Key role of Dkk3 protein in inhibition of cancer cell proliferation: an in silico identification

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Abstract

Dkk3 is a member of Dkk family proteins, regulating Wnt signaling. Dkk3 plays different roles in human and mouse tumors. Dkk3 predominantly act as a tumor suppressor, however several reports revealed that Dkk3 could accelerate cancer cell proliferation. Herein, we aimed at launching an in silico study to determine Dkk3 structure and its interactions with Kremen and LRP as Wnt signaling receptors as well as EGF receptor. Using various softwares a model was built for Dkk3 molecule. Different protein modeling approaches along with model refinement processes were employed to arrive at the final model. To achieve the final complex of Dkk3 with Kremen, LRP and EGFR molecules protein-protein docking servers were employed. Model assessment softwares indicated the high quality of the finally refined Dkk3 3D structure, indicating the accuracy of modeling and refinement process. Our results revealed that Dkk3 is capable of interacting with Kremen, LRP and EGFR with comparable binding energies. Dkk3 efficiently interacts with LRP, Kremen and EGF receptor and may be a promising protein in cancer therapy by blocking Wnt and EGFR downstream signaling.

Keywords: Bioinformatics; Wnt signaling; Dkk; EGFR; Cancer

1. Introduction

Wnt signaling pathway is involved in a wide range of physiological and pathological processes such as cell fate determination, cell migration and tumorigenesis (Giles et al., 2003). The extracellular signal of Wnt agonists and antagonists stimulate several intracellular cascades including canonical and non-canonical pathways (Kawano and Kypta, 2003). The canonical pathway is started by accumulation and translocation of β -catenin into the nucleus, which in turn is activated by binding of Wnt agonist and antagonist proteins to their receptors such as Frizzles, LRP5/6 and Kremen. In the context of extracellular matrix, a number of secreted proteins including Dkk, Wnt inhibitor protein and Cerebrus regulate the Wnt pathway and prevent Wnt interaction with its receptors (Luu et al., 2004).

One of the main Wnt pathway regulators is Dkk family proteins that compose of Dkk1-4 and Soggy. This family is a secreted glycoprotein family that is involved in a wide range of disease including cancers, retinal degradation and cerebral ischemia (Niehrs, 2006). Dkk3 is the last characterized member of the Dkk family that plays pivotal roles in regulating Wnt pathway and determining cell fate during embryonic development (Mizobuchi et al., 2008).

Dkk3 protein interacts with Kremen 1 and 2 receptor proteins. The interaction takes place in a membrane vesicle of the cells. The Kremen protein composed three extracellular domains containing kringle domain, CUB domain and WSC domain. Generally, Kremen receptor acts as a negative regulator of Wnt/ β -catenin (Nakamura and Hackam, 2010).

Reducing and/or silencing of Dkk3 has been reported in various cancers including lung cancer, gastric cancer, ovarian cancer, acute lymphoblastic leukemia, prostate cancer and bladder cancer (Chim et al., 2008; Kawauchi et al., 2012; Kurose et al., 2004; Ueno et al., 2011; Veeck et al.,

2008; Yue et al., 2008). In contrast, some researches showed that Dkk3 potentiates Wnt signaling and diminish cell death and apoptosis in normal cells as well as hydrogen peroxidase and chemotherapy exposed cells (Katase et al., 2008; Mühlmann et al., 2010; Nakamura et al., 2007). Contemplating controversial data on Dkk3s functions and structure, we exploited bioinformatics tools to model and predict structure of Dkk3 protein explaining its various interaction with Kremen, LRP and EGFR proteins in the cell surface.

2. Methods

2.1. Study design

The study of Dkk3 molecule requires the determination of its 3D structure, to be docked on the receptor molecules. The model should be built employing various methods and assessed by quality scores to choose the best model. Selecting a qualified model would ensure the reliability of the following analysis. Since even the best selected models are spurious, there should be a refinement step to achieve a more native like model. Loop refinement, full atomic refinement and molecular dynamics are among the possible model refinements which could be helpful to rectify the accompanying errors within the selected model. Ultimately, molecular docking of the Dkk3 3D model with its receptors would clarify the orientation of protein-protein interactions. The steps, should be taken to achieve the final complexes, are depicted in the Figure 1.

2.2. Sequences and homology analyses

UniProt (Universal Protein Resource) knowledgebase (<http://www.uniprot.org/>) was employed to obtain protein sequence for Dkk3 protein. NCBI protein BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to gain a suitable template structure for homology modeling predictions. The BLAST was done against Protein Data Bank proteins while the search was restricted for Homo sapiens only and all other parameters was set as default.

2.3. Protein modelling

Since there was not a suitable template for Dkk3 homology modelling, we used both fold recognition and ab initio modeling approaches for model construction. I-TASSER server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) was used for the Dkk3 structure prediction. This server, ranked as the No 1 server for protein structure prediction in recent CASP7, CASP8, CASP9, and CASP10 experiments, builds its 3D models based on multiple-threading alignments by LOMETS and iterative template fragment assembly simulations (Roy et al., 2010; Yang et al., 2015; Zhang, 2008). Robetta at <http://robetta.bakerlab.org/>, predicting protein domain structures based on both ab initio and comparative modeling approaches, was the other software employed for 3D structure prediction. Rosetta de novo protocol was used to model domains without a detectable PDB homolog while locally installed versions of HHSEARCH/HHpred, RaptorX, and Sparks-X were used to build comparative models.

2.4. Model quality assessment

As a vital step in protein structure prediction process obtained models was fed as input files into QMEAN model quality assessment server (<http://swissmodel.expasy.org/qmean/cgi/index.cgi>) (Benkert et al., 2009). QMEAN provides

access to a composite scoring function capable of deriving both global and local error estimates on the basis of one single model. Moreover packing quality of the model was assessed by the atomic empirical mean force potential ANOLEA (Melo and Feytmans, 1997) (<http://swissmodel.expasy.org/>) along with its quality evaluation by QMEAN quality plot. TM-align server (<http://zhanglab.ccmb.med.umich.edu/TM-align/>) was employed to superimpose the predicted structures. The superimposition would reveal if they are structurally similar.

2.5. Model refinement and molecular dynamics analyses

To rectify modeling errors, model refinement process was executed on selected best model (the model built by Robetta server). Three loops spanning 1-10, 1-20 and 135-150 regions of the model indicating high residue error peaks (according to QMEAN residue error plot) were remodeled using UCSF Chimera, production version 1.9 software and ModLoop server (<http://modbase.compbio.ucsf.edu/modloop/>) (Fiser et al., 2000). Resulted modified model was further refined by two consecutive runs of 3Drefine server (<http://sysbio.rnet.missouri.edu/3Drefine/>) (Bhattacharya and Cheng, 2013). This server modifies protein structures by optimizing hydrogen-bonding network and atomic-level energy minimization. To further refine the model, molecular dynamics run was conducted on refined model using CHARMMING server (<http://charmming.org/>) (Miller et al., 2008). The molecular dynamics run was set to 1000 steps, starting temperature of 210.15, final temperature of 310.15, temperature increment of 10, steps between temperature increments of 100 and bath temperature of 310.15. The structure was minimized and solvated by the CHARMMING server beforehand. Stereochemical quality of the predicted models were assessed using Procheck software (<http://swissmodel.expasy.org/>) (Laskowski et al., 1993) to evaluate geometry of the

residues in a given protein structure. Moreover the model quality was reassessed by QMEAN server.

2.6. Protein-protein interaction study

2.6.1 Shape complementarity principle based docking

Three docking servers listed in international protein–protein docking experiment, Critical assessment of Predicted Interactions (CAPRI) were employed to predict the best interaction of the Dkk3 model with LRP5/6, EGFR and Kremen structures. The 3D structures of LRP5/6, EGFR were obtained from PDB server (<http://www.rcsb.org/pdb/home/home.do>) while Kremen molecule was modeled in our lab (MOHAMMADPOUR et al., 2015). Cluspro server (<http://cluspro.bu.edu/>) (Kozakov et al., 2006) uses an automated rigid-body docking and discrimination algorithm to filter docked conformations with good surface complementarity and rank them based on their clustering properties. ZDOCK at (<http://zdock.umassmed.edu/>) (Pierce et al., 2014) aiming at finding efficient global docking search on a 3D grid, utilizes a Fast Fourier Transform algorithm while the scoring is done based on a combination of shape complementarity, electrostatics and statistical potential terms.

2.6.2. Interaction refinement

FireDock (<http://bioinfo3d.cs.tau.ac.il/FireDock/>) (Mashiach et al., 2008) is a fast flexible induced-fit backbone and side chain refinement server utilizing a coarse refinement method to optimize the interaction in molecular docking studies. The initially docked protein complex files were subjected to restricted side-chain optimization followed by soft rigid-body minimization using FireDock.

2.6.3. Identifying low-energy conformations

As the last step of protein interaction prediction FireDock, results were subjected to rigid-body orientation and side-chain conformation optimization using RosettaDock server (<http://rosettadock.graylab.jhu.edu/>) (Lyskov and Gray, 2008).

3. Results

3.1 Sequence and homology analyses

The Dickkopf-related protein 3 under UniProtKB ID Q9UBP4 was retrieved for in silico analyses. Its 350 amino acids in length, containing a C-terminal cysteine-rich domain. The protein contains a 21 amino acid signal peptide which would be further processed into a mature form. The BLAST search against PDB using this sequence as query returns similar sequences best of which have 21% query coverage and 32% sequence identity.

3.2. Obtaining protein 3D model

Both I-TASSER and Robetta successfully modeled the Dkk3 protein 3D structure. Both servers provide 5 top predicted models based on their scoring algorithms. Table 1 listed the quality assessment z-scores calculated for the best models predicted by QMEAN server. As obtained data clearly implied, Robetta server predicted a model with highest quality. The following analysis would be performed on the model built by this server. Moreover, superimposition results indicates that getting TM score of 0.267 and RMSD of 6.42 Å, two predicted structures have random structural similarity.

3.3. Model refinement

Remodeling of the three loops spanning 1-20, 1-10 and 135-150 regions on the best model resulted in resolving two major residue error peaks. Loop modeling together with refinements

performed by 3Drefine server improved the quality z-core for QMEAN server (Table 1). The molecular dynamics analyses, performed on the refined model resulted in a final model (Figure 2). Figure 3 shows ANOLEA results for finally refined model, indicating the most of the amino acids are in their favorable energy environment with acceptable QMEAN scores. The quality score of the final model is as -0.32 for Procheck total G-factor. Figure 4 is the Ramachandran plot for the finally achieved structure, revealing more than 90% of residues are in the allowed regions.

3.4. Protein-protein interaction results

Feeding the results of each docking step to the following one resulted in three finally refined complexes with lowest binding energies and highest docking scores for each pair of docked structures. As illustrated in Figure 5 initial docking using Zdock and Cluspro and refinement by Firdock and Rosettadock results in complexes with different possible orientations of Dkk3 with EGFR, LRP and Kremen interaction. Figure 6 is illustrating the positions of EGF (under PDB ID: 1NQL) and Dkk3 (modeled in the present study) molecules in interaction with EGFR molecule. The predicted structure indicates that both Dkk3 and EGF molecules are in interaction with N-terminal domain of the EGFR molecule. Table 2 listed the Rossie total scores for the best structures of each docked pair with lowest energy.

4. Discussion

Costly and time consuming nature of structural studies is an ongoing hurdle to scrutinize functional features of biological molecules and complexes. In silico studies are among credible alternatives for conventional structural and immunological studies (Jahangiri et al., 2012; Khalili et al., 2015; Khalili et al., 2014; Sefid et al., 2013), circumventing inevitable tough challenges lie

1 ahead of experimental methods. Moreover these methods could help avoiding ethical concerns
2 associated with in vivo studies. In the present study we harnessed bioinformatics tools to predict
3 the 3D structure of Dkk3 molecule and its interaction with EGFR, LRP and Kremen molecules
4 upon complexation.

5 Sharing less than 30% identity makes the homology prediction more likely to fail accurate
6 modeling, affected by alignment errors (Kopp and Schwede, 2004). Therefore, according to
7 BLAST search results and due to lack of any reliable template for Dkk3 molecule to be modeled
8 by homology modelling approach, we employed fold recognition and ab initio approaches to
9 model the Dkk3 molecule. Significantly more appropriate Z-scores ascribed for model built by
10 Robetta convince us to choose its result as the best modeled structure. The high quality of the
11 predicted model was buttressed by ANOLA plot, indicating favorable energy environment for
12 most amino acids of the predicted structure. To bolster the obtained model into a more qualified
13 model with more native like coordinates different quality refinements were performed on the
14 model. Accurate loop modeling, hitting existing high residue error picks according to QMEAN
15 energy profile, could be a major factor improving model quality. Moreover a full-atomic
16 refinement simulation would determine the global topology and local details of the predicted
17 model. Molecular dynamics analysis provides an opportunity for the atoms to interact for a
18 period of time. This would help the predicted structure to get a more accurate coordinates. Since
19 a good quality model would be expected to have over 90% of its residues in the most favored
20 regions of Ramachandran plot, our refinement approach seems to bring the finally achieved
21 model into the category of high quality structures. While existing wide sequence-structure gap
22 reflects that experimental methods are still costly and time consuming to resolve biological
23 structures and their correct orientation in protein complexes (Persson and Argos, 1996), protein

docking would be a proper alternative to find out the correct association of the interacting molecules upon complexation. Protein-protein complexes play pivotal roles in a wide range of key biological processes such as cell signaling, enzyme inhibition and immune recognition. Coming to grips with atomic details of residue-residue contacts within protein-protein interactions, would elucidate the intricacies of their binding and hopefully would bring about a better understanding of their roles in biological pathways. Despite the ongoing attempts to solve the problem of predicting protein-protein interactions, they could still be spurious. This problem would be more daunting in the case of automated docking without any further information about the interacting amino acids or even interacting faces of the proteins. Although the surface complementarity is one of the essential components of any protein-protein interactions, the production of too many possible rotations and docking positions is a limiting factor faced in this approach. However, this problem could be addressed by including other terms like hydrogen bonding, electrostatic energy, solvation or hydrophobicity. In this regard, the employed docking servers utilize a combinatorial approach to find the best interacting faces. Z-dock server uses a combination of shape complementarity, electrostatics and statistical potential terms for scoring (Pierce et al., 2014), while ClusPro follows a three step processing including: (1) rigid-body docking using the FFT correlation approach; (2) root mean square deviation (RMSD)-based clustering of the structures, generated to find the largest clusters that will represent the most likely models of the complex; and (3) refinement of selected structures (Kozakov et al., 2013). Our results apparently indicate that Dkk3 interacts with EGFR and Kremen molecules with comparable scores, while the interaction with LRP molecule gets a significantly higher score. This high score could be due to lower ΔG of Dkk3/LRP interactions and far bigger nature of LRP molecule in comparison to EGFR and Kremen molecules, involving far more atoms. It's

1 noteworthy that the orientation in which Dkk3 interacts with EGFR molecule completely
2 occupies the EGF binding domain of the EGFR molecule. Both EGF and Dkk3 molecules are in
3 tight interaction with the N terminal domain of the EGFR molecule. This fact could be
4 imperative regarding the possibility that the Dkk3 molecule could actually inhibit EGFR/EGF
5 interaction. These results suggested that, through Wnt and EFGR signaling interruption, Dkk3
6 molecule could plays a crucial role for inhibiting cancer proliferation.

7 Based on previous reports, Dkk3 regulates the Wnt pathway depending on the cell type. Some
8 studies reported that Dkk3 inhibits Wnt signaling through Kremen receptors and induces cell
9 death and apoptosis following endocytosis of Dkk3/Kremen complex (Wang et al., 2008).
10 Controversially, other reports showed that cell proliferation and survival were increased by
11 Dkk3. Different models are proposed by Nakamura et al to justify the functions of Dkk3. The
12 first model states that Kremen receptors are inhibitors of the Wnt pathway and binding of Dkk3
13 to Kremen receptors would led to a reduction in surface expression of Kremen receptors. The
14 second model proposed that Dkk3 competes with Kremen receptors and blocks their ability to
15 prevent the Wnt pathway. These models suggested that Dkk3 is an activator of Wnt/ β -catenin
16 signaling (Nakamura and Hackam, 2010). In contrast, recent data demonstrated that Dkk3
17 strongly inhibits the cancer cell proliferation and migration as well as induces the apoptosis
18 through prevention of the β -catenin accumulation (Romero and Kypta, 2013). Moreover, many
19 researchers have been shown extensive collaboration between EGFR and Wnt signaling manners
20 in malignancies to maintain the neoplastic properties (Paul et al., 2013).

21 Herein, based on bioinformatics findings, we proposed the following model regarding the
22 functional roles of the Dkk3 molecule. Generally, Dkk3 interactions with Kremen and LRP
23 proteins determine the cell fate. Dkk3 could interact with either or both of the kringle and CUB

domains of the Kremen receptor protein (Mohammadpour et al., 2015). CUB domain plays distinct roles in proteins regulation development and cancer invasion. We hypothesized that the interaction of Dkk3 with just CUB domain or down regulation of Dkk3 would led to actuation of TCF-4 reporter activity, C-myc and Cyclin D1 expression (Uekita and Sakai, 2011). In corroboration with our hypothesis, a recent investigation showed β -catenin promotes cancer cells proliferation using cyclin D1(Jang et al., 2012). Interaction of Dkk3 with CUB domain may potentiate the Wnt pathway and could facilitate the cell invasion and migration. However, the interaction of Dkk3 with kringle domain or both of the kringle and CUB domains could prevent Wnt signaling through β -catenin nuclear translocation. This hypothesis is supported by many studies, reporting distinct roles played by kringle domain to induce cell death, apoptosis and anti-angiogenic properties (Kim et al., 2012). Moreover, complete interaction of Dkk3 with Kremen would led to apoptosis process through activation of caspase 3 and 9. Furthermore, Dkk3 induces apoptosis using C-JUK phosphorylation and endoplasmic reticulum stress depending manner (Veeck and Dahl, 2012).

On the other hands, some investigations demonstrated that Dkk3 is an immunomodulator that can suppress T cell proliferation and cytokine production. Based on these data, different functions of Dkk3 in various cancer cell lines and tumors are depend on the ability of tumor cells to induce immune response. In low immunogenicity, interaction of Dkk3 with Kremen and other receptors led to inhibition of Wnt signaling and suppresses the cell proliferation and survival. But in high immunogenicity, Dkk3 disrupts the balance of immune responses and suppresses T cell proliferation which would head to cancer cell proliferation and faster tumor growth, despite the inhibition of Wnt signaling in cancer cells (Lu, 2014; Papatriantafyllou et al., 2012). Furthermore, our data revealed that Dkk3 has occupied EGFR binding domain with a similar

orientation observed in EGF/EGFR complex. Thus, Dkk3 could functionally inhibits the interaction between EGF and EGFR which in turn would blocks the EGFR signaling downstream. EGFR signaling plays crucial roles in progression of variety of cancers through crosstalk between EGFR, Wnt and TGF- β signaling pathways. EGFR trans-activated by Wnt in diverse tumors. Also, it has been reported that EGFR activation results in induction of β -catenin signaling in some cancers which EGFR acts as an important regulator of cancer cell proliferation and progression (Paul et al., 2013). Regarding the aforementioned data, Dkk3 may functionally inhibited EGFR signaling and cell proliferation.

In conclusion, we extensively proposed that Dkk3 inhibits cancer cells proliferation and migration through the blocking of Wnt and EGFR signaling pathway.

Conflict of interest: no conflict of interests declared

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1 **Legends of figure**

2 **Figure 1.** The workflow chart. The order in which the necessary steps for the final complex
3 formation are depicted.

4 **Figure 2.** 3D structure of finally refined Dkk3 molecule. Residues are colored from blue to red
5 in the order of increasing residue energy, the red regions correspond to problematic or erroneous
6 parts of the structures.

7 **Figure 3.** ANOLEA and QMEAN model quality estimation plots. Negative ANOLEA values
8 represents favorable energy environment for a given amino acid. Small QMEAN values
9 correspond to regions in the model being potentially more reliable.

10 **Figure 4.** Ramachandran plot. The plot indicates that only 0.4% of residues are in disallowed
11 regions.

12 **Figure 5.** 3D structure of docked complexes. Upper row belongs to the prediction results
13 primarily docked by Cluspro while the lower row belongs to prediction results primarily docked
14 by Zdock. From left to right complexes are EGFR/Dkk3, LRP/Dkk3 and Kremen/Dkk3.
15 Residues are colored from blue to red in the order of increasing residue energy, the red regions
16 correspond to problematic or erroneous parts of the structures.

17 **Figure 6.** The complexes of EGFR with EGF (left) and Dkk3 (right) molecules. In both
18 complexes the EGF and Dkk3 molecules are in green, the N-terminal domain of EGFR molecule
19 is in blue and the rest of EGFR molecule is in Red.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Table 1: Quality assessment scores. Model quality scores of modeled structures along with quality scores of refined model are listed.

Quality assessment	QMEAN Zscore
ITASSER best model	-5.46
Robetta best model	-3.57
Refined Robetta model	-3.19

Table 1: Rossie total scores for both Dkk3/EGFR and Dkk3/LRP coplexes.

	Dkk3/EGFR	Dkk3/LRP	Dkk3/Kremen
Initial docking by			
Cluspro	-645.893	-1399.164	-579.237
Initial docking by			
Zdock	-467.543	-1331.438	-578.669