CHAPTER 5

Unveiling the transient Protein-Protein interactions that modulate the activity of Estrogen Receptor(ER)-α by Human Lemur Tyrosine Kinase-3 (LMTK3) domain: An *in silico* study

Unveiling the transient Protein-Protein interactions that modulate the activity of Estrogen Receptor (ER)-α by Human Lemur Tyrosine Kinase-3 (LMTK3) domain: An *in silico* study

5.1 Abstract

In majority of human breast cancer, the interactions between kinases and the ERα are considered to be critical in signaling pathway. There are many kinases known to regulate ERα activity. Recently Lemur tyrosine kinase-3 (LMTK3) was identified as predictive oncogenic ERα regulator with a vital role in endocrine resistance in ERα positive breast cancer. To understand the role of LMTK3 in ERα regulation, it is critical to study the interactions between them. So we demonstrate here transient interactions between ERα and LMTK3 using computational techniques. We modelled ERα-LMTK3 complex structure from PatchDock server. The approximate interface area of ERα-LMTK3 complex was found to be 3175 $Å^2$ with atomic contact energy (ACE) of 191.77 kcal/mol. The interacting residues and interface area between ERα and LMTK3 were identified using PDBsum. The analysis revealed that some of the residues in C-terminal region of LMTK3 displayed non-bonding interactions with the residues in the phosphorylation sites (Ser104 and Ser106) of ERα. We noticed the total number of interface residues in ERα-LMTK3 complex to be 50 and the total interface area at ERα-LMTK3 interface to be more than 2380 $Å^2$. We also studied conformational dynamics of ERα-LMTK3 complex and found the complex structure to be stable. The outcomes of the current study enhance the understanding of interactions between ERα and LMTK3 which are thought to be critical in signaling pathway in majority of human breast cancers.

5.2. Introduction

Breast cancer is the most common dreadful disease and the leading cause of cancer mortality in females [284]. ERα is a member of nuclear receptor superfamily of transcription factor whose activity is mainly regulated by binding of estrogen [323].

Endocrine therapy has been shown to have a positive effect on ER positive breast cancer treatment [324, 325]. The endocrine therapy using tamoxifen (anti-estrogen, estrogen receptor modulator), aromatase inhibitors that block estrogen biosynthesis, or fulvestrant which induces ERα degradation have been shown to improve disease-free survival. However, initial or acquired resistance to these therapies commonly occurs in breast cancer [286]. ERα is a modular protein having a number of functional domains that includes an N-terminal domain, two transcriptional activation functions (AF-1, AF-2), a centrally located DNA binding domain, hing region, and a C-terminal ligand binding domain. A schematic representation of the location of various domains in ERα and phosphorylation sites has been shown in **Figure 5.1**.

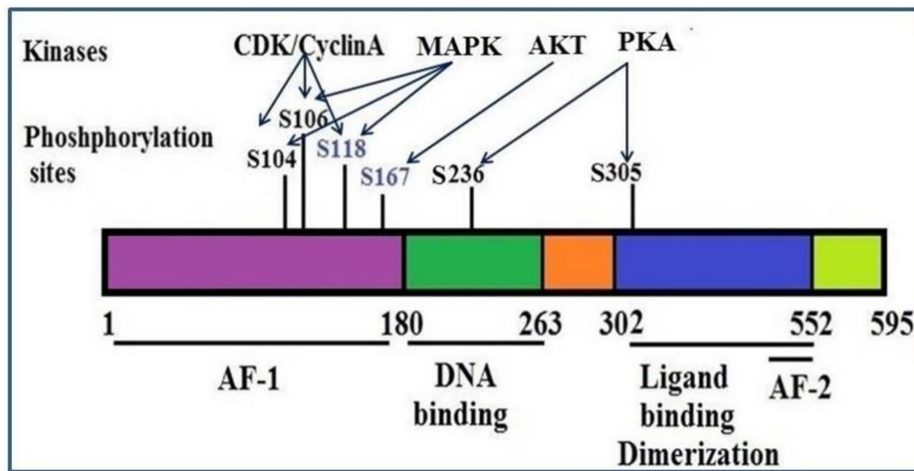

Figure 5.1. *ERα structure and phosphorylation sites: Activation function-1 (AF-1), DNA-binding domain (DBD), hinge, ligand binding domain (LBD), dimerization, and activation function-2 (AF-2) domain. Modified from* [37]

The C-terminal domain of ERα is highly conserved and structured upon ligand binding to its domain, whereas the N-terminal domain is poorly conserved and is less structured. N-terminal domain gets activated by both ligand-dependent and ligand-independent mechanism [31-33]. ERα is the cause for some of the effects of estrogen on normal mammary and breast cancer tissue that occurs through ligand induced transcriptional mechanism [326]. ERα function is also known to be regulated by ligand-independent mechanism through various kinases, such as mitogen activated protein kinase (MAPK) [34], CDK2-CyclinA [35], and protein kinase A (PKA) [36]. It is

normally seen that protein kinases share a conserved catalytic core that consists of 250-300 amino acids common with both serine/threonine and tyrosine protein kinase [291]. MAPK has been reported to phosphorylate ERα at Ser104, Ser106 and Ser118 [34] while CDK2-CyclinA complex phosphorylate ERα at Ser104, and Ser106 [35] which are considered to be important for ERα AF-1 activity. It is well known that protein kinases regulate cell growth, cell proliferation, transcriptional, and translational regulation through phosphorylation to its substrates [36].

Recently, it has been reported that LMTK3 regulate ERα activity by phosphorylation. Some of the recent studies have highlighted that LMTK3 interact and phosphorylate ERα, thus protecting ERα from proteasomal degradation [5]. LMTK3 is also known to regulate *ESR1* by positively regulating FOXO3, a known transcriptional activator of *ESR1* [327]. Modification of ERα by these kinases, promote ligand-independent activation of ERα and tamoxifen resistance [328]. So to have a control over the ERα activity, it is important to understand the interaction between ERα and LMTK3. Literature study reveals that, number of critical key protein-protein interactions participate in disease-associated signaling pathways and represent novel targets for therapeutic intrusion [329].

There are only a few studies on LMTK3 and ERα interaction [5], but the molecular interactions between them are not studied well. So here we made an attempt to investigate on the interacting residues and the interface area between ERα and LMTK3 domain. We obtained the complex structure of ERα-LMTK3 using PatchDock [229] online server. The protein-protein interaction was then studied using PDBsum server [252]. We also compared LMTK3-ERα interaction profile with another well-known kinase that is MAPK interaction with ERα. We observed MAPK has more or less similar interaction profile with ERα as that of LMTK3. Using Molecular dynamics simulation, the stability and energetics of the ERα-LMTK3 complex was also studied.

Our results provide significant insights into the interacting interface area across ERα-LMTK3 complex, interacting residues, bonded and non-bonded interactions. These observations may enhance our understanding of structure-function relationships

of LMTK3 and ERα interaction. Thus, controlling protein-protein interactions will be helpful for the discovery of new drug targets.

5.3. Materials and Methods

5.3.1. Structural modelling and validation of ERα and LMTK3 domain

Since the experimental structure of LMTK3 domain and whole ERα structure is not available, we obtained their 3D model structure using I-TASSER (Iterative Threading ASSembly Refinement) [99] server by submitting their corresponding amino acid sequence. I-TASSER protein prediction server is an automated server which is reported to be the best by CASP experiments. The structural modelling and validation of LMTK3 domain has been described in **Chapter 4.** We retrieved the amino acid sequence of human ERα (595 amino acid, accession number: P03372) from UniprotKB database [296]. The best model for ERα was identified based on the C-score shown in **Figure 5.2**. In addition, other parameters such as Template Modelling (TM) score and Root Mean Square Deviation (RMSD) were used to evaluate the best model. The validation of the model structure of ERα carried out using RAMPAGE [303] and ProSA server [300].

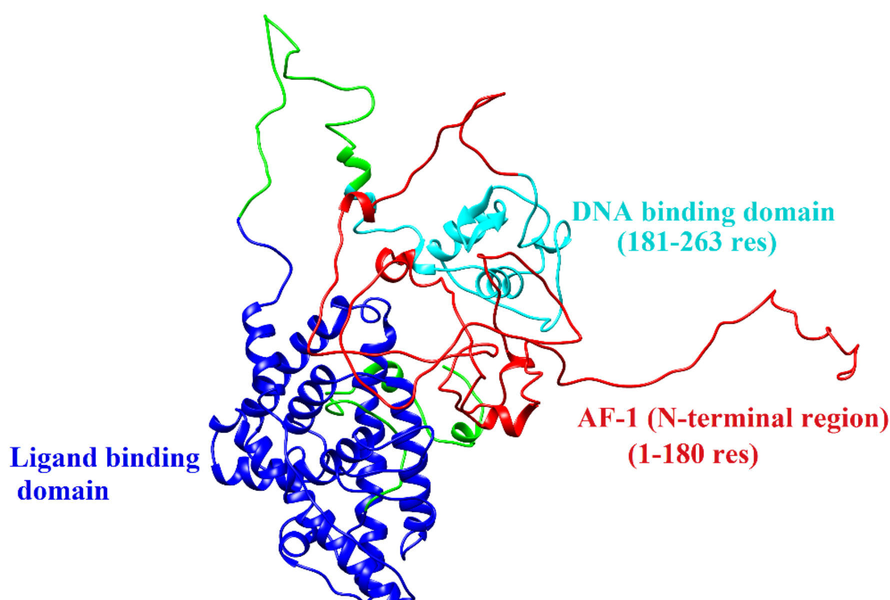

Figure 5.2. *Modelled structure of ERα*

5.3.2. Protein – Protein Docking

5.3.2.1. Rigid docking

We have used the PatchDock [299] server, a molecular docking tool to generate the rigid docking model of ERα with LMTK3. In PatchDock, the docking is carried out by computing the 3D transformations of one of the given molecules with respect to the other one with the objective of maximizing surface shape complementarity while reducing the number of steric clashes.

5.3.2.2. Refinement of Complex Structure

To refine the rigid docking model structures of ERα-LMTK3 obtained from PatchDock, we used FireDock algorithm [330]. The FireDock algorithm refines the complex structures by optimizing the side chain conformation and rigid body orientation. The resultant refined model structures were then given score based on an energy function. The score includes atomic contact energy [242], softened van der Waals interaction, electrostatics, hydrogen bonding and additional estimations of the binding free energy.

5.3.4. Prediction of Interface residues between ERα and LMTK3 domain

We used PDBsum online server to determine the information about interacting interface area across ERα-LMTK3 complex. We have selected the best docking solution from PatchDock based on highest geometric surface area and submitted to PDBsum [252]. PDBsum server summarizes the information about bonded, non-bonded contacts and the interacting residues involved between ERα and LMTK3.

5.3.5. ERα-MAPK interaction:

To support and cross-check our computational study on ERα-LMTK3 interactions, we have further analyzed the interaction of ERα with another known kinase, MAPK. MAPK is also a protein kinase which phosphorylates ERα at Ser104 and Ser106. For this study we have used the complex structure of ERα-MAPK that has been obtained from PatchDock. And the interacting residues between ERα-MAPK were

studied from PDBsum server. PDBsum provide the information about the residues involved in interactions.

5.3.6. Molecular Dynamics simulation of ERα-LMTK3 complex

In order to study the stability and dynamics of the ERα-LMTK3 complex, we have performed the molecular dynamics simulation. The ERα-LMTK3 complex structure with lower energy obtained from FireDock has been used as input structure for the Molecular Dynamics (MD) simulation. The complex was then subjected to MD simulations using Particle Mesh Ewald Molecular Dynamics (PMEMD) [305] module of AMBER12 [**30**] software and AMBER ff99SB force field [306] protein parameters on the ERα-LMTK3 complex. The ERα-LMTK3 modelled complex structure was subjected to implicit solvation and the corresponding topology and co-ordinate files were prepared using xleap module of AMBER package. The resultant structure was then pursued 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. The minimized structure was then subjected to 100 ps of MD using 2 fs timestep for integration. During the MD, the system was gradually heated from 0 to 300 K and ensured slow relaxation of the built initial structure. In addition, shake constraints were imposed with a geometric tolerance of 5×10^{-4} Å on all the covalent bonds involving hydrogen atoms [165]. Subsequently MD was performed under constant pressure-temperature conditions (NPT) with temperature regulation achieved using Berendsen weak coupling method [166] (0.5 ps time constant for heat bath coupling and 0.2 ps pressure relaxation time). This was followed by another 20 ps of equilibration step. Finally for the analysis of structures and properties we carried out MD for 14 ns at NPT conditions using a heat bath coupling time constant of 1ps.

5.4. Results and Discussions

5.4.1. Validation of ERα and LMTK3 structures

The best model for both ERα was identified based on the C-score calculated from the comparative clustering structural density and consensus significance. The C-score for and ERα is -2.90. The overall quality of model was determined by parameters

like TM score of 0.38 ± 0.13 and RMSD of -15.0 ± 3.5 Å). Then we carried out structural validation for the model structures by constructing Ramachandran plot using RAMPAGE server. In the modeled structure of ERα, 75% residues are seen in the most favored region, 20 % in allowed region and 4 % in disallowed region. The overall quality and protein folding energy of both the model structures were validated using ProSA server with the quality index represented by Z-score of -7.23 for ERα as shown in **Figure 5.3.**

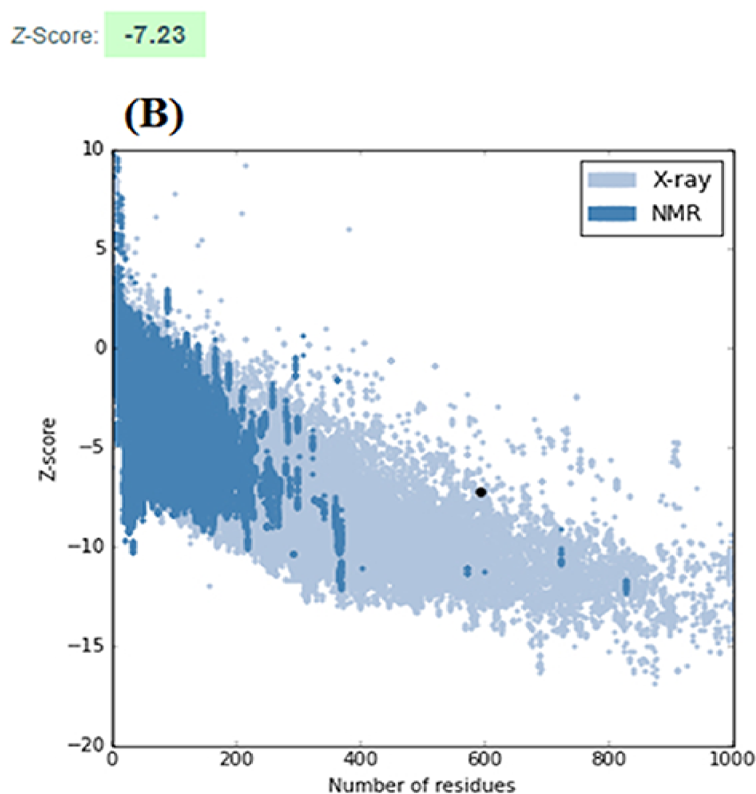

Figure 5.3 *Quality index for the model structure of ERα generated by ProSA server. The index value was represented by Z-score of -7.23.*

5.4.2. Protein-Protein interaction study

It has been known that LMTK3 regulate ERα activity through phosphorylation and protects ERα from proteasomal degradation *in vitro*. In one of the study, co-immunoprecipitation and co-immunofluorescence assay suggested that LMTK3 and ERα are able to interact *in vivo* [**18**]. But the interaction between ERα and LMTK3 at

the molecular level is not studied well. In order to study the interactions between ERα and LMTK3 at molecular level, we docked LMTK3 with ERα using PatchDock, a molecular docking algorithm based on geometric shape complementary score. Geometric scoring here refers to good molecular shape complementarity between the docked structures due to optimal fit with wide interface area and lesser steric clashes [299]. We obtained number of docked structures for ERα-LMTK3 complex and they were ranked based on geometric shape complementary score, interface area and atomic contact energy as shown in **Figure 5.4**. The best ranked structure was observed to have a geometric shape complementarity score of 18806, approximate interface area of 3175 Å2 and atomic contact energy of (ACE) of 191.77 kcal/mol (Solution Structure 1 from **Figure 5.4**).

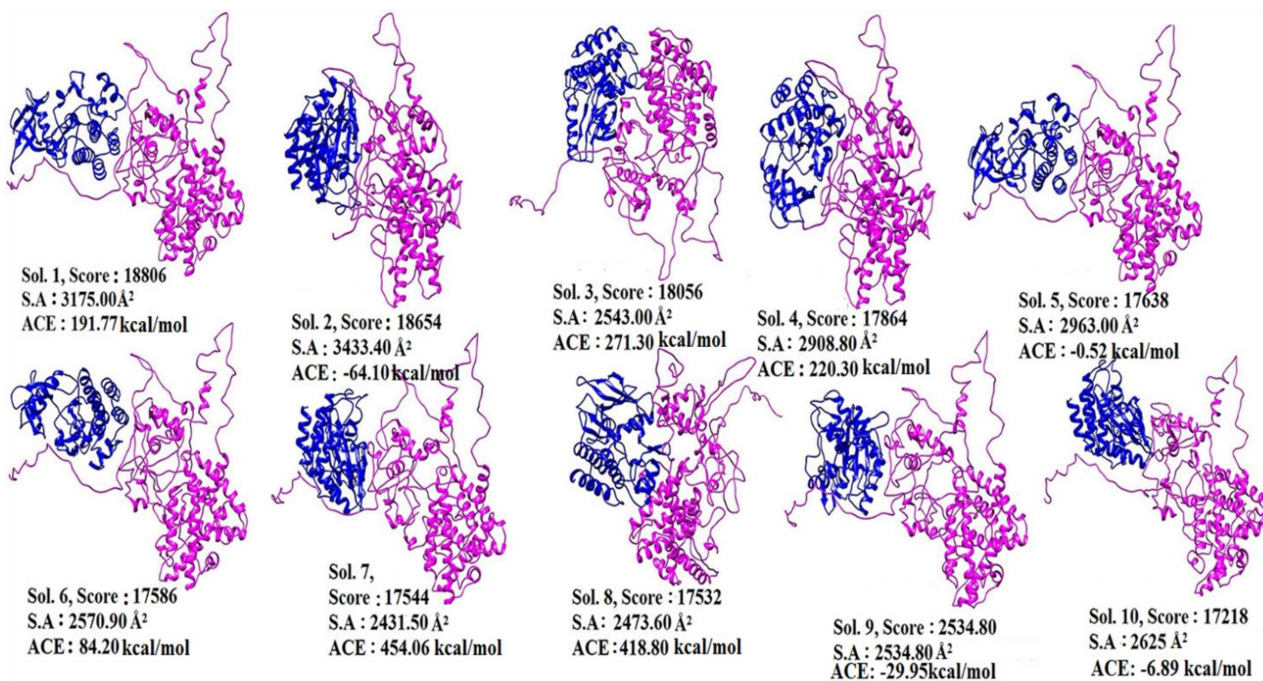

Figure 5.4. *ERα-LMTK3 complex model structures obtained from patchdock server based on high geometric shape complementary score*

We then determined the interacting residues and the interface area of ERα-LMTK3 complex using PDBsum [252]. In the PDBsum server, we submitted the ERα-LMTK3 complex structure having high geometric shape complimentary score. The total number of interface residues in ERα-LMTK3 complex was found to be 50 (**Figure 5.5**) and the

interface area for each chain involved in the interaction was observed to be more than ~2380 Å^2. The docked complex was stabilized by molecular interactions involving number of salt bridges, hydrogen bonds and non-bonded contacts. The interface statistics was summarized in Table **5.1**

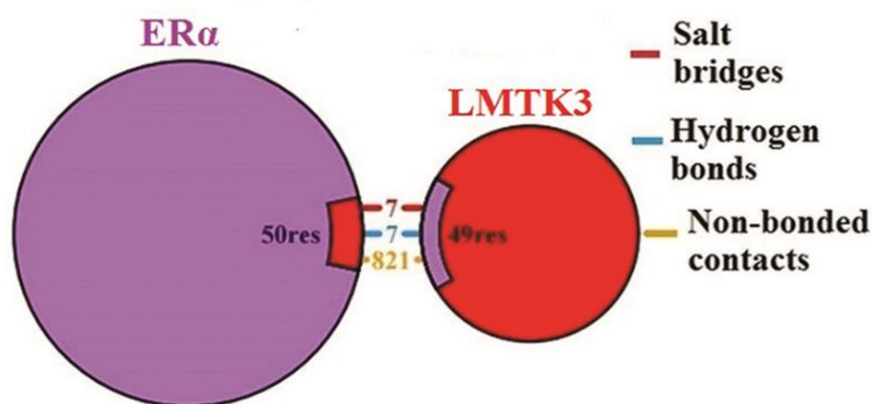

Figure 5.5. *Summary of the total number of residues involved in different types of interactions between ERα and LMTK3 domain*

Table 5.1. Interface statistics for ERα-LMTK3 complex

Chain	Number of interface residues	Interface area (Å^2)	Number of salt bridges	Number of disulphide bonds	Number of hydrogen bonds	Number non-bonded contacts
A (ERα)	50	2437	7	-	7	821
B (LMTK3)	49	2384				

The interface amino acid residues of ERα and LMTK3 involved in the interaction are shown in **Figure 5.6**. From **Figure 5.6 and Figure 5.7,** we observed most of the C-terminal residues of LMTK3 showing non-bonded interaction with the residues present in the AF-1 domain (N-terminal region) of ERα. Some of the amino acid residues (Lys238, Asp254, Gln257, Asp258, Arg261 and Lys238, His236, Arg261, Pro262) in LMTK3 found to be forming non-bonded interactions with Ser104 and Ser106 (which are considered to be common phosphorylating sites) of ERα. Our results show the

probable interaction interface area and the residues involved in ERα-LMTK3 complex which are thought to play a significant role in regulation of ERα in breast cancer.

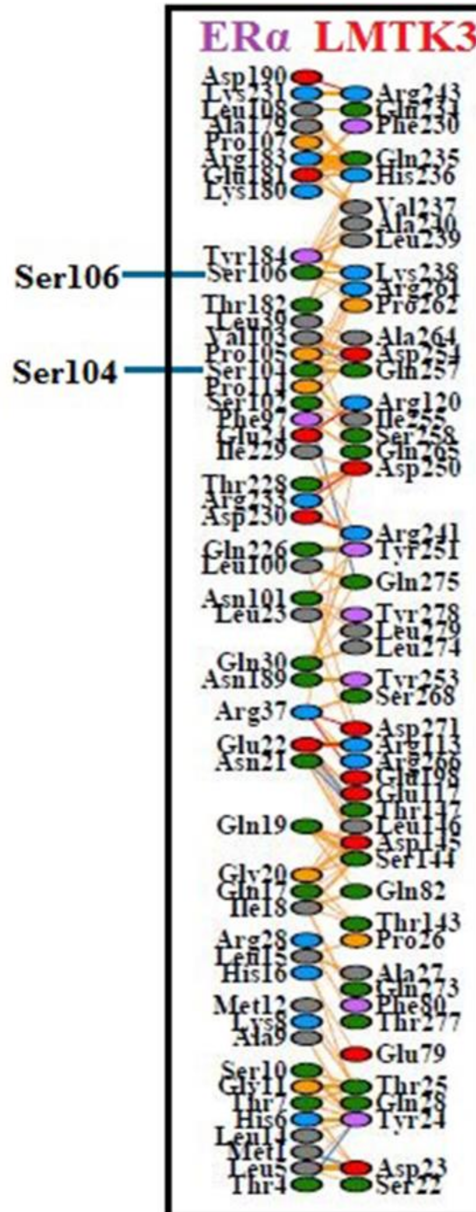

Figure 5.6. *Summary of interacting residues that are involved in bonded and non-bonded interactions between ERα and LMTK3 interface.*

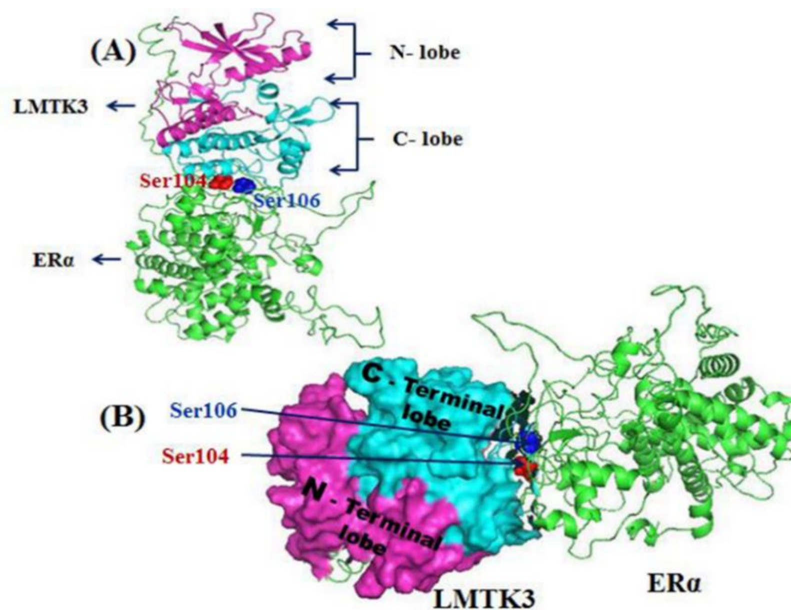

Figure 5.7 *Representation of LMTK3-ERα interaction: C-terminal lobe of LMTK3 interacting with N-terminal region of ERα at Ser104 and Ser106*

5.4.3. Interaction profile between ERα and MAPK

To support and cross check our computational study on ERα-LMTK3 interaction, we have also studied the interaction of ERα with another well studied kinase such as MAPK. MAPK is also a protein kinase which phosphorylates ERα at Ser104 and Ser106 [34]. Best docking solution of ERα-MAPK was selected from PatchDock and submitted to PDBsum in order to obtain interface residues involved in ERα and MAPK interactions. The summary of different types of interactions at the ERα-MAPK interface was shown in **Figure 5.8**. We see 56 residues of ERα interacting with 41 residues of MAPK. The specific residues involved in the ERα-MAPK interface area and their interactions were shown in **Figure 5.9**. Interface statistics for ERα-MAPK complex is summarized in Table **5.2**. We can see that mostly C-terminal residues of MAPK interacting with ERα AF-1 region and also involved in the non-bonded interactions with Ser104 and Ser106. From these observations, it is evident that the interaction profile we observed between ERα and LMTK3 to be nearly the same as that of the well-studied kinase protein MAPK. Hence we may expect LMTK3 to phosphorylate ER-α in a similar manner as that of MAPK.

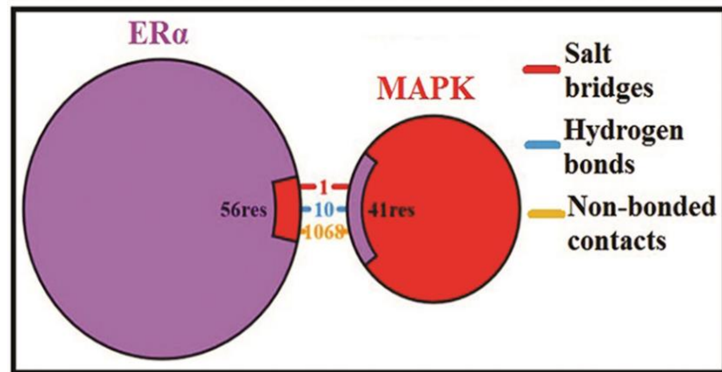

Figure 5.8. *Summary of the total number of residues involved in different types of interactions between ERα and MAPK.*

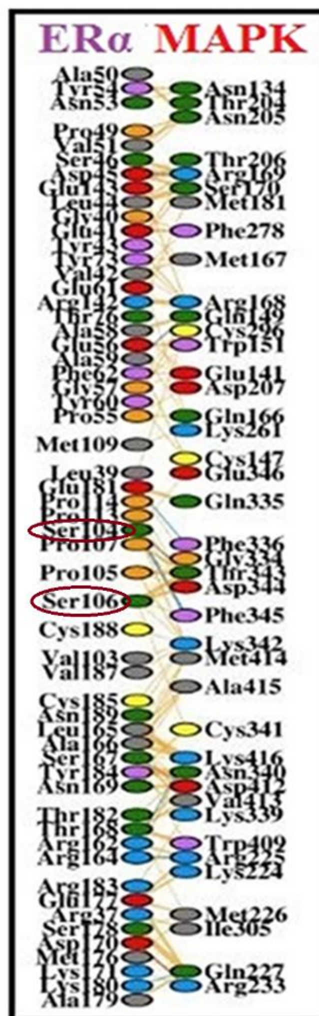

Figure 5.9. *Summary of interacting residues that are involved in bonded and non-bonded interactions between ERα and MAPK interface.*

Table 5.2. Interface statistics for ERα-MAPK complex

Chain	Number of interface residues	Interface area (Å^2)	Number of salt bridges	Number of disulphide bonds	Number of hydrogen bonds	Number non-bonded contacts
A (ERα)	56	2520	1	-	10	1068
B (MAPK)	41	2876				

5.4.4 MD simulation study on the ERα-LMTK3 complex

In the molecular dynamics study, we analyzed the stability and convergence of the model structure of ERα-LMTK3 complex as a function of time. The equilibrated model structure of the complex was used as a reference structure for the RMSD analysis. The RMSD profile for the ERα-LMTK3 complex is shown in **Figure 5.10**. In the complex structure, the RMSD value settles well below 2.5 Å after 1 ns in the case of LMTK3 while in the case of ERα the RMSD value settles around 12.5 Å after 7 ns. From the RMSD analysis, the stability of the complex structure can be seen. The snapshots of ERα-LMTK3 complex at different intervals of simulation time were shown in **Figure 5.11**.

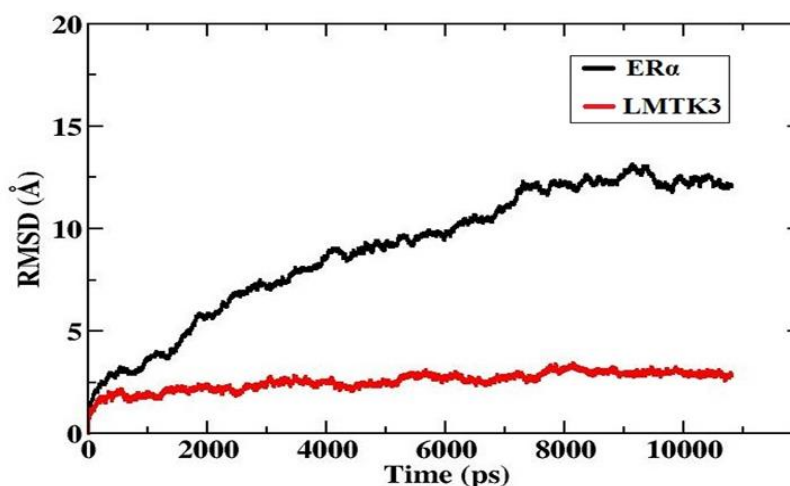

Figure 5.10. *Root Mean Square Deviations (RMSD) of ERα and LMTK3 as a function of simulation time*

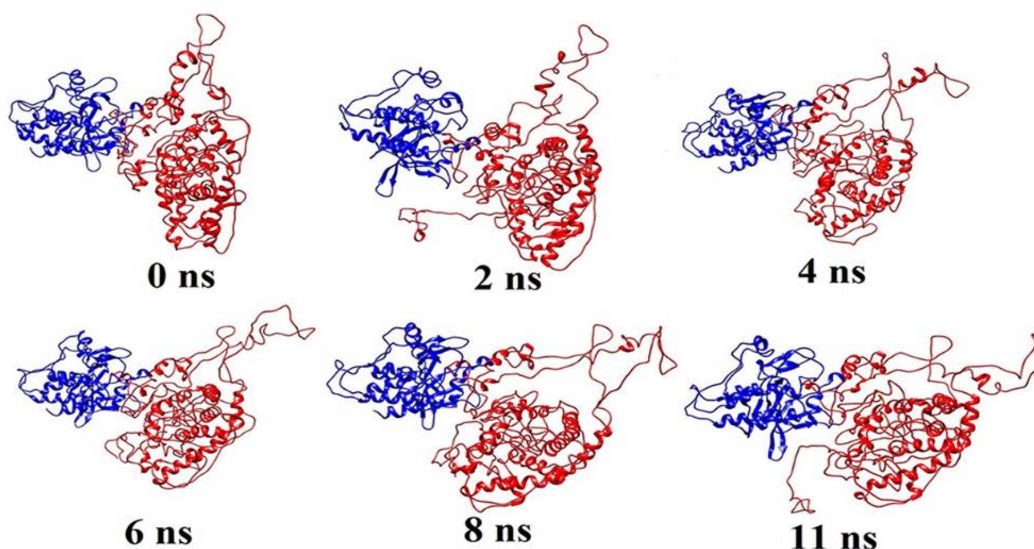

***Figure 5.11**. Snapshots of ERα-LMTK3 complex at different time intervals of simulation*

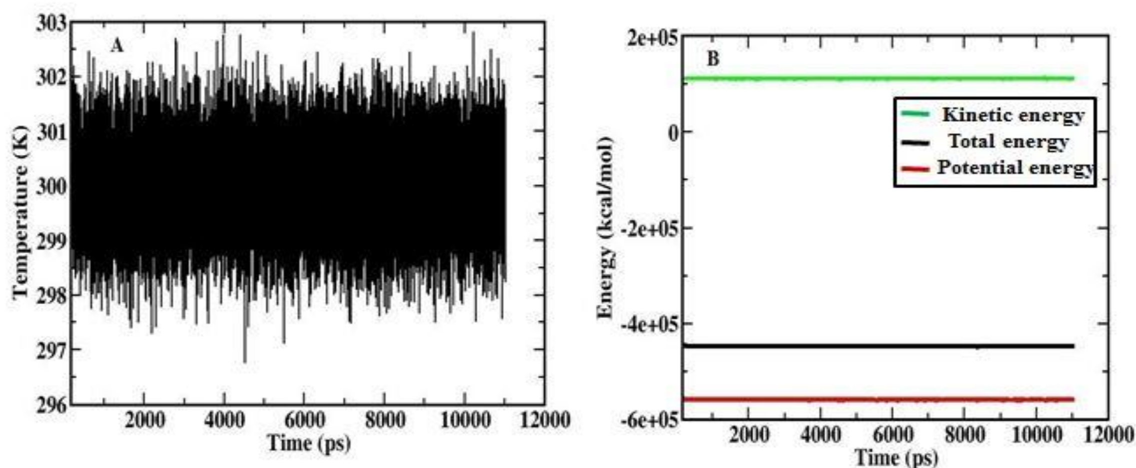

***Figure 5.12**. Temperature and energy of ERα-LMTK3 complex as a function of time*

From the temperature and energy plots **(Figure 5.12)**, we see that our modelled complex structure has reached equilibration without any problem just like that of any other stable complex structure. We also analyzed the intermolecular distance **(Figure 5.13)** and the number of inter-molecular hydrogen bonds **(Figure 5.14)** between ERα and LMTK3 as a function of time. We observed the intermolecular distance between ERα and LMTK3 to hold a value around 45 Å (**Figure 5.13**). For calculating the hydrogen bond, the cut-off for angle and distance was set to 120° and 3.5 Å respectively. From **Figure 5.14** we see the number of intermolecular hydrogen bonds in

the complex to be oscillating around 12-15. Thus, we can infer the intermolecular hydrogen bonds play the significant role in stabilizing the ERα- LMTK3 complex.

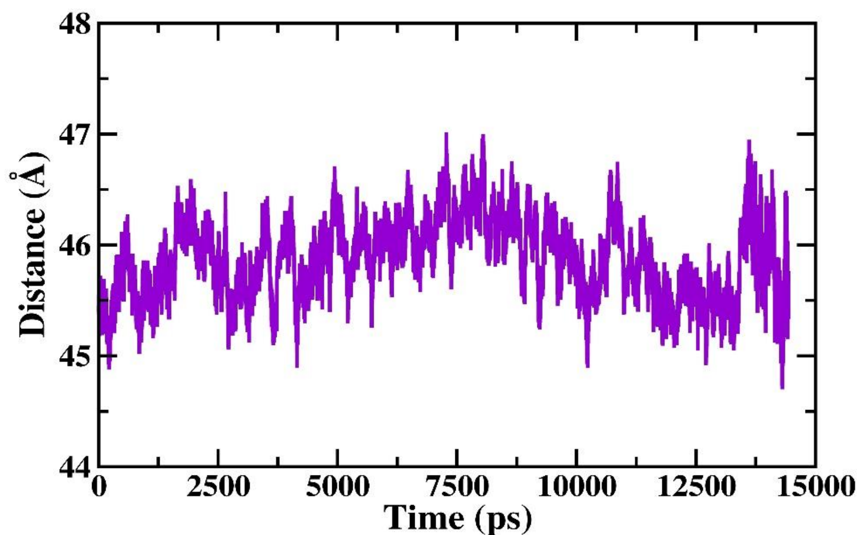

Figure 5.13. *Intermolecular distance between ERα and LMTK3 domain as a function of time*

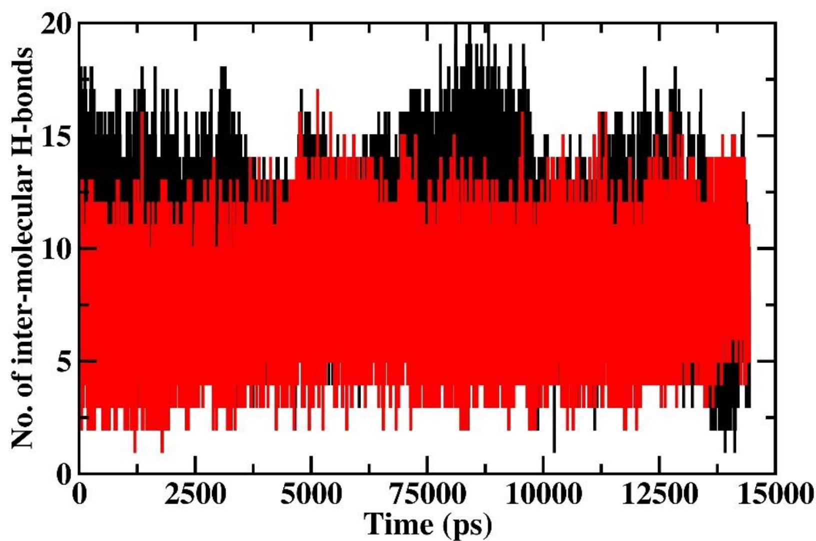

Figure 5.14. *Number of inter-molecular hydrogen bonds in ERα- LMTK3 complex model structure as a function of simulation time period. Here we have considered inter-molecular hydrogen bonds involving two cases: ERα as acceptor (A) and LMTK3 as donor (D) (black line) and the other one, ERα as donor and LMTK3 as acceptor (red line).*

5.5. Conclusions

The present computational study on the interactions between ERα and LMTK3 is essential to understand the role of LMTK3 in modulating the ERα activity. In this work, we modeled ERα-LMTK3 complex and obtained salient features about the interacting residues and the interface area. We have identified mostly C-terminal region of LMTK3 interact with N terminal region of ERα. We observed that some of the residues near the C-terminal region of LMTK3 (Lys238, Asp254, Gln257, Asp258, and Lys238, His236, Arg261, Pro262) involve in forming bonded and non-bonded interactions with N-terminal region of ERα at Ser 104 and Ser106 (common phosphorylating sites). We also cross checked the interaction results by studying with another known kinase that is MAPK which modulate ERα activity through phosphorylation. We observed, MAPK has more or less similar interaction profile with ERα. Our findings in this work highlighted the probable interactions and interface area between LMTK3 and ERα that may play an important role to understand ERα phosphorylation. From this study we suggest that the probable interacting interface regions should be targeted to control ERα activity. Thus this study enhances the understanding of interactions between ERα and LMTK3 which are thought to play significant role in the signaling pathway in ERα positive breast cancers. With the knowledge of interacting domain and residues in LMTK3, it is quite possible to have control over the role of LMTK3 on the ERα activity.