RESEARCH ARTICLE

AN *IN SILICO* ANALYSIS OF FOLLICULIN AND FOLLICULIN INTERACTING PROTEINS RESPONSIBLE FOR SKIN TUMORS AND BIRT HOGG DUBÉ SYNDROME

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ABSTRACT

Birt–Hogg–Dube´ (BHD) syndrome is an inherited and autosomal disease characterized by skin and kidney tumors, as well as cystic lung disease, which results from mutation in *BHD* gene / *FLCN* gene. The product of FLCN gene is folliculin protein which has been implicated in numerous signalling pathways like mTOR, AMPK signalling, HIF signalling, TGF-β signalling and the JAK-STAT signalling pathway. From literature it was clear that there were two major consequences of mutated folliculin. First is hindrance in formation of FLCN, FNIP1 and FNIP2 complex, which ultimately interacts with TGF-β protein of TGF-β signalling pathway and inactivates tumor suppression function of that pathway. The other major consequence is activation of HIF1A. From HIF-reporter cell-based screening studies of a natural product-like chemical library it is suggested that KC7F2 is an inhibitor of HIF1A. Thus here we have focused on another inducible factor HIF2A interaction with KC7F2 to carry out *insilico* analysis. A previous study also suggests ability of TSA to reverse the biological and molecular defects of BHD cells but they have also suggested that further work will be needed to elucidate the nature of these effects. So we have carried out the work to study the nature of these effects and the mode of interaction between TSA and TGF-β protein. We found that TSA binds with TGF-β protein at same site where the non-mutated folliculin protein was binding and thus the TGF-β protein works normally, as a result anti-proliferation will takes place via p15 protein and ultimately the tumor suppression will takes place.

Keywords: *FLCN, TSA (Trichostatin A), KC7F2, Folliculin, TGF-β (Transforming Growth Factor- beta), HIF2A (Hypoxia Inducible Factor-2 Alpha).*

INTRODUCTION

Birt–Hogg–Dubé (BHD) syndrome is an autosomal, dominantly inherited, monogenic condition, characterized by the development of fibrofolliculomas (benign skin tumors) on the face, head and upper torso, pulmonary cysts and pneumothorax (collapsed lung), and predisposition to kidney cancers with clear cell, chromophobic or oncocytic features [1]. Birt–Hogg–Dubé syndrome affects the skin and increases the risk of certain types of tumors. The condition is characterized by multiple noncancerous tumors of the hair follicles, particularly on the face, neck, and upper chest. These growths typically first appear in a person's twenties or thirties. People with Birt–Hogg–Dubé syndrome also have an increased risk of developing cancerous or noncancerous kidney tumors (chromophobe renal cell carcinoma and oncocytoma, respectively) and possibly tumors in other organs and tissues. Additionally, affected individuals frequently develop cysts in the lungs that are at risk to rupture and cause an abnormal collection of air in the chest cavity (pneumothorax) that may result in the collapse of a lung. In 2001, a BHD-associated gene locus was localized to chromosome 17p11.2 [2, 3] and a novel gene, *Folliculin* (*FLCN)*, was subsequently identified as being inactivated in individuals with BHD syndrome [4].

BHD gene is also known as *FLCN* gene. This gene codes for a protein called Folliculin (FLCN), which has a putative tumor suppressor function [5, 6, 7, 8]. *FLCN* is predicted to encode the 579 amino acid protein FLCN (64kDa) [9]. Folliculin naturally interacts with two proteins – Folliculin interacting protein 1(FNIP1) and Folliculin interacting protein 2 (FNIP2) [10, 11, 12]. Binding of FLCN to FNIP1 and FNIP2 is mediated specifically through the C-terminal region of FLCN [10, 11, 12]. In BHD syndrome, the majority of mutations were predicted which introduces a premature stop codon into *FLCN*, and therefore result in a protein truncation [13]. However, it is presently unclear whether this truncated FLCN is targeted for nonsense-mediated decay, or remains in the cell with an altered function but these mutations remove the ability of FLCN to interact with FNIP1 and FNIP2, which suggests that this interaction is functionally important. Recently, Baba and colleagues identified a novel folliculin-interacting protein, FNIP1, by co-immunoprecipitation studies in mammalian cells. They found that FNIP1 binds to 5'-AMP activated protein kinase, a negative regulator of mTOR suggesting that folliculin and its interacting partner may be involved in the AMPK and mTOR signalling pathways [10].

FLCN has an essential role in the regulation of Transforming Growth Factor-beta (TGF-β) signalling [6]. FLCN appears to regulate apoptosis through TGF-β-dependent transcription, which could help to further explain the renal cancer phenotype observed in BHD syndrome. In nut shell FLCN has been implicated in numerous signalling pathways including mTOR and AMPK signalling [14, 10, 15] Hypoxia Inducible Factor (HIF) signalling [3], TGF-β signalling [5, 6] and the JAK-STAT signalling pathway [16]. Mutation in *FLCN* gene increases activity of three other genes *HIF1A*, *HIF2A*, and *TFE3* and cause proliferation of cell, a typical causative of cancer. According to research in past a disulfide compound KC7F2 shown to inhibit the proliferation of cancer cells and decreases Hypoxia Inducible Factor-1 Alpha (HIF-1A) levels by down regulating HIF-1A protein synthesis [17].

From literature it was clear that there were two major consequences takes place because of mutated folliculin protein. First is hindrance in formation of FLCN, FNIP1 and FNIP2 complex which ultimately interacts with TGF-β protein of TGF-β signalling pathway and inactivates tumor suppression function of that pathway. The other major consequence is activation of HIF1A. From HIF-reporter cell-based screening studies of a natural product-like chemical library it is suggested that KC7F2 is an inhibitor of HIF1A [17]. Thus here we have focused on another inducible factor Hypoxia Inducible Factor-2 Alpha (HIF2A) interaction with KC7F2 to carry out in silico analysis. A previous study also suggests ability of TSA to reverse the biological and molecular defects of BHD cells but they have also suggested that further work will be needed to elucidate the nature of these effects. So we have carried out the work to study the nature of these effects and the mode of interaction between Trichostatin A (TSA) and TGF-β protein. We found that TSA binds with TGF-β protein at same site where the non-mutated folliculin protein was binding and thus the TGF-β protein works normally as a result, anti-proliferation will takes place via p15 protein and ultimately the tumor suppression will takes place. Understanding the structure as well as interaction will help in treatment. In this study, we investigated structure of FLCN, interacting proteins and potential ligands which may help in overcoming from the BHDS.

MATERIALS AND METHODS

Sequence retrieval

The sequences of FLCN and HIF2A were retrieved from one of the largest sequence repository NCBI with sequence ID NP_659434.2 and NP_001421.2, respectively.

Protein Structure modeling & Validation

Structure of TGF-β is obtained from the Protein Databank but structural search of FLCN and HIF2A proteins suggested that structures of both proteins were not present in Protein Databank (PDB). But we found the modeled structure of FLCN by using different template in Protein Model Portal. According our studies of Ramachandran plot of modeled folliculin protein by 2pziA we found that the ramachandran plot revealed 63.0% amino acids in favoured, 6.2% amino acids in allowed and 1.8% amino acids in generously allowed regions which can be improved by modeling the protein using other template.

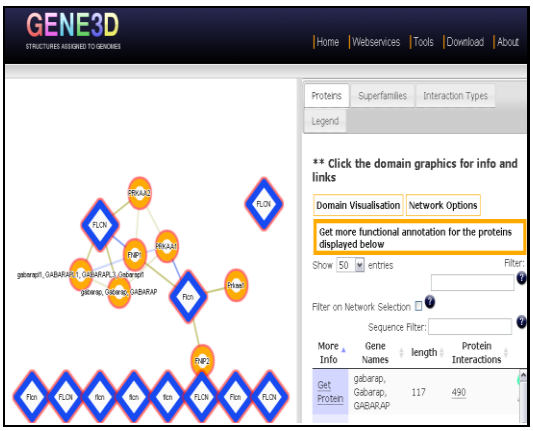


Figure 1: GENE3D representing GABARAP family and PRK family as interacting proteins of FLCN

Thus template based modeling approach using Swiss-PDB Viewer (SPDBV) was applied to model the protein structure of FLCN. 2p82 (Human cysteine protease ATG4A) template was used to model FLCN protein. Same template was used to model HIF2A as the sequence similarity was good between the HIF2A and 2p82. Further to check the quality of the modeled structure, structure verification server PROCHECK was utilized and analysis of molecular surface and binding cavities of different volume and area was done using SPDBV.

Ligand Finding

The ligand TSA for TGF-β is retrieved from Drug Bank with **DB04297** (EXPT03123). Other suggested ligand KC7F2 for HIF2A was produced by MarvinSketch by using the basic information about it like IUPAC name and chemical formula from the literature survey as it was not present in any drug databases. It satisfied majority of all the Lipinski's rule of 5.

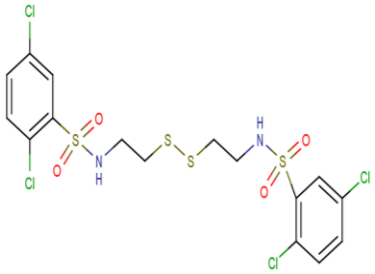


Figure 2: Produced structure of KC7F2 by using MarvinSketch

Interaction studies

After identification of binding cavities and the appropriate putative ligand, protein-protein interaction and ligand-receptor interactions was done by using PATCHDOCK and FireDock server.

RESULTS AND DISCUSSION

The structures of protein FLCN and HIF2A were modeled by template based modeling using SPDBV tool. With the use of PDB specific BLAST appropriate template structure was searched which resulted in 6 template structures with less than 35% similarity were obtained. These 6 structures were used as a template but the structure modeled with these templates failed in structure validation by ramachandran plot. After this, GENE3D gave information about the interacting protein partners of Folliculin. Analysis of interacting proteins of folliculin confirmed GABARAP family and PRK family as its interacting partners. Both the above protein structures showed high sequence similarity with Folliculin (Sequence similarity: 54%, Query coverage: 21%) and were used as a template to model the folliculin protein. But data of model validation represented that folliculin modeled using PRKAA1 (pdb id: 2H6D), had ramachandran plot is only 22.3% which represents a poor structure.

On the other hand by using GABARAP protein-cysteine protease ATG4A (pdb id: 2p82), modeling of folliculin show ramachandran plot which represents model of acceptable quality. Analysis of ramachandran plot revealed 88.4% in favoured, 8.4% in allowed and 2.0% in generously allowed regions which is also better than the modeled structure available in the protein model portal.

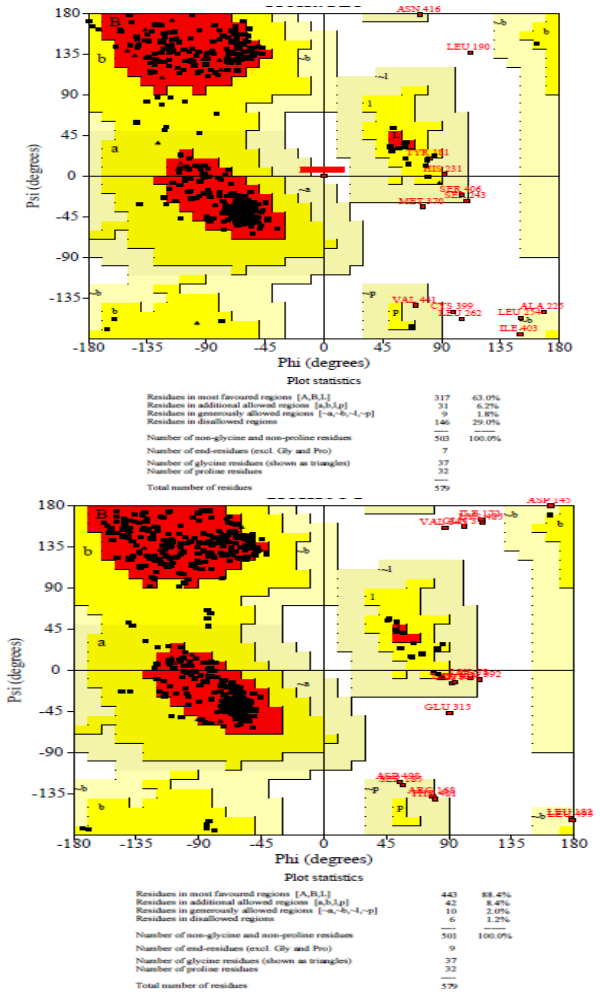


Figure 3: Comparison of Ramachandran plot results of 2p82 and 2pziA templates for folliculin isoform1

The modeled structure of folliculin (fig3) and HIF2A are deposited in international modeled protein structure repository named Protein model database (PMDB) with PMID: PM0078009 and PM0078067, respectively.

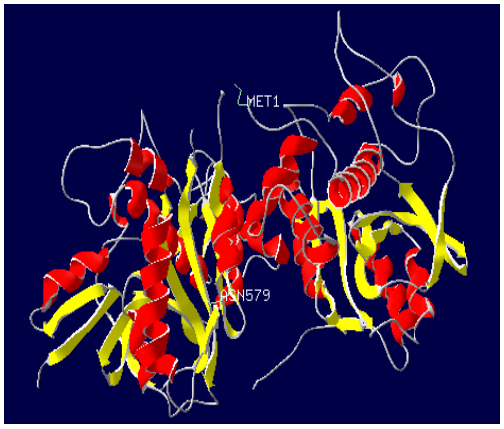


Figure 4: Modeled structure of folliculin isoform 1 using 2p82 template

The validation of modeled HIF2A protein through Ramachandran plot gave the following results i.e. 88.3% amino acids in favoured region, 8.9% amino acids in allowed region and 1.8% amino acids in generously allowed region.

The sole purpose of modeling the proteins were to study their interactions. The interactions are of TGF-β with FLCN and HIF2 with KC7F2, respectively. FLCN in its active form works as a tumor suppressor through TGF-β pathway but when FLCN is in inactive form because of the mutation, it hinders TGF-β pathway and its normal function of tumor suppression. Therefore we focused on protein-protein interactions study of FLCN with TGF-β protein have tried to propose a putative ligand that can bind TGF-β at same binding pocket of FLCN and thus can reinforce the normal function of TGF-β as tumor suppressor. Val441 of FLCN was interacted with ser73 of TGF-β and the bond length is 1.1A° between them. The global energy of this interaction is -63.72 kcal/mol generated by FireDock. We have found ligand TSA, which is HDAC inhibitor and an organic compound used as an appropriate ligand to activate the TGF-β signalling pathway. We have to treat TGF-β protein with TSA to make the TGF-β signalling pathway normal functioning and thus it mediate tumor suppressor.

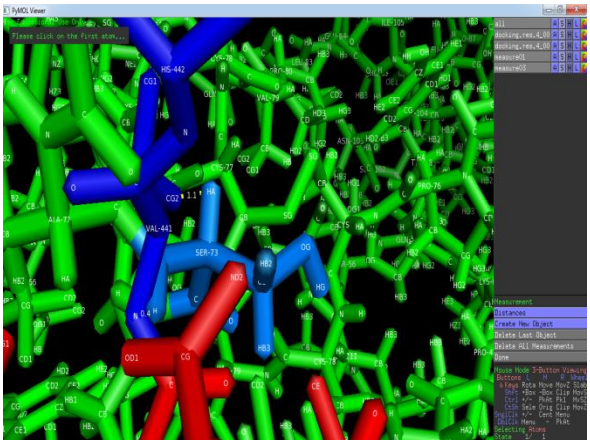
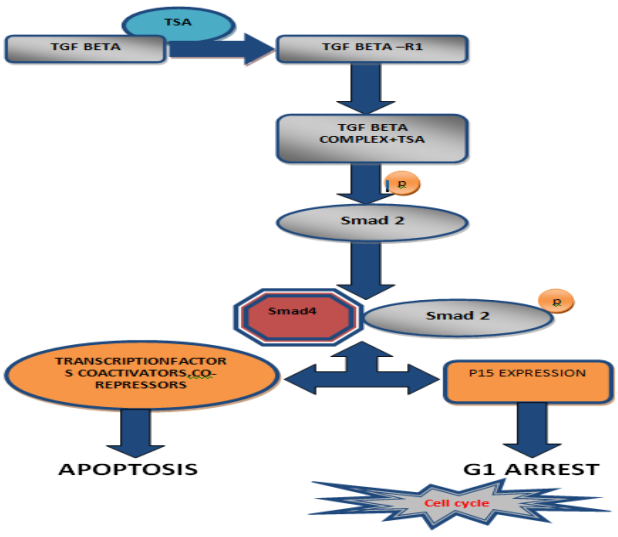


Figure 5: screenshot of FLCN and TGF-β interaction

TGF-β signalling pathway

TSA binds with TGF-β protein and then this will further binds with TGFβR1. Now this complex will bind with smad2 and it phosphorylates smad2 by doing this the activity of p15 became active and the level of Bim proteins were increased. So, that the work of anti proliferation and pro apoptosis is done by p15 and Bim protein, respectively.



Proposed-Pathway: TGF-β signaling pathway treating with TSA

Ser73 of TGF-β is interacting with TSA. The bond length between them is 0.4A°. The global energy of this interaction is -44.70kcal/mol. TSA bound on the same amino acid where FLCN binds with TGFβ. From this analysis, TSA can be considered as a drug to treat the BHDS syndrome.

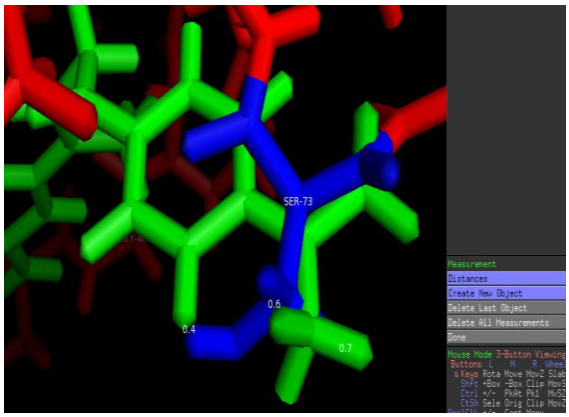


Figure 6: Screenshot of interaction between TGF-β protein and TSA. The ligand (TSA) is in green color and the protein which was bound with ligand is in blue color

According to the natural function of FLCN it inhibits the activity of HIF2A protein. But because of mutation, FLCN became inactive and this protein became activated which is responsible for uncontrolled cell proliferation. Therefore it is required to inhibit this protein, as blocking of this protein will control the activity of cell proliferation. KC7F2 is a chemical drug which is used to block the hypoxia response (low oxygen tension). So, we have tried to use as a ligand to block the HIF2A protein.

Interaction of HIF2A with KC7F2 was performed (fig7). The interaction shows that Lys503 of HIF2A was interacted with KC7F2 with 0.4A° bond length. And the global energy produced by FireDock result of this interaction is -60.69kcal/mol.

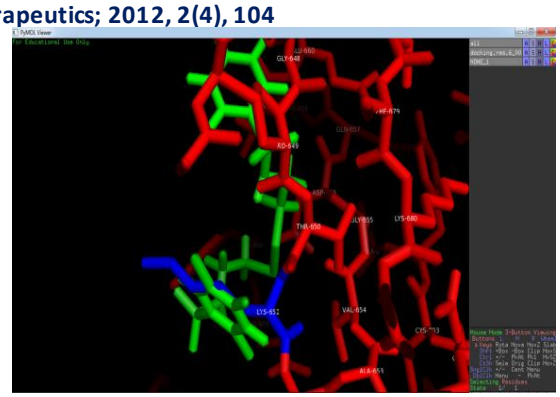


Figure 7: Screenshot of interaction between HIF2A-KC7F2 done by PATCHDOCK. Amino acid with blue color is interacting amino acid of HIF2A with KC7F2 ligand

CONCLUSION

From the present studies we can conclude that the function of folliculin protein is to suppress formation of tumor by different pathways. From pathway analysis, different reactions were studied to block the proliferation of the cells which form tumors. By providing the external drug like TSA, the process of phosphorylation of SMAD2 is done. It activates Bim protein and p15 protein which work as pro-apoptotic and anti- proliferative, respectively. HIF2A is blocked by KC7F2 treatment. So by doing this, we may cure the disease. Further web lab analysis of this can be done for better understanding of the functioning of the suggested treatments.

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