rab substrate. They induce large conformational changes in switches I and II. Switch I opens and displaces an aromatic residue that normally stabilizes GDP. The DENN domain does not protrude into the Rab nucleotide-binding pocket, although steric clashes between DENND1B (Helix H7) and portions of switch I may contribute to switch I refolding as the Rab/GEF complex forms. The folliculin segments, homologous to switch I-interacting DENN1B segments, are loops, Sheet 3–Helix 5, Sheet 2–Helix 6, and Sheet 7–Helix 7 (Nookala et al., 2012). These segments may interact with switch I region and induce desirable conformational changes required for nucleotide release. However, a clearer picture can only be revealed by the folliculin-CT–Rab35 crystal structure. This study reveals that mutations in folliculin bring significant conformational changes, especially in the d-DENN region which may be the reason for loss of folliculin GEF activity or interaction with partner proteins Fnip1/2 and further progression of renal cell carcinoma.

**Principal component analysis**

The MD trajectory of system was inspected with the principal components to better understand the conformational changes of the folliculin, of all wild and mutant forms. Correlated motion plot shows how atoms move relative to each other. Motions can be positively correlated, anti-correlated, or uncorrelated. The positive and negative limits are shown in Supplementary Figures 1 and 2.

Covariance analyses revealed that most of the dominant motions were in d-DENN part of the protein. The wild folliculin showed anti-correlated motions in the DENN domain, while correlated motions were found negligible or very weak (Supplementary Figure 1 (A)). All the mutant forms showed both correlated and anti-correlated motions (Supplementary Figures 1(B) and 2). These motions, especially in d-DENN domain, drive the structural changes in mutants as revealed by RMSF profile before.

**Conclusion**

The findings of this study concluded that folliculin-CT mutations, Ser386Asp, Leu418Trp, and His429Pro, lead to significant change in the overall structure, especially in d-DENN domain. The changes in d-DENN region were found significant, though the mutation sites are distant. This indicates that each and every residue participates in the structural integrity of folliculin-CT. All mutations lead to a more compact structure as revealed by $R_g$ and structural analysis. These changes might be responsible for lesser affinity for Rab35 and for the binding protein partners such as Fnip1/2. The folliculin–Rab35 complex structure is crucial to completely understand the whole interaction scenario and mechanism of folliculin GEF activity.

**Author contribution statement**

SV, CT, SG, and AG conceived the experiment, SV, CT, SG, BP, and AS conducted the experiment and were assisted by SJ and AS. All authors reviewed the manuscript.

**Abbreviations**

- BHD: Birt–Hogg–Dubé
- DENN: differentially expressed in normal cells and neoplasia
- GEF: guanine nucleotide exchange factor
- FLCN: folliculin
- Fnip1 and 2: folliculin-interacting proteins 1 and 2
- Folliculin CT: folliculin C-terminal
- RMSD: root mean square deviation
- RMSF: root mean square fluctuation
- $R_g$: radius of gyration
- Rab 35: Ras-related protein 35
Mutations induce conformational changes in folliculin C terminal

Supplemental material
The supplemental material for this paper is available at http://dx.doi.org/10.1080/07391102.2016.1188728.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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