WEB TOOL

ANGDelMut – a web-based tool for predicting and analyzing functional loss mechanisms of deleterious angiogenin mutations causing amyotrophic lateral sclerosis [version 1; peer review: 1 approved, 1 not approved]

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Abstract

ANGDelMut is a web-based tool for predicting the functional consequences of missense mutations in the angiogenin (ANG) protein, which is associated with amyotrophic lateral sclerosis (ALS). Missense mutations in ANG result in loss of either ribonucleolytic activity or nuclear translocation activity or both of these functions, and in turn cause ALS. However, there are no web-based tools available to predict whether a newly identified ANG mutation will be ALS causative. More importantly, no web-implemented method is currently available to elucidate the mechanisms of loss-of-function(s) of ANG mutants. In light of this observation, we developed the ANGDelMut web-based tool, which predicts whether an ANG mutation is deleterious or benign. The user selects certain attributes from the input panel, which serves as a query to infer whether a mutant will exhibit loss of ribonucleolytic activity or nuclear translocation activity or whether the overall stability will be affected. The output states whether the mutation is deleterious or benign, and if it is deleterious, gives the mechanism(s) of loss-of-function. This web-based tool, freely available at http://bioschool.iitd.ernet.in/DelMut/, is the first of its kind to provide a platform for researchers and clinicians, to infer the functional consequences of ANG mutations and their association with ALS ahead of experimental findings.
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Introduction
Amyotrophic lateral sclerosis (ALS) is a rapidly progressive, invariably fatal neurodegenerative disorder that causes the selective destruction of motor neurons, primarily the voluntary muscles. As a result of this degenerative process, most patients usually die from respiratory failure within 3–5 years from the onset of symptoms\(^1,2\). The etiology and mechanisms underlying this debilitating disease are not fully understood, and hence, there is currently no successful therapy for this disorder. Among the genetic factors, about 110 genes have documented association with ALS to date\(^3,4\). Since its first discovery by Greenway et al.\(^5\), the ANG gene has emerged as one of the most frequently mutated genes found in ALS patients of diverse ethnic groups. A total of 19 missense mutations in ANG have been associated with ALS\(^6\). In addition, several rare mutations have also been discovered in the ANG gene; however, it is not yet known whether these are instrumental in causing ALS\(^7\). The human ANG gene encodes a 14.1 kDa long monomeric ANG protein that induces neovascularization, maintains the physiology and health of motor neurons by inducing angiogenesis, stimulates neurite outgrowth and path-finding and protects motor neurons from hypoxia-induced death and hence acts as a neuroprotective factor\(^8-12\). ANG binds to its target cells and undergoes nuclear translocation due to the presence of two functional sites such as the receptor-binding site (\(^5\)NKGNPHREN\(^6\)) and the nuclear localization signal (\(^5\)IMRRGL\(^5\)) respectively. Another important function of ANG is the ribonucleolytic activity governed by the catalytic triad residues His13, Lys40 and His114\(^13\). Several reports on functional assay experiments\(^8-13\) and molecular dynamics (MD) simulations\(^13,14\) have shown that loss of either ribonucleolytic activity or nuclear translocation activity or both of these functions due to missense mutations in ANG cause ALS. However, no web-based tool is currently available that can establish the functional consequences of ANG mutations and precisely predict the loss-of-function(s) mechanisms. In light of these observations, we developed and hosted the ANGDelMut web-based tool, available at http://bioschool.iitd.ernet.in/ DelMut/, which utilizes a MD simulation-based protocol to capture certain structural and dynamic features of simulated mutant proteins to predict mechanisms of functional loss.

The method implemented in ANGDelMut has been previously tried and tested, and in addition, the functional loss predictions of mutants have been correlated with known experimental reports\(^6,7,13\). When a user submits a mutation, the mutant protein is prepared in silico by replacing the target residue with the desired amino acid residue. Subsequently, a standard MD simulation-based protocol is followed based on certain optimized parameters, which performs extensive MD simulations for 25 ns. The simulated trajectories are then visualized and analyzed for the presence of certain global attributes\(^6,7,13\), in which turn indicate whether a mutant will exhibit loss-of-function(s). The output of ANGDelMut suggests the nature of the mutation; whether it is ALS causative or not, and highlights the molecular mechanisms which were responsible for the loss-of-function(s) to the user (Figure 1).

Materials and methods
Technical details and implementation
The ANGDelMut web-based tool was developed in MySQL and is hosted on an APACHE http server located in the computer service centre of the Indian Institute of Technology, Delhi, India. The following are the minimum hardware and software requirements to run this tool –

- At least 64 MB of available random-access memory (RAM) (256 MB recommended).
- Internet Explorer 7.0 or higher (IE8.0 or higher recommended), Firefox 3.x, Chrome 9, 10.
- JavaScript and cookies enabled, recommend ActiveX enabled for Internet Explorer.
- Flash player installed.

The ANGDelMut algorithm is executed in four steps, namely, mutation selection, implicit-solvent molecular dynamics simulation, analysis of simulation trajectory, and data output (Figure 1). The source code is freely and permanently available at: 10.5281/zenodo.7478.

**Step 1: Mutation selection.** In the first step known as “Mutation selection”, the user submits a missense mutation as the input. Once the mutation is submitted, the mutated protein is prepared in silico (Figure 1). The X-ray structure of human angiogenin (PDB code: 1B1I\(^5\)) is used as a starting structure to prepare the mutant, from which the crystallographic water and cofactor, citric acid (CIT) is removed while keeping the secondary structure intact.

**Step 2: Implicit-solvent molecular dynamics simulation.** Before performing MD simulations, the mutated protein is checked for accuracy and missing atoms, if present, are fixed. Following this, hydrogen atoms are added using the Xleap tool of AMBER 11\(^15\) and if required, the system is neutralized with counter ions (Figure 1). The SANDER module of the AMBER 11 package with the “ff99SB” force field is used for all MD simulations\(^16\). A standard protocol for MD simulations is then implemented that consists of an energy minimization (2500 steps of steepest descent followed by 1000 steps of conjugate gradient method), an equilibration phase involving gradual heating from 0 to 300 K in 200 ps followed by a constant temperature equilibration for 1000 ps at 300 K\(^17\). Finally, 25 ns production MD simulations are carried out with periodic boundary conditions in the isothermal–isobaric (NPT) ensemble at a temperature of 300 K with Berendsen temperature coupling and a constant pressure of 1 atm with isotropic molecule-based scaling\(^17\).

The commonly used SHAKE algorithm and particle-mesh Ewald (PME) method are used to constrain bond lengths involving hydrogen atom(s) and in the calculation of long range electrostatic forces, respectively\(^18,19\). An implicit-solvent based MD simulation method which employs the Generalized Born (GB) model to describe the solvation effects implicitly, executes the 25 ns simulation in about 42 hours compared to 625 hours taken by the explicit method. For this, the GB model proposed by Onufriev, Bashford, and Case [generalized Born solvent (igb) = 5] and the analytical linearized Poisson–Boltzmann (ALPB) approximations are used\(^20,21\). The Born radii were adopted from Bondi with modification (mBondi2). For analysing the simulated trajectories, the trajectory of each system is recorded at every 1 ps. Analysis of simulation results are carried out using PTRAJ module implemented in AMBER 11. All the simulations are performed on a 320 processor SUN Microsystems cluster.
Step 1: Mutation selection

Wild-type ANG → ANG mutant

Step 2: Implicit-solvent molecular dynamics simulation

Hydrogens are added, energy minimized and equilibrated

Start → 25 ns

Step 3: Analysis of simulation trajectory

RMSD versus Time

His114 conformational switching

Local folding of $^{31}$RRR$^{33}$ residues

SASA versus Time

Hydrogen bond interaction path

Step 4: Data Output

The snapshot (PDB file) extracted from the MD simulation is uploaded into the server, which can be downloaded and visualized by the user. ANGDelMut is available at http://biosci.iitd.ernet.in/DelMut/

Figure 1. ANGDelMut protocol. Steps involved to predict whether an ANG mutation will be ALS causative or not, with a brief understanding of the mechanism(s) of its loss-of-functions, implemented in the ANGDelMut web-tool. In Step 1, the user submits a missense ANG mutation to the web-tool and the mutated ANG protein is prepared. Next, in Step 2, an implicit-solvent MD simulation for 25 ns is performed after adding hydrogen atoms, and executing energy minimization, equilibration. After completion of the simulation, in Step 3 certain structural and dynamic markers/attributes from the MD simulation trajectory are analyzed, namely, the RMSD versus time, conformational switching of catalytic residue His114, presence of hydrogen bond interaction path from the site of mutation to His114 mediated through Leu115, local folding of nuclear localization signal residues $^{31}$RRR$^{33}$ and change in SASA versus time. After analysis in Step 3, the PDB file extracted from the MD simulation and a text file containing the RMSD data are uploaded into the web-tool.
at the Supercomputing Facility (http://www2.fc.up.pt/PortoBio) of the Indian Institute of Technology, Delhi, India. As a quality control measure, each simulation trajectory is visualized using visual molecular dynamics (VMD)\(^2\) and monitored by calculating certain parameters, such as, total energy and the root mean square deviation (RMSD) at regular intervals.

**Step 3: Analysis of simulation trajectory.** Once the simulation is completed, each trajectory is analyzed for the presence of several structural and dynamic attributes. Broadly, these attributes are indicators of overall stability of the mutant, residue-specific conformational changes during the dynamics run, presence of a hydrogen bond interaction path, and changes in solvent-accessible surface area (SASA) values (Figure 1). The overall stability of the mutant is assessed by calculating the RMSD of the backbone atoms, sampled once every picosecond, using the PTRAJ module of AMBER\(^1,\)\(^15\) (Figure 1). Conformational changes of the catalytic triad residues are visualized and monitored using VMD\(^3\). After this, the hydrogen bond interaction paths are computed using UCSF CHIMERA\(^2\) and visualized using Cytoscape\(^1\) to examine the ribonucleolytic activity. The nuclear translocation activity of the submitted ANG mutant is examined by calculating the SASA of nuclear localization signal residues \(^3\)RRR\(^1\) using VolArea (http://www2.fc.up.pt/PortoBio-Comp/Software/Volarea/Home.html), a VMD plug-in.

**Step 4: Data output.** The output panel shows whether the user-submitted ANG mutation exhibited conformational switching of His114 and if it possessed a hydrogen bond interaction path mediated through Leu115, to predict the loss of ribonucleolytic activity. Next, it is determined if the mutated protein exhibited reduction in SASA and local folding of nuclear localization signal residues \(^3\)RRR\(^1\) to ascertain loss of nuclear translocation activity. Finally, the back bone RMSD data is examined to evaluate its stability (Figure 1).

In addition to all of this information, a Protein Data Bank (PDB) file from the simulation trajectory and the backbone RMSD data computed from the simulated trajectory is made available to the user for downloading, visualization and analyses. Moreover, the functional loss mechanism data interpreted by the host is also suggested and the user is notified via an e-mail.

**Results and discussion**

**Features of ANGDelMut**

The main interface of ANGDelMut provides the user with a brief description about the tool, how mutations in ANG cause ALS and web links to certain important related articles. Further, the interface has an information panel that gives a brief overview of the methodology employed in ANGDelMut and the various stages involved in query processing and in analysing and obtaining the output data. To submit a mutation, the user selects the source amino acid residue which needs to be mutated with the desired residue. In order to understand the functional loss mechanisms of mutants, the user picks certain attributes from the input panel, such as: Conformational switching of His114, Hydrogen bond interaction path mediated through Leu115 - for loss of ribonucleolytic activity), Reduction of SASA, Local folding of nuclear localization signal residues \(^3\)RRR\(^1\) - for loss of nuclear translocation activity and RMSD - for stability), which are analyzed from the simulation trajectory to predict whether a mutation is deleterious or benign. Once all the information is uploaded to the server, the processing pipeline is allowed to run. In addition, the host is notified by a confirmatory e-mail and with the corresponding job ID-used to track the job. After checking the accuracy of the input data, the web-tool starts processing the ANG mutant protein and performs the simulations. The simulated trajectories are then visualized, analyzed, and the results are uploaded to the server. The user can download the output file, which is a PDB file, that has certain structural and dynamic attributes, and if those attributes are detected, is then used to illustrate if a mutation is deleterious or benign. Further, the user is notified through an e-mail, once the result is available. The detailed working principle of ANGDelMut, illustrated using two ANG mutations, K17I (found in ALS patients, and known to cause loss of ribonucleolytic activity)\(^6,\)\(^7\) and L35P (not yet identified in ALS patients but predicted to cause loss of both ribonucleolytic activity and nuclear translocation activity)\(^7,\)\(^13\) and WT-ANG as control, according to the four steps (Figure 1) is described below.

**Case study: Prediction of functional loss mechanisms of K17I and L35P-ANG mutants**

**K17I and L35P mutant preparation.** When the user submits an ANG mutation, for example K17I and/or L35P, the PyMOL molecular graphics system\(^2\) is used to mutate residue K17 to I17 and L35 to P35 in the crystal structure of ANG (PDB ID: 1B1I without the heteroatoms) and the modified file is then saved in PDB format (Figure 1). For WT-ANG, only the heteroatoms are removed from the structure and saved in PDB format. Before setting up the simulations, the PDB file of the mutant is inspected to check whether the mutation has affected its secondary structure. We noticed that before simulations, the K17I and L35P mutant had structurally aligned well with the WT-ANG.

**Performing implicit-solvent molecular dynamics simulation and analysing the trajectory.** The K17I, L35P mutants and WT-ANG prepared from the above step were subjected to implicit-solvent MD simulations for 25 ns (Figure 1). The trajectories of the K17I and L35P mutants and WT-ANG were then visualized using VMD\(^3\). First, the ribonucleolytic activity was investigated. We had earlier established that the conformational switching of catalytic residue His114 is responsible for loss of ribonucleolytic activity of certain ANG mutants, and the predictions were correlated with experimental reports\(^6,\)\(^7,\)\(^13\). The whole trajectories of the K17I and L35P mutants, and WT-ANG were scanned and it was observed that the conformation of catalytic residue His114 changed significantly in the K17I and L35P mutants, while the WT-ANG did not show any His114 conformational alterations (Figure 2A). Further, it was observed that although the site of mutation (Ile17 and Pro35) is distal from the catalytic residue His114, this affected the His114 conformation. In our previous reports, we have determined that in certain mutants, a conserved hydrogen bond interaction path mediated through Leu115 is responsible for conformational switching of His114\(^5,\)\(^11\). The hydrogen bond interactions among pairs of amino acid residues were computed from the MD trajectory using UCSF Chimera\(^2\) based on a distance cut-off ≤ 3.2 Å and visualized using Cytoscape\(^1\). It was found that the K17I mutant possessed a hydrogen bond interaction path Ile17-Asp15-Ile46-His13-Leu115-Gln117-Asp116-His114 while the L35P mutant possessed a Pro35-Lys40-Gln12-His13-(Thr44 and Leu115)-Gln117-Asp116-His114.
compared to WT-ANG and the K17I mutant, as a result of local folding and close packing of nuclear localization signal residues \(31\text{RRR}33\) (Figure 2C and 2D). The two attributes, such as local folding of nuclear localization signal residues \(31\text{RRR}33\) and reduction of SASA, derived from the simulation data suggest that the L35P mutant will exhibit loss of nuclear translocation activity while WT-ANG and K17I mutant will retain this activity (Figure 2).

The nuclear translocation activity of K17I and L35P mutants was also studied. Earlier studies conducted by us have established that local folding and close packing of nuclear localization signal residues \(31\text{RRR}33\) are responsible for loss of nuclear translocation activity of ANG mutants\(^6\),\(^7\),\(^13\). As a result of this local folding, the three successive arginine residues, \(31\text{RRR}33\), exhibit a reduction in the SASA value. For the K17I, L35P mutants and WT-ANG, SASA of \(31\text{RRR}33\) residues was calculated from their respective MD trajectories. It was observed that L35P had a reduction in SASA compared to WT-ANG and the K17I mutant, as a result of local folding and close packing of nuclear localization signal residues \(31\text{RRR}33\) (Figure 2C and 2D). The two attributes, such as local folding of nuclear localization signal residues \(31\text{RRR}33\) and reduction of SASA, derived from the simulation data suggest that the L35P mutant will exhibit loss of nuclear translocation activity while WT-ANG and K17I mutant will retain this activity (Figure 2).

The overall stability of the K17I and L35P mutants and WT-ANG was studied by calculating the backbone RMSD values from the MD simulations. It was observed that the RMSD value of the K17I and L35P mutant was comparable to WT-ANG, suggesting the mutation from Lys17 to Ile17 and Leu35 to Pro35 did not affect the overall stability of the K17I and L35P mutants during the simulations (Figure 2E). It is also important to notice that due to such minor alterations in conformations of certain residues, such as the His114 path, which is responsible for the observed conformational change of His114 (Figure 2B). The two attributes, such as conformational switching of His114 and presence of a conserved hydrogen bond interaction path mediated through Leu15, derived from the simulation data suggest that the K17I and L35P mutants exhibit loss of ribonucleolytic activity while WT-ANG retained this activity (Figure 2).
and 31RRR, the overall stability of the mutant remained unaltered.

Data output. The K17I mutant exhibited loss of ribonucleolytic activity due to conformational switching of catalytic residue His114 and presence of a conserved hydrogen bond interaction path mediated through Leu115 while retaining its nuclear translocation activity (Figure 2). However, the L35P mutant exhibited loss of both ribonucleolytic activity and nuclear translocation activity (Figure 2). Although the L35P mutant has not yet been identified in ALS patients, from the simulation results using the ANGDelMut web-tool, it seems that L35P would be a deleterious mutation. The PDB files of the K17I and L35P mutants available for download and visualization show the presence of the above attributes and show the mechanisms for the loss-of-functions. The user also gets a text file which has information on the backbone RMSD value for the K17I and L35P mutants, which can be downloaded and analyzed by the user in order to infer the overall stability of the mutants. Broadly, the users get to know whether the mutation is ALS causative or not. A notification e-mail is also sent to the user informing them of the completion of job and the final output of the query.

Conclusion

ANGDelMut is an easy-to-use web-based tool that incorporates a methodology to accurately predict whether an ANG mutation will be ALS causative or not. A set of global attributes are usually investigated from MD simulations to predict whether a mutation will be deleterious or benign. Therefore, it is the first tool of its kind to provide the user with details of the mechanisms of functional loss of an ANG mutant. The user can get this information relatively quickly ahead of experiments. We hope that the ANGDelMut web-tool, freely available at http://bioschool.iitd.ernet.in/DelMut/, will help clinicians and researchers to understand the pathogenesis and progression of ALS, due to an increase in the number of newly discovered ANG mutations.

Author contributions

A.K.P., B.J. and J.G. conceived and designed the experiments. A.K.P. carried out all the experiments. S.V.V. created the web server. A.K.P., B.J. and J.G. analyzed the results and wrote the manuscript; all the authors read and checked the manuscript for content.

Competing interests

No competing interests were disclosed.

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References


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The authors have created a web-based tool to predict the effect of mutations on the angiogenin protein that utilizes Molecular Dynamics simulations to evaluate if the selected mutation is benign or otherwise deleterious. I have several criticisms regarding this work both in terms of: 1- The implementation of the web-tool described here and more importantly, 2- The methodology behind the web-tool. These are discussed below:

1. Web-tool implementation and function.

The web-tool seems to only be accessible online intermittently. On several occasions I could not access it. When I could, using Firefox or Safari browsers in Mac OS 10.9, the interface seemed badly designed, not allowing the visual inspection of the selected potential mutations in a 3D model of the protein. The authors could for example, use Jmol for that. 48 hours after submitting a query I am still waiting for any results. The MD protocol implemented by the authors calls for a 25 ns simulation, in our experience we observed that a 25 ns simulation takes approximately 5 days using 48 cores. I would therefore expect the MD simulations performed by the authors to take less than one day. While the language used in the manuscript is not sufficiently precise, the authors mention that “each trajectory is analyzed for the presence of several structural and dynamic attributes”. To me this suggests that there is possibly manual human intervention in the analysis of each query as part of the results and as such, the method would be impractical unless using mechanisms such as Amazon's Mechanical Turk. I cannot comment further on any potential outputs for the submitted queries as I never obtained a result to my first submitted query.

2. The methodology behind the web-tool

Despite the authors having published 3 papers in peer-reviewed journals using the same methodology as proposed in this web-tool, the reliability of the method in my opinion remains to be proven. I will proceed here to describe the reasons why. The first publication by the authors (PLoS ONE 7(2): e32479. doi:10.1371/journal.pone.0032479) analyzes 6 mutations (K17I, S28N, P112L, L35P, K60E and V113I). In this work, the authors observe computationally several changes.
Notably a conformational change of the catalytic H114 residue side chain and decrease in solvent accessible surface area of the $^{31}$RRR$^{33}$ signal residues. Later (Scientific Reports, January 2013, doi:10.1038/srep01225), the authors considerably expanded their analysis to other mutations and predicted the effect of several other mutations entirely (I46V, K17E, R31K) or partially (R121H and K54E). This was followed by a third paper (FEBS Letters, Volume 587, Issue 12, 19 June 2013, Pages 1762–1766 doi: 10.1016/j.febslet.2013.04.022) where some other, rarer mutations are analyzed using the methodology. While the authors present compelling computational results in all these three papers, these results should be viewed as a hypothesis for the mechanism by which such mutations lead to loss of activity. These mechanisms remains to be proved experimentally. It is important to always keep in mind that correlation does not mean causation. As these suggested mechanisms have not been proven to cause the loss of function, their correlation with loss-of-function cannot be used to for ‘predicting and analyzing functional loss mechanisms of deleterious angiogenin mutations casing amyotrophic lateral sclerosis”.

In summary, I have several reservations regarding the usability of the web-tool presented in this manuscript and I object to the use of the mechanisms suggested by the MD simulations, no matter how compelling their likelihood based on computational evidence, as if they were accepted mechanisms without having ever been experimentally validated. The fact that the authors have already published three papers in which the reviewers did not seem to point out this issue, makes this issue all the more important.

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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**Author Response 26 Nov 2013**

**James Gomes**, Indian Institute of Technology Delhi, New Delhi, India

To the best of our knowledge, the web-tool always opens from different browsers and works fine in different operating systems such as, Windows and Mac. We do not know why the reviewer was unable to access it because during this period our servers were not down. The Jmol JAVA visualizer needs a PDB file for 3D visualization. If a mutation is incorporated, the new coordinates of the C, N, H, O-atoms have to be updated in the PDB file. This is possible only after MD simulations. Therefore, the reviewer’s expectation of a visual inspection of the potential mutations in 3D model in Jmol before MD simulations is unrealistic. As a part of our output, we provide the user with the PDB file for visualization.

The results of the reviewer submitted query were available within 48 hours after the start of the simulation and are still available on the web server (see [here](#) for a screenshot) . However, after manual inspection of the output file for accuracy, completeness and interpreting the results (deleterious or benign mutation), the output files were uploaded to the web-server within the next 12-14 hours and an e-mail was sent to the reviewer informing this. The user was notified of the completion of the job by e-mail (a screenshot of the email is available [here](#)). A Word file, named “Reply_JobID-D00018” was also sent as part of the email to his e-mail ID on 22nd November 2013 at 4:31 PM, Indian Standard Time, to provide further information on the interpreted results.

We are not sure how the reviewer got the impression that a 25 ns-simulation could be completed in approximately five days using 48 cores. We would expect the reviewer to be aware that simulation
times vary depending on the size of the protein and the processors available for computation. With the computational facility available in our institute, a 25 ns time duration implicit-solvent based MD simulation for a mutant Angiogenin protein takes about 42 hours in 16 processors out of the 320 processor SUN Microsystems clusters (please see the text in the manuscript “An implicit-solvent based MD simulation method which employs the Generalized Born (GB) model to describe the solvation effects implicitly, executes the 25 ns simulation in about 42 hours compared to 625 hours taken by the explicit method” and “All the simulations are performed on a 320 processor SUN Microsystems cluster at the Supercomputing Facility (http://www.scfbio-iitd.res.in) of the Indian Institute of Technology, Delhi, India). Once the simulations are completed, the RMSD and PDB files are uploaded to the web-server.

As we use two different servers (one where the web-server is located and another, the supercomputing facility, where the queries are transferred and simulations are performed), for initial query processing and data completeness, human intervention is a mandatory step. In addition, after the completion of the simulations, we visualize and analyze the requested query to check for accuracy and to avoid data incompleteness. This was stated clearly in our manuscript. We hope this answers the reviewer’s comment on this issue. If we submit the reviewer’s e-mail ID or Job-ID into the output panel of the web-tool, even at this point, the output result is still visible.

In response to the reviewer’s comment about the methodology behind the web-tool we would like to state the following. We are not sure that the reviewer has read the Scientific Reports (doi: 10.1038/srep01225) and FEBS Letters (doi: 10.1016/j.febslet.2013.04.022) manuscripts in detail. In the Scientific Reports paper, the last paragraph of the Introduction clearly states that “The close match between simulation results and reported experimental data establishes the strength of this method and its application as a predictive tool to determine if an ANG mutation is deleterious or benign, and if deleterious, the probable mechanism of its loss-of-functions”, rather than only expanding the number of mutations. Also, we would like to suggest that the reviewer checks Table 1 in this article, where we have shown that our predictions matched with all the reported experimental data.

In the FEBS Letters paper, we have clearly stated in the abstract that “We present here a fast molecular dynamics based method for determining the mechanisms of functional loss caused by mutations, and attributes to ascertain whether a mutation causes ALS”, rather than highlighting the rare mutations. We have employed the same method in this web-tool as well to understand the functional loss mechanisms. As our predictions match with the reported experimental data, it provides a step forward to use this methodology to predict and analyze the functional loss mechanisms for other new mutations and to predict whether they are deleterious or benign. Thus, we are well aware of the fact that “correlation does not mean causation” as stated by the reviewer. At present, no experimental technique exists that can be used to validate these mechanisms, such as conformational changes of residues at the molecular level, only a time dependence dynamics method, such as, MD simulation can give insights into atomic level details.

In response to the reviewer’s final comment, we would like to emphasize that all our previously published papers have been extensively peer reviewed by the respective referees, with some of them even undergoing 3 or 4 rounds of revision. We feel that the reviewer’s comments that call into question the scientific and intellectual ability of peers across different parts of the world will only discourage authors to publish in open, peer-reviewed journals like F1000Research.

**Competing Interests:** No competing interests were disclosed.
I thank the authors for their extensive response to my original review. It seems that some of the points I made were not sufficiently clear. But before I continue, I would like to acknowledge that I did indeed receive the results in an email but only after I had submitted the original report. I waited for as long as I thought reasonable before submitting it and therefore to be complete, my statement should have read that I did not receive any results up to the time of writing my report.

The results are available online at the time of writing this response. With respect to the PDB file provided, it would be useful if the authors would adhere more strictly to PDB naming nomenclature, in particular, the authors use the 3-letter code WAT for water molecules instead of HOH. It would also be useful if the authors would provide the possibility to download a Pymol .pse file of the MD trajectory where the regions of interest used in their manual analysis are differentially displayed and also include the possibility to download the raw MD trajectory. The only results that are provided online are a PDB file and an RMSD file, the latter very poorly documented. Additionally, the conclusions of their manual (Human) analysis of the results are displayed but no data are given online as to the fraction of time that systems spends out of the 25 ns simulation in any of the local conformations that lead to their proposed mechanism of loss of ribonucleolytic or nuclear translocation activity mechanisms. Additionally the authors should state online as part of the results given what threshold values are used to conclude based on the dynamics that any of these proposed mechanisms are not favoured in the mutant under study. It is clear that even in the wild type such conformations will be present at least a fraction of the time. This again alludes to my criticism of the human interventions in concluding that any of these proposed mechanisms are prominent during the simulation.

Now, the first point about a Jmol visualization, perhaps I didn't express myself clearly enough, I didn't mean to suggest that the Jmol visualization would show the resulting mutation, of course that requires some form of minimization at the very least. What I would like to see in Jmol is the position of the selected mutation (with the original amino acid).

Regarding the speed of MD simulations, it is not relevant as this discussion was part of my original report in trying to quantify when I could expect the results to arrive as no calculation progress report is given. For example, an email could be sent for a query still running with a link to a results page where the percentage to completion is displayed while the results are not available.

The author may rest assured that I do indeed fulfill their expectation that I am aware that simulation times vary depending on the size of the protein and processors available. The 5 days for a 25 ns simulation using 48 cores represents my subjective experience. I thank the authors for clarifying that several steps require manual intervention. I still think that manual intervention in the generation of results (be it transferring files across servers, checking the output file for accuracy, completeness and interpretation of results), as noted in the original report, is neither dependable not scalable and therefore is not acceptable. What will happens in five years when the postdoc or student responsible for the manual intervention leave? Or when they go on vacation next time?

Contrary to what the authors suggest, I did indeed carefully read their three previous published manuscripts before writing my report on this manuscript. The authors cite in their current response some of their conclusions in these previous reports. While the language used in the Scientific
Reports manuscript is correct, stating 'probable mechanism' in the FEBS Letters paper it becomes 'determining the mechanism'. In this manuscript it turns to even more emphatic language saying 'elucidating the mechanisms'. I am glad that the authors are aware that correlation does not imply causation. Irrespective of what experimental techniques can or cannot be used to prove the veracity of the proposed mechanisms, until experimental validation confirms the mechanisms, the proposed mechanisms will remain proposed mechanisms only. I encourage the authors to be clear about this and I remain convinced that this point should have been more clearly stated by the authors in the other papers. Unfortunately, precisely because the peer reviews of these other papers are not openly accessible, I cannot judge the quality of the peer review. Nowhere did I call into question the 'scientific and intellectual abilities' of previous peer reviewers. I said that the fact that it had been missed makes emphasizing this issue here all the more important.

As much as the problems with manual intervention are in my mind sufficient to object to the current manuscript, I am willing change my classification to 'accept the with reservations' as long as the manuscript is extensively rewritten, including changing the title to remove any suggestions, implicit or otherwise, that the mechanisms used to classify the mutations are in any way proven as well as to clearly state the limitations of the manual intervention steps. Furthermore, the proposed hybrid Human/Web-tool presented should be modified to give users access to a job progression report page that is frequently updated and that can be accessed at any time after submission until the results are available.

Competing Interests: none

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Author Response 23 Dec 2013

James Gomes, Indian Institute of Technology Delhi, New Delhi, India

- "I would like to acknowledge that I did indeed receive the results in an email but only after I had submitted the original report. I waited for as long as I thought reasonable before submitting it and therefore to be complete, my statement should have read that I did not receive any results up to the time of writing my report."
  
  Our record shows that the e-mail was sent to the reviewer on 22nd November 2013 at 4:31 PM, Indian Standard Time (as shown in the attached screen shots of our earlier response) and the output result files were available in time.

- "The results are available online at the time of writing this response. With respect to the PDB file provided, it would be useful if the authors would adhere more strictly to PDB naming nomenclature, in particular, the authors use the 3-letter code WAT for water molecules instead of HOH."
  
  Our web-tool uses the AMBER software package for MD simulations and thus the PDB file, which was retrieved from the MD trajectory, has the WAT code for water molecules instead of HOH. As this has no affect in the interpretation and visualization of the output PDB file, we did not replace WAT with HOH.

- "It would also be useful if the authors would provide the possibility to download a Pymol .pse file of the MD trajectory where the regions of interest used in their manual analysis are differentially displayed and also include the possibility to download the raw MD trajectory. The only results that are provided online are a PDB file and an RMSD file, the latter very poorly documented."
  
  We agree with the reviewer that uploading a Pymol .pse session file can help the user in
directly visualizing the regions of interest. Therefore, we have now accepted the above suggestions of the reviewer. As the raw MD trajectory files are usually very large in size, it is not possible to provide the user an option of downloading these files. For the reviewer’s comment about the RMSD data, we are now providing an excel file of RMSD for downloading.

- "Additionally, the conclusions of their manual (Human) analysis of the results are displayed but no data are given online as to the fraction of time that systems spends out of the 25 ns simulation in any of the local conformations that lead to their proposed mechanism of loss of ribonuclease activity mechanisms. Additionally the authors should state online as part of the results given what threshold values are used to conclude based on the dynamics that any of these proposed mechanisms are not favoured in the mutant under study."

As suggested by the reviewer, we are now incorporating the data on the time spent by the mutant out of 25 ns in the local conformations in the file sent to the user through e-mail. We are also adding the threshold values used to predict whether a mutation will exhibit any loss-of-functions.

- "What I would like to see in Jmol is the position of the selected mutation (with the original amino acid)." We are working on the Jmol visualization at present and will be adding this feature as soon as possible.

- "Regarding the speed of MD simulations, it is not relevant as this discussion was part of my original report in trying to quantify when I could expect the results to arrive as no calculation progress report is given. For example, an email could be sent for a query still running with a link to a results page where the percentage to completion is displayed while the results are not available."

We have incorporated a progress bar for the query progression. Once the user submits his/her Email-ID or Job-ID into the Output section of the web-tool, the output displays “Query in queue” or a “progress bar” showing the step number which has been completed.

- "I thank the authors for clarifying that several steps require manual intervention. I still think that manual intervention in the generation of results (be it transferring files across servers, checking the output file for accuracy, completeness and interpretation of results), as noted in the original report, is neither dependable nor scalable and therefore is not acceptable."

We agree with the reviewer that a completely automated process flow is desirable. However, our institute LAN does not permit us to access the Supercomputing facility (http://www.scfbio-iitd.res.in) servers without log-in. Therefore, the two steps – uploading the jobs and downloading the results are inevitably manual. Even if the next version of the web-tool is hosted in the Supercomputing facility, we will need to manually download the result, analyse the trajectory and link it to the output page of the web-tool.

- "The authors cite in their current response some of their conclusions in [their] previous reports. While the language used in the Scientific Reports manuscript is correct, stating 'probable mechanism' in the FEBS Letters paper it becomes 'determining the mechanism'. In this manuscript it turns to even more emphatic language saying 'elucidating the mechanisms'."

As per the reviewer’s comments, we have made the necessary changes in this version of
our manuscript. We have replaced the word “elucidate” with “predict” and made the required changes as suggested by the reviewer. Regarding validation of the proposed mechanisms, we would again like to emphasize that apart from a time dependence dynamics method, such as MD simulations, no other experimental technique exists at present that can be used to validate these mechanisms. However, if the reviewer reads our previous paper (Padhi et al, 2012), he can note that we have validated the proposed mechanism using proper computational control. We suggest the reviewer reads the “MD Simulations” subsection of the “Results” section - “We also validated the 50 ns period by performing a simulation of………which was then used for subsequent simulation experiments and analyses”.

- "As much as the problems with manual intervention are in my mind sufficient to object to the current manuscript, I am willing change my classification to 'accept the with reservations' as long as the manuscript is extensively rewritten, including changing the title to remove any suggestions, implicit or otherwise, that the mechanisms used to classify the mutations are in any way proven as well as to clearly state the limitations of the manual intervention steps. Furthermore, the proposed hybrid Human/Web-tool presented should be modified to give users access to a job progression report page that is frequently updated and that can be accessed at any time after submission until the results are available."

We have addressed the reviewer’s major concerns and modified the title of our manuscript. After reading, we have modified and added text in several sections of the manuscript. We have also indicated that we require manual intervention in providing the output to the user. We hope the reviewer finds this revised version of our manuscript suitable for changing the current status to “Approval”.

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 07 November 2013

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**Ammar Al-Chalabi**
Kings College London, London, UK

In this interesting paper, Aditya Padhi and colleagues describe a mechanism for predicting the effect of mutations in the ANG gene on the risk of ALS. The paper is well written, the title is appropriate, the abstract provides a good summary and there is an excellent explanation of the study design, methods and analysis.

I have only two minor recommendations:

First, the authors state that there is no successful therapy for ALS. This completely depends on what is meant by successful; there are many therapies and it is the only neurodegenerative disease for which a
disease-modifying therapy exists - riluzole. I agree there are no curative or disease-halting therapies. Perhaps this sentence could be rephrased.

Secondly, the authors state that functional assays have been used to predict whether certain mutations cause ALS. I would argue that this is not possible. They can only show a laboratory-based mechanism that is consistent with ALS causation.

It would be helpful if the authors could provide the URL for the ALSoD database at first citation. (http://alsod.iop.kcl.ac.uk).

**Competing Interests:** I help to curate the ALSoD database.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.