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Effect of Activation Loop Phosphorylation on Lemur Tyrosine Kinase 3 (LMTK3) activity: A Molecular Dynamics Simulation Study

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ABSTRACT: Protein kinases catalyze the phosphorylation reaction, and they themselves become catalytically activated through phosphorylation of their activation loop. LMTK3 is an oncogenic kinase, reported in various types of cancer. Recent study highlights LMTK3 phosphorylation by CDK5 results in breast cancer tumourogenesis. We determined the probable activation loop in LMTK3 and carried out *in silico* phosphorylation at probable phosphorylation site (Thr189) in activation loop and studied the effects of phosphorylation on conformational dynamics. We substituted Glu for phosphorylated Thr189 and noticed Glu does not mimic the effect of phosphorylation. From Molecular dynamics analysis, phosphorylated, unphosphorylated and mutated LMTK3 found to be stable. But phosphorylated loop region shows much fluctuation. Thereby ATP binding mode was observed to be different in phosphorylated as compared to unphosphorylated LMTK3. Phosphorylation mediated conformational change in the ATP binding site of LMTK3 may facilitate phosphoryl transfer reaction to its substrates, and may leads to breast cancer progression.

Keywords: Breast cancer tumourogenesis; Phosphorylation sites; Molecular docking; Activation segment; Conformational dynamics.

INTRODUCTION

Eukaryotic protein kinases are the largest gene family that regulates several important cellular processes, such as cell growth and differentiation (Ban *et. al.,* 2011; Waldrop, 2014). Protein kinases share a conserved core consisting of two lobes, the N-terminal (small N-lobe) and C-terminal (large C-lobe). These two lobes form a deep pocket that accommodates an ATP molecule (Kornev *et. al.,* 2010). The N-lobe consists of five β-strands and an α-helix (called αC-helix). The C-lobe contains α-helices and includes the activation segment which is 20-35 residues stretch located between a conserved DFG motif and APE motif that is conformationaly very flexible and its conformation can influence both substrate binding and catalytic efficiency (Huse *et. al.,* 2002; Nolen *et. al.,* 2004).

Phosphorylation is the commonest posttranslational modification of proteins in eukaryotic cells (Olsen *et. al.,* 2013). Eukaryotic protein kinases (EPKs) are the family of enzymes that catalyse the phosphorylation reaction, and themselves regulated by phosphorylation (Beltrao *et. al.,* 2012; Nolen *et. al.,* 2004). The regulatory phosphorylation event occurs in most EPKs at activation loop of kinases, which is part of the activation segment (Beltrao *et. al.,* 2012; Nolen *et. al.,* 2004). Phosphorylation of the activation loop is a key mechanism that induces a dynamic changes in activation process (Kornev *et. al.,* 2006; Kornev *et. al.,* 2015; Meharena *et. al.,* 2013; Taylor *et. al.,* 2012), and leads to structural changes which stabilize the active conformation. This mechanism catalyses the transfer of γ-phosphate of an ATP molecule to the phosphoacceptor site of substrate (Beltrao *et. al.,* 2012; Kornev *et. al.,* 2015; Johnson *et. al.,* 1996). Therefore activation-loop phosphorylation is crucial because it is required for the interconversion from an inactive to an active conformation of kinase. Generally the protein kinase domain is a structurally conserved protein domain containing the catalytic function of all protein kinases (Hanks *et. al.,* 1991; Hanks *et. al.,* 1995; Scheeff *et. al.,* 2005).

Lemur tyrosine kinase-3 (LMTK3) is an oncogenic Serine Threonine Tyrosine kinase implicated in various types of cancer including breast (Giamas *et. al.,* 2011) lungs (Xu *et. al.,* 2014) and colorectal (Shi *et. al.,* 2014).

Recently LMTK3 has been reported to regulate ERα through phosphorylation with a significant role in endocrine resistance (Giamas *et al* 2011; Sebbing *et. al.,* 2012; Stebbing *et. al.*,2013). In triple-negative breast cancer, LMTK3 elevation in cytoplasm, promotes breast cancer cell motility, migration and invasion through transcriptional activation of intigrins (Xu *et. al.,* 2014). In addition, LMTK3 also been seen to promote cancer progression through chromatin remodelling (Xu *et. al.,* 2015). It is obvious from these studies that LMTK3 is a new therapeutic target in breast cancer therapeutics (Xu *et. al.,* 2014).

Recently one of the *in vitro* studies confirmed the ability of CDK5 to phosphorylate LMTK3 and results in breast cancer tumourogenesis (Lucchiari *et. al.*,2016). CDK5 is a cytoplasmic proline-directed serine/threonine kinase which is expressed in many solid tumours (Keaton, 2017). But phosphorylation of LMTK3 in detail at molecular level has not been studied yet. In our computational study, we modelled 3D structure of LMTK3 using I-TASSER (Iterative Threading Assembly Refinement) (Roy *et. al.,* 2010) as the crystallographic structure of LMTK3 is not available.Then we predicted the activation segment in LMTK3 by comparing the LMTK3 structure with well-known kinases (PKA, Pdb id- 1ATP; ERK2, Pdb id-2ERK) and based on conserved motifs (DFG and APE) (Huse *et. al.,* 2002; Nolen *et. al.,* 2004) activation segment was predicted. It has been reported that phosphorylation of these kinases in activation loop at threonine regulate the kinase activity (Adams, 2003; Cheng *et. al.,* 2006; Steichen *et. al.,* 2012; Canagarajah *et. al.,* 1997). So with this comparative study we predicted, threonine 189 (Thr-189) to be the phosphorylation site in LMTK3 which is present near to the APF motif in activation segment of LMTK3. To cross check our predicted phosphorylating site (Thr-189), we used NetPhos3.1 (Blom *et. al.,* 2004) server where it shows, the Thr-189 to be a probable phosphorylation site on LMTK3. Thereby in this study we carried out *in silico* phosphorylation of LMTK3 in activation loop at Thr-189 and studied its effect on conformational dynamics upon phosphorylation and also studied ATP binding mechanism. MD simulation study demonstrated that, phosphorylation stabilize the structure of LMTK3 but facilitate significant conformational change in phosphorylated activation segment and also affect ATP binding site of LMTK3.

Proteins that require phosphorylation for its activation, there are many examples of constitutively active mutantin which the phosphorylatable residue is substituted with either aspartate or glutamate (Charbon *et. al.,* 2004; Kassenbrock *et. al.,* 2004; Huang *et. al.,* 1994) Klose *et. al.,* 1993; McCabe *et. al.,* 2000). We substituted glutamic acid (Glu) for phosphorylated Thr-189, and predicted that Glu does not mimic the effect of phosphorylation. We noticed mutated LMTK3 has no conformational change in the activation loop thus the mutated LMTK3 is more or less similar to that of unphosphorylated LMTK3. From molecular docking study we noticed that due to phosphorylation mediated conformational change in ATP binding site of LMTK3, the binding mode of ATP to be different in phosphorylated LMTK3 as compared to unphosphorylated LMTK3. The conformational change in ATP binding site may facilitate to catalyze the phosphoryl transfer reaction in LMTK3. Overall from our computational study we infer that the phosphorylation leads to localized conformational changes in LMTK3 and affects the binding mechanism of ATP. Our idea from this study can be used to create hypotheses about mechanisms of regulation by phosphorylation that can be tested further experimentally.

RESEARCH METHOD

A. Prediction of Activation segment and phosphorylation site in LMTK3: A comparative study with other kinases (PKA and ERK2)

We modelled the 3D structure of LMTK3 domain from I-TASSER. Then the equilibrated structure of LMTK3 was superimposed with X-ray crystallographic structure of PKA (PDB ID: 1ATA) and ERK2 (PDB ID: 2ERK) to determine the probable activation segment and phosphorylation site in LMTK3.

B. In silico Phosphorylation of LMTK3 at Thr-189 and substitution of Glutamic acid (Glu) for the phosphorylated Thr-189 in the activation segment of LMTK3

Using xleap module of AMBER12 we added phosphate PO_4^{3-} group to the equilibrated structure of LMTK3 at Thr-189 of C-β atom by removing hydrogen (H) atom from hydroxyl group (OH). In addition wealso substituted Glu for the phosphorylated Thr-189 using Chimera in order to check whether Glu mimic the effect of phosphorylation or not, as Glu is a negatively charged amino acid.

C. Molecular Dynamics (MD) simulation of Phosphorylated, Unphosphorylated and Mutated LMTK3

LMTK3 structures were subjected to MD simulation. We used the Particle Mesh Ewald Molecular Dynamics (PMEMD) (Joseph *et. al.,* 2013) module of AMBER12 software package (Case *et. al.,* 2012).

AMBER ff99SB force field (Honark *et. al.,* 2006) protein parameters were used on the LMTK3 structures. Using implicit solvation method the corresponding topology and co-ordinate files were prepared for the modelled structures of phosphorylated, unphosphorylated and mutated LMTK3 domain. The resultant structures were then subjected to energy minimization by using 500 steps of steepest descent and another 500 steps of conjugate gradient. During energy minimization, we did not fix any restraint to hold the protein system. In the course of MD simulation, the systems were gradually heated from 0 to 300 K for slow relaxation of the built initial structure. In addition shake constraints (Ryckaert *et. al.,* 1997) using a geometrical tolerance of 5 x 10^{-4}Å were imposed on all the covalent bonds involving hydrogen atoms. Subsequently MD was performed under constant pressure-temperature conditions (NPT) with temperature regulation achieved using Berendsen weak coupling method (Berendsen *et. al.,* 1984) (0.5 ps time constant for heat bath coupling and 0.2 ps pressure relaxation time). This was followed by another 200 ps of equilibration step. After equilibration of the system with constant temperature and pressure, the production MD runs were carried out for 30 ns in order to do the analysis of structure and properties.

D. Molecular Docking with ATP

We utilized AutoDock4.2 (Morries *et. al.,* 2009) for our molecular docking study. This provides valuable insight into the interaction of ATP with phosphorylated and unphosphorylated LMTK3. For docking we used last LMTK3 trajectory files for docking. AutoDock program with the Lamarckian genetic algorithms (LGA) was used to perform docking experiments. The Lamarckian GA parameters used in the analysis consist of 10 independent runs. Docking was carried out with the grid size of 40, 40, and 40 along the X, Y, and Z-axis with 0.481 Å spacing.

E. Trajectory analysis

Trajectory files from MD simulations were analyzed using cpptraj (Daniel *et. al.,* 2013) module of AMBER12 package. Quality assurance such as Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF) and Radius of gyration (Rg), were performed. All the graphs were generated using xmgrace plotting tool. For inspecting the 3D structure of the molecule, we used UCSF Chimera (Pettersen *et. al.,* 2004), and VMD (Humphery *et. al.,* 1996).

RESULTS AND ANALYSIS

A. Activation segment and phosphorylation site prediction

By comparing the LMTK3 structure with ERK2 and PKA protein kinase, we predicted the probable activation segment and phosphorylation site in LMTK3. From Fig. 1 and Fig. 2 we see LMTK3 has an APE motif as that of ERK2 and PKA. There by with the visual inspection we predicted that the Tyr164 to Ser196 region to be the activation segment (shown in navy blue colour) in LMTK3, which may be important for catalytic activation of LMTK3.Within the probable activation segment we assumed Thr-189 to be the phosphorylation site. We cross checked our predicted phosphorylation site using NetPhos3.1 server, and we observed that Thr-189 to be the probable phosphorylation site in LMTK3.

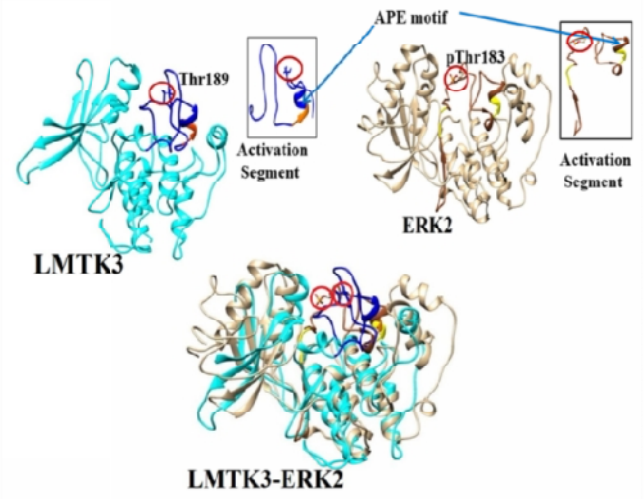

Fig. 1. Superimposed structure of LMTK3 with ERK2.

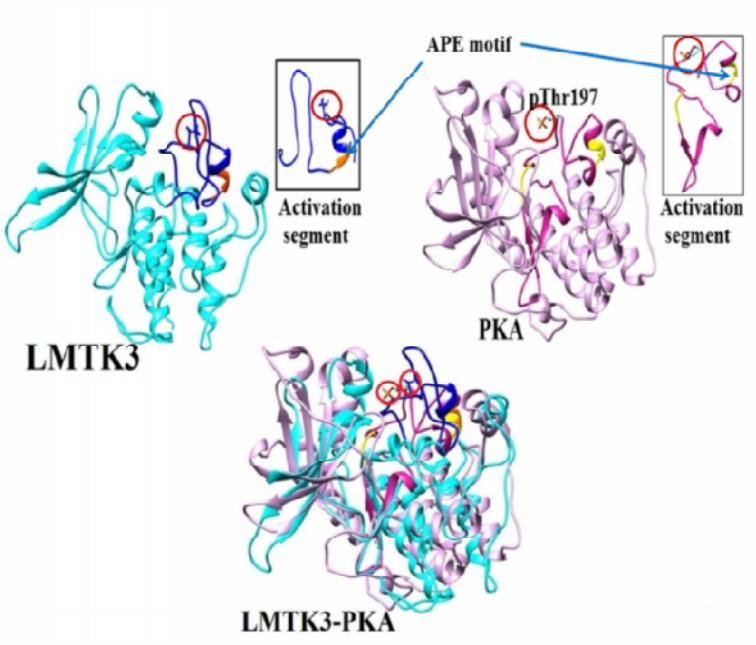

Fig. 2. Superimposed structure of LMTK3 with PKA.

B. Molecular Dynamics (MD) Simulation

To examine the conformational changes, the phosphorylated, unphosphorylated and mutated LMTK3 structure, was subjected to MD simulation upto 30 ns. The snapshots of LMTK3 structures are depicted in Fig. 3.

Stability and Flexibility. Overall structural stability of phosphorylated LMTK3 was analysed from Root Mean Square Deviation (RMSD) and compared with unphosphorylated and mutated LMTK3. The RMSD analysis was done based on C-α atoms, depicted in Fig. 4, we noticed, the structures started converging from its reference structure after 5 ns in LMTK3 and attained the stable conformation after 15 ns with RMSD near 2.5Å (in phosphorylated LMTK3), 3.5Å (in unphosphorylated LMTK3) and 4 Å (mutated LMTK3) during the course of MD run. The lack of gross structural changes throughout the MD simulation confirmed that all the respective structures were stable. However RMSD of loop region (Gly184 to Ser196) in phosphorylated, unphosphorylated and mutated region is observed to be, 2.8Å, 2.5Å and 1.4Å respectively shown in Fig. 5. Phosphorylated loop region shows high RMSD value (2.8 Å) than the unphosphorylated and mutated loop region.

For the loop region, we also analyse the potential energy as a function of RMSD shown in Fig. 6. A,B,C. The Phosphorylated loop region initially change its conformation from its initial structure during the course ofMD simulation from 1Å to 3 Å with a potential energy of -7500 kcal/mol, however we observed the lowest energy conformers settles within RMSD value 1 to 1.5 Å with the potential energy of -10700 kcal/mol depicted in Fig. 6B. Whereas unphosphorylated and mutated loop region does not show much conformational changes and shows the RMSD value ranges between 1 to 1.5 Å with a potential energy of -7500 kcal/mol (Fig. 6. A and C) and the lowest energy conformers settles for both the structures around 1.5 Å with the same potential energy as that of phosphorylated loop region (-10700 kcal/mol). We see there is a less conformational change in unphosphorylated and mutated loop region from the initial structure throughout the MD simulation (Fig. 6 A,C). Whereas phosphorylated loop region undergoes much conformational changes from the initial structure throughout the MD simulation shown in Fig. 6B. In order to check the flexibility of phosphorylated-LMTK3 we analyse Root Mean Square Fluctuation (RMSF) for the C-α atoms and compared with unphosphorylated and mutated LMTK3.

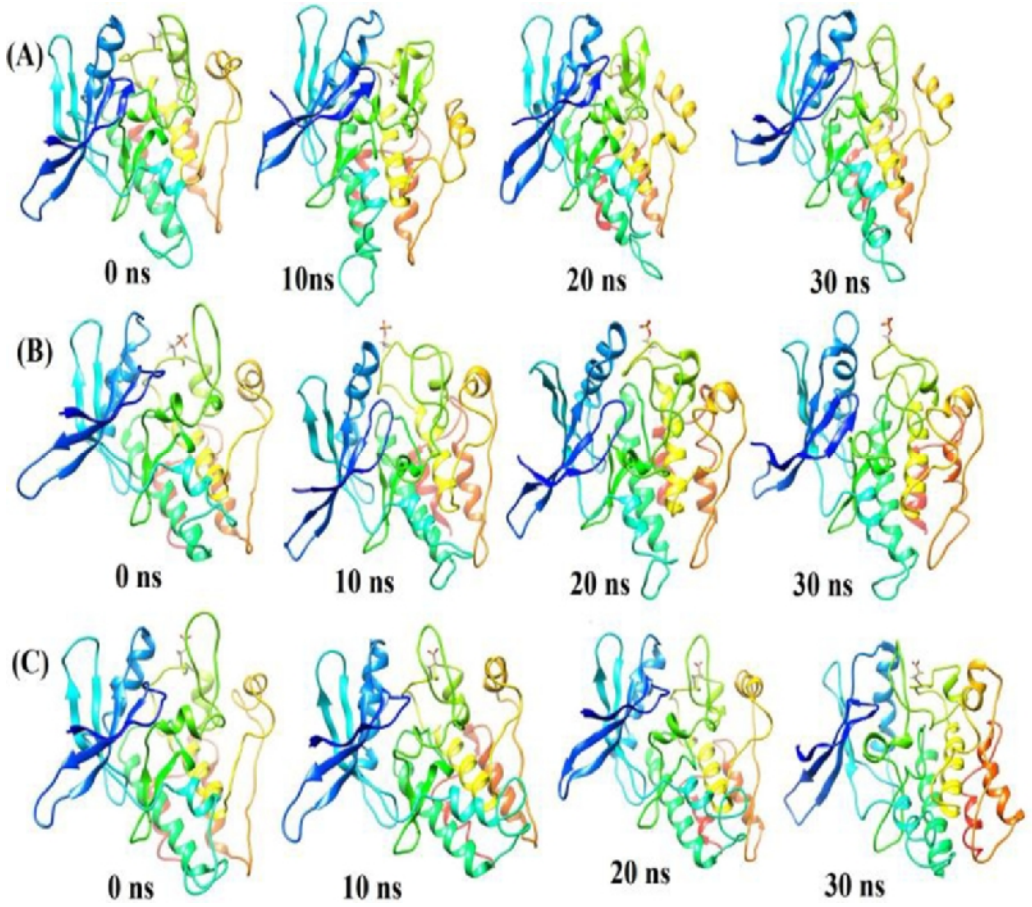

Fig. 3. Snapshots of (A) Unphosphorylated (B) Phosphorylated (C) Mutated LMTK3.

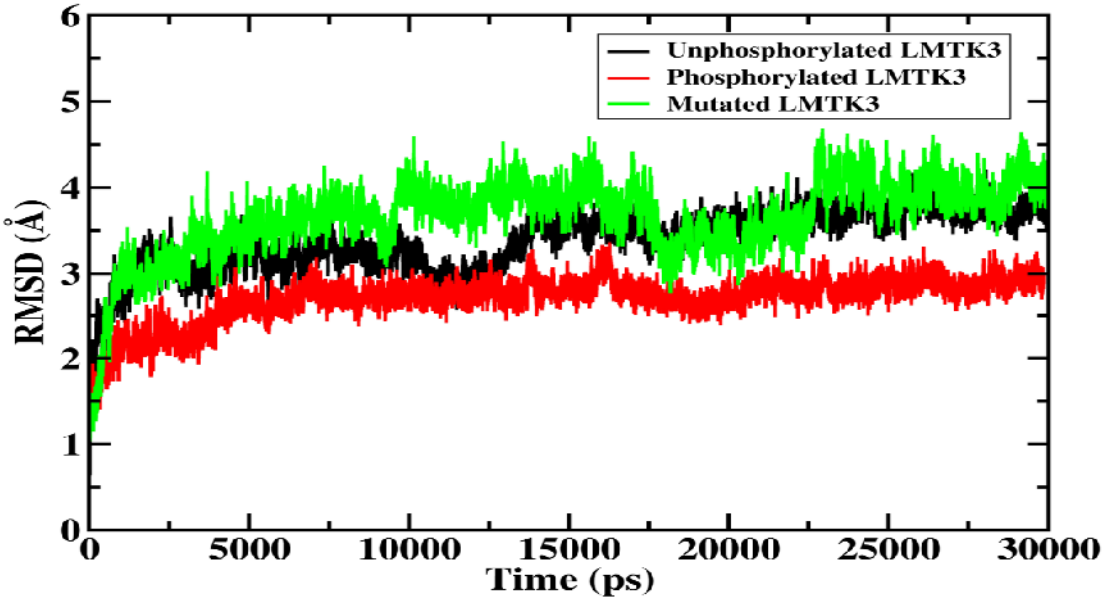

Fig. 4. RMSD plot of Cα atom over time course of MD simulation for phosphorylated (red) and unphosphorylated (black) and mutated (green) LMTK.3.

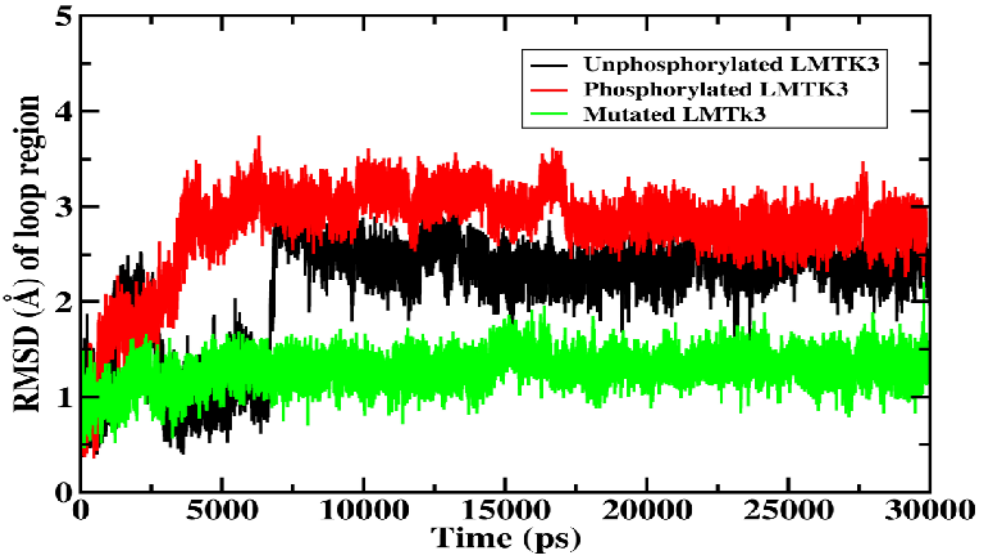

Fig. 5. Loop region RMSD plot of Cα atom over time course of MD simulation for phosphorylated (red) and unphosphorylated (black) and mutated (green) LMTK3.

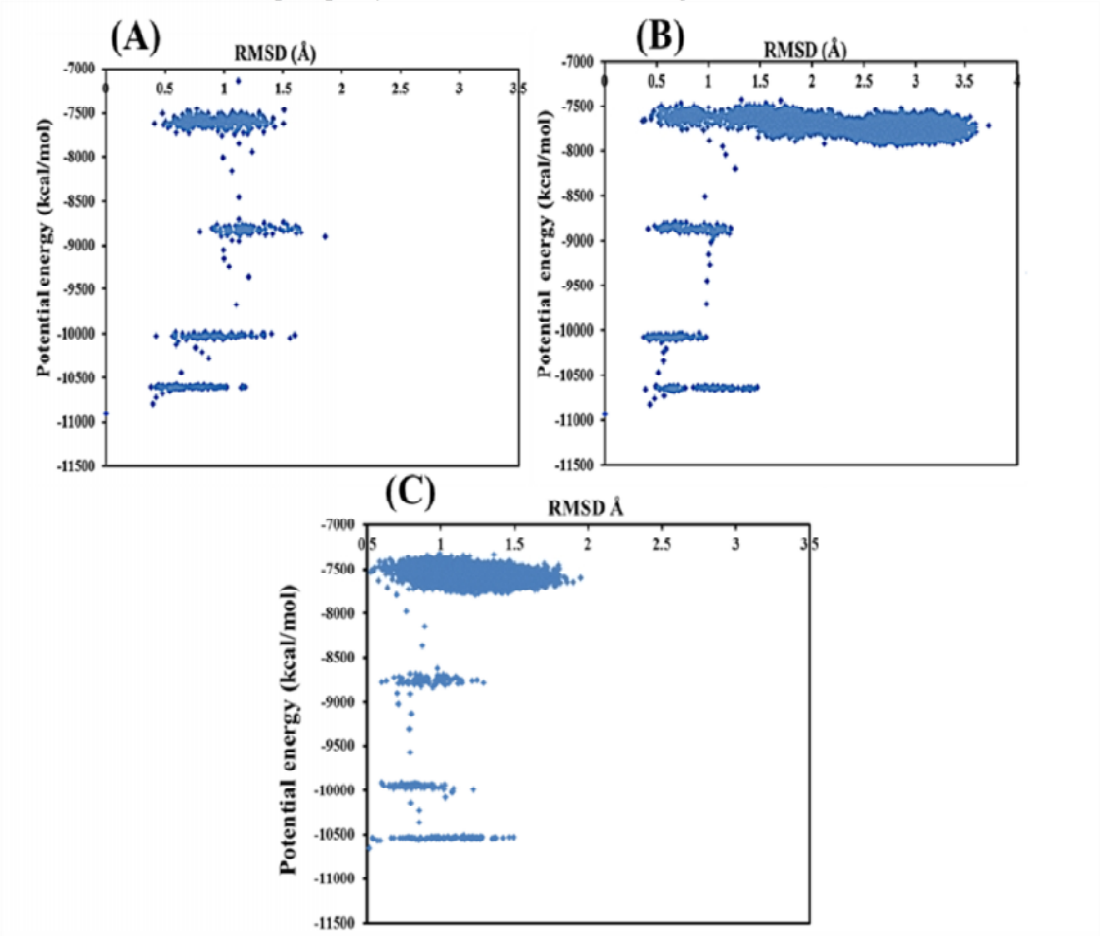

Fig. 6. Potential energy as function of RMSD:(A) Unphosphorylated (B) Phosphorylated (C) Mutated LMTK3.

From the Fig. 7, we see there is a fluctuation near the phosphorylated residue (Thr-189) between residue indexes 150 to 200 with 3 high peaks (2.8Å, 3.5Å and 3.8Å), but there is less fluctuation is case of unphosphorylated LMTK3 region, and there is no fluctuation in the mutated LMTK3 near the mutated (Glu-189) region, but shows fluctuations far from the mutated region (200-250). From this observation we can say Glu does not mimic the effect of phosphorylation in terms of stability and fluctuation. The fluctuation for phosphorylated, unphosphorylated mutated residue for C-β atom observed to be 16 Å, 10 Å and 9Å respectively throughout the MD simulation. Similarly we have also analyse the fluctuation in the loop region (Gly184-Ser196) for C-β atom, the phosphorylated, unphosphorylated and mutated loop fluctuates from its initial position by 14.5Å 11Å and 10 Å respectively throughout the MD simulation. The fluctuations in the loop region depicted in Fig. 8.

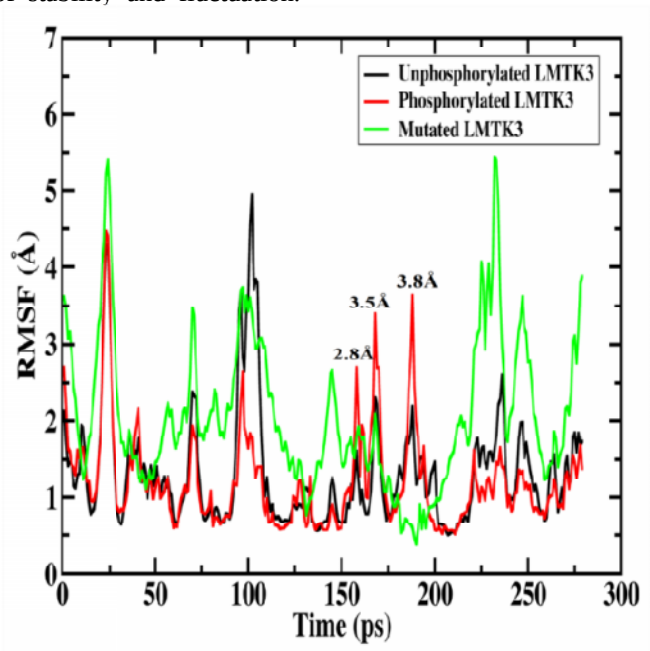

Fig. 7. RMSF plot of Cα atom over time course of MD simulation for phosphorylated (red), unphosphorylated (black) LMTK3 and mutated (green) LMTK3.

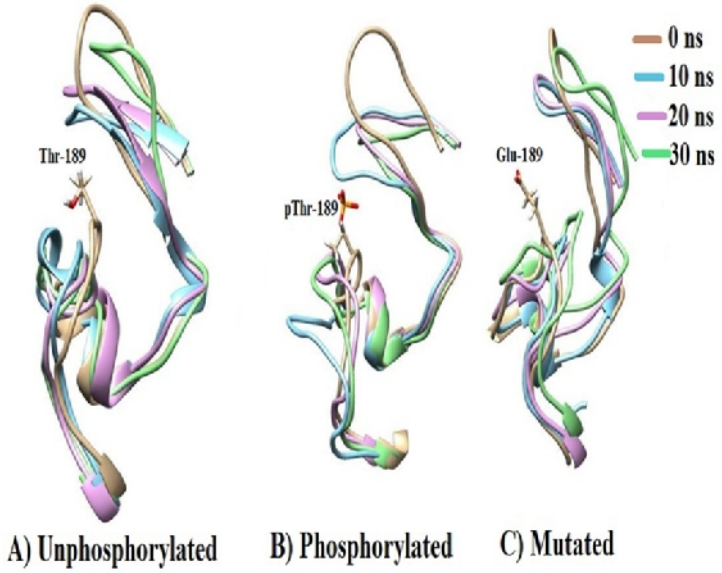

Fig. 8. Flexibility and Conformation change in activation segment of (A) Unphosphorylated (B) Phosphorylatedand (C) Mutated LMTK3 during the course of MD simulation.

Radius of gyration. In order to check the compactness of phosphorylated, unphosphorylated and mutated LMTK3 we performed radius of gyration (Rg) analysis. From Fig. S1, in all the three structures we see Rg value showed a drift between 19.7 Å and 18.7 Å and reached a stable conformation after 18.75 Å. From the plot we can infer that there is not much degree of compactness in the respective structures during the course of MD simulation, thereby confirming the folding of the protein structures.

Energetics. From the potential energy plot (Fig. 9), we see the phosphorylated and unphosphorylated LMTK3 structure have a potential energy value of around -7900 kcal/mol and -7800kcal/mol respectively while in case of mutated LMTK3, potential energy value is around -6600 kcal/mol during the course of MD simulation. We can say that the phosphorylated LMTK3 structure is more stable than the other structures.

For all the three system electrostatic interactions were analysed from the last trajectories as a function of time, electrostatic interactions for unphosphorylated LMTK3 found to oscillate around -18500 to -20500 kcal/mol whereas electrostatic interactions in phosphorylated and mutated LMTK3 found to oscillate around -18400 to -20000 kcal/mol as a function of time **(**Fig. 10).

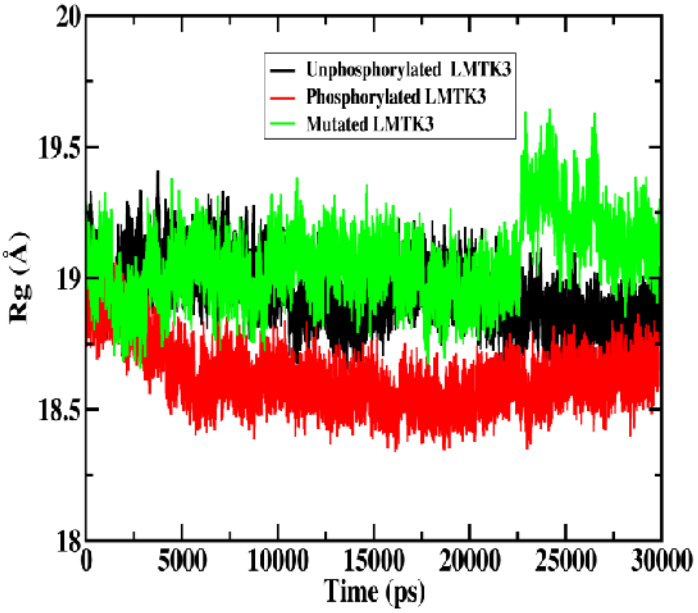

Fig. S1. Radius of Gyration as a function of time.

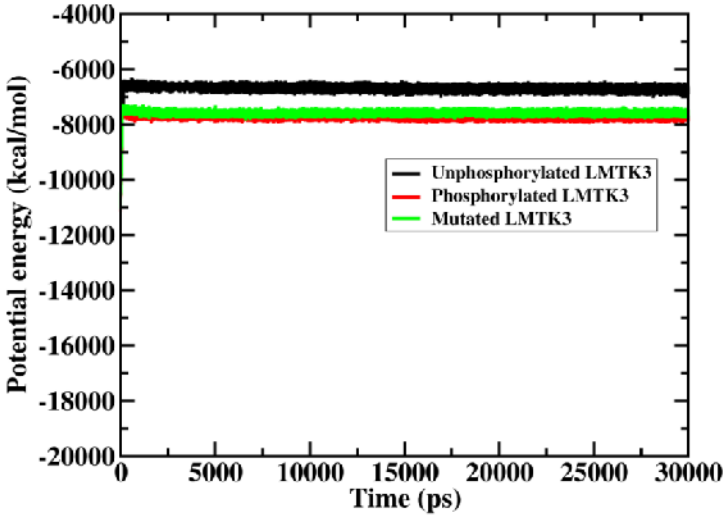

Fig. 9. Potential energy plot as a function of time for Unphosphorylated (black), Phosphorylated (red) and Mutated LMTK3 (green).

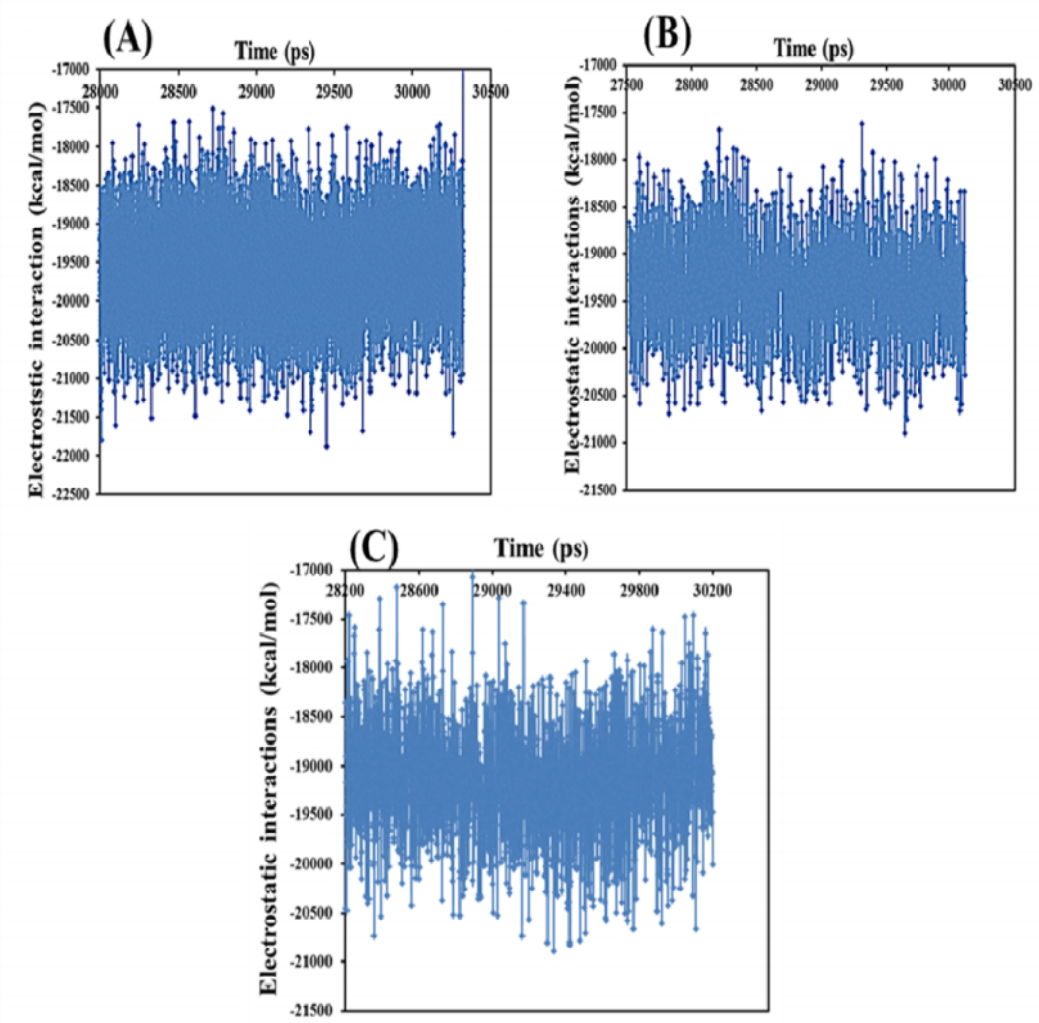

Fig. 10. Electrostatic interaction for (A) unphosphorylated (B) Phosphorylated and (C) Mutated LMTK3 as a function of time.

We also calculated the energies for phosphate group in threonine with different orientation in LMTK3. The variation in energy with rotation of phosphate group is depicted in Fig. S2.

C. Molecular Docking

From the molecular docking study the ATP binding pocket of phosphorylated LMTK3 is found to be wider than unphosphorylated LMTK3 (depicted in Fig. S3), this is due to the flexibility of phosphorylated activation loop which changes its conformation during MD simulation (Fig. 11). Due to the conformational change of activation loop we observed the binding mode of ATP is quite different in phosphorylated LMTK3 (Fig. 12). The flexibility and conformational change in ATP binding site may facilitate the phosphoryl transfer reaction and may activate LMTK3 which might lead to the breast cancer tumourogenesis.

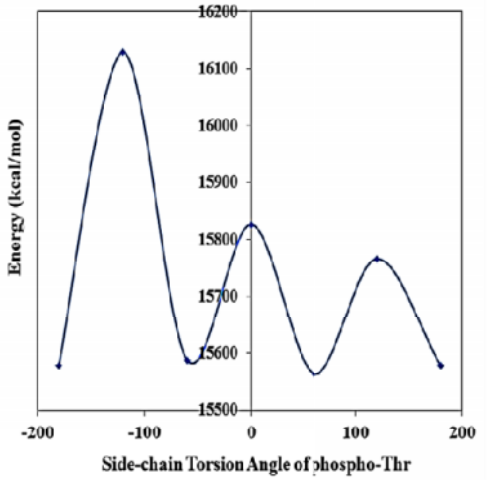

Fig. S2. Energies of the phosphate group in different orientation.

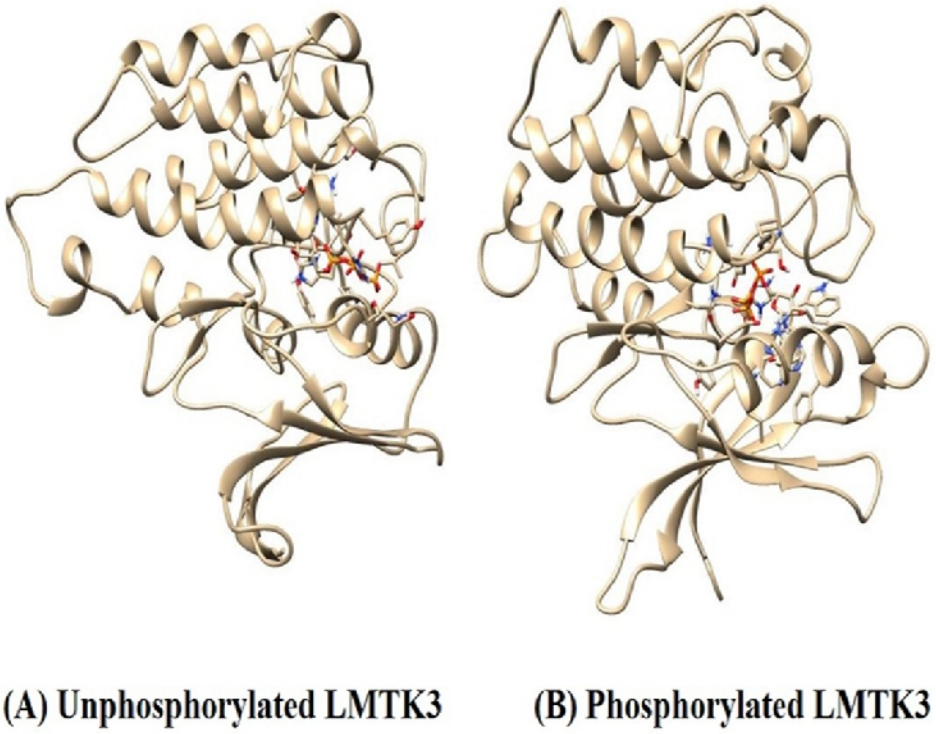

Fig. S3. Docked structure of (A) Unphosphorylated and (B) Phosphorylated LMTK3 with ATP.

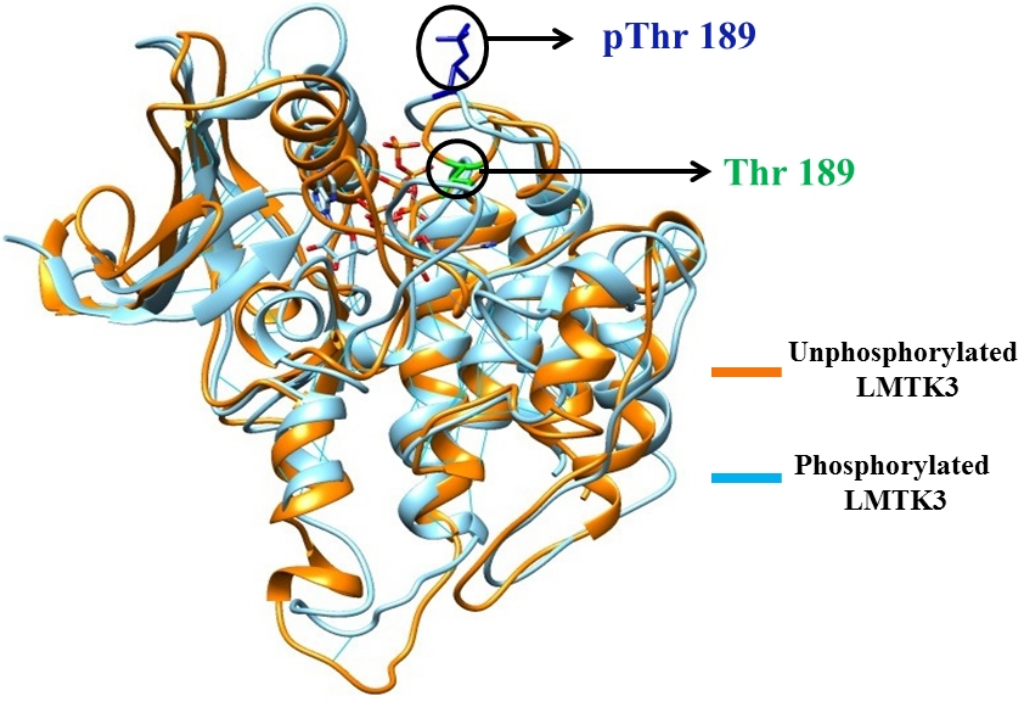

Fig. 11. Superimposed structure of Phosphorylated and Unphosphorylated LMTK3.

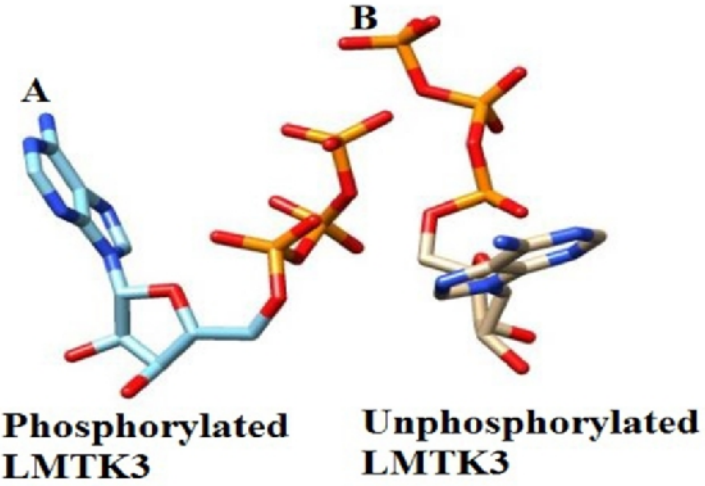

Fig. 12. Binding mode of ATP in (A) Phosphorylated LMTK3 and (B) Unphosphorylated LMTK3.

CONCLUSION

In this computational study we determinedthe probable activation segment in LMTK3. Then we studied the dynamics of LMTK3 upon phosphorylation and compared its dynamics with unphosphorylated and mutated LMTK3 structures. RMSD and potential energy analysis reveal that phosphorylated, unphosphorylated and mutated LMTK3 structures are stable during the MD simulation. Despite the stability of the structures we see mainly the phosphorylated activation segment undergoes much conformational changes as compared to unphosphorylated and mutated one. We also inferred that Glu(189) Thr mutationin phosphorylated LMTK3 does not mimic the effect of phosphorylation. The conformational change in the phosphorylated activation segment of LMTK3 was found to have significant impact on the ATP binding site. As a result the binding mode of ATP found to be different in phosphorylated LMTK3 as compared to unphosphorylated LMTK3. The conformational change in ATP binding site may facilitate the catalysis of phosphoryl transfer reaction in LMTK3 to its substrates and may leads to the breast cancer tumourogenesis. In this work, we have restricted our efforts in predicting relatively modest, localized conformational changes in phosphorylated LMTK3, we have determined the LMTK3 undergoes significant conformational change mainly those portions closest to the phosphorylated threonine-189. This modelling technology may thus be used to create hypotheses about mechanisms of regulation by phosphorylation in LMTK3 that can be further studied experimentally.

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