TSHR169 Antigen Specifically Binds to the Thyroid-stimulating Autoantibody, Representing an Effective Biomarker for Graves' Disease

Zulkarnain Zulkarnain[1,2], Hidayat Sujuti[3], Djoko Wahono Soeatmadji[4], Didik Huswo Utomo[5,6], Aulanni'am Aulanni'am[3,7*]

[1]*Biomedical Sciences Department*
Brawijaya University
Malang, Indonesia

[2]*Physiology Department, Syiah Kuala University*
Banda Aceh, Indonesia
E-mail: zulkarnain_md@unsyiah.ac.id

[3]*Biochemistry and Biomolecular Department, Brawijaya University*
Malang, Indonesia
E-mail: hidayatsujuti@yahoo.com, aulani@ub.ac.id

[4]*Internal Medicine Department, Saiful Anwar General Hospital*
Malang, Indonesia
E-mail: djokowsoeatmadji@yahoo.com

[5]*Research and Education Center for Bioinformatics,*
Nusantara Institute of Science and Technology
Malang, Indonesia

[6]*Biology Department, Brawijaya University*
Malang, Indonesia
E-mail: didik.huswo@gmail.com

[7]*Faculty of Veterinary Medicine, Brawijaya University*
Malang, Indonesia
E-mail: aulani.fkhub@gmail.com

[*]*Corresponding author*

Received: March 01, 2018 **Accepted: January 08, 2019**

Published: March 31, 2019

Abstract: *The thyroid-stimulating hormone receptor (TSHR) is one of the thyroid antigens responsible for Graves' disease and acts as a biomarker for early detection. The purpose of this study was to computationally compare the effectiveness of TSHR56 and TSHR169 fragments in binding to the thyroid-stimulating human monoclonal autoantibody (M22) at the molecular level. The 3D model of M22 was obtained from RCSB (ID 3G04), while the TSHR antigen models were submitted to homology modeling using SWISSMODEL and PhyreV2.0 server to predict the protein structures. The model was validated by generating a Ramachandran plot with the RAMPAGE server. Prediction of the molecular interaction between TSHR and M22 was performed using the HADDOCK web server (version 2.2). Analysis of the binding affinity was conducted using the PRODIGY server. Interactions within the TSHR-M22 complex were analyzed using DIMPLOT. The antigen models had reliability scores of 100% and 97.7% for TSHR56 and TSHR169, respectively. The results of the molecular docking analysis revealed a better HADDOCK score for TSHR169 (-144.7 ± 2.4)*

compared to TSHR56 (-53.3 ± 10.1). However, the affinity of TSHR56 (-10.1 Kcal/mol) and TSHR169 (-10.2 Kcal/mol) were not significantly different. The results of the TSHR-M22 interaction specificity analysis suggested that TSHR169 is superior to TSHR56, as the number of interacting amino acids was comparable to the control (TSHR260-M22). It can be concluded that TSHR169 represents a specific full-length TSHR antigen and can be developed as biomarker for Graves' disease.

Keywords: *Graves' disease, Molecular docking, Thyroid-stimulating antibody, TSHR56, TSHR169.*

Introduction

Graves' disease is the most common cause of hyperthyroidism, characterized by an enlarged thyroid gland (goitre) with an increase in diffuse toxic activity (thyrotoxicosis). Extrathyroid manifestations such as protrusion of the eye (ophtalmopathy) and skin lesions (dermopathy) can be found in Grave patients [2, 6]. Report on Result of National Basic Health Research in Indonesia, revealed that hyperthyroid disorders are still present on the top five of non-communicable diseases, with a prevalence of about 0.4-0.7% [17].

The presence of thyroid-stimulating autoantibodies (TSAb) that interact with the thyroid-stimulating hormone receptor (TSHR) underlies the development of Graves' disease [16]. The TSHR antigen has two subunits, namely A and B. Subunit A is encoded by exons 1-8, consisting of the extracellular domain and part of leucine-rich repeat domain (LRRD), which represent the main areas of TSAb binding [10]. Subunit A of TSHR has been reported to be more effective at inducing a TSAb response compared to when it is part of a holoreceptor [3].

The interaction of the TSHR subunit A (residues 1-260) in the thyroid-stimulating human monoclonal autoantibody (M22) complex had been previously reported by Sanders et al. [20] and had also been crystallized (PDB ID: 3G04). However, it is still unknown whether the subunit A fragment of TSHR (e.g., TSHR56 or TSHR169) influences its recognition of and interaction with TSAb. Bioinformatics approaches provide sophisticated applications to design functional antigen peptides that are capable of interacting specifically with target proteins and antibodies [12, 19].

The computational approach will guide the specific and accurate design of antigen (TSHR). The structure of the TSHR subunit A fragment was cut to create two peptides (TSHR56 and TSHR169). Both peptides were expected to have the ability to recognize and interact with the Fab region of M22 both in vitro and in vivo. The purpose of this study was to compare the effectiveness of TSHR56 and TSHR169 antigen fragments in binding to M22 via a computational modeling approach.

Materials and methods

Sample collection

The TSHR sequence was obtained from the UniProt proteomic database (P16473; http://www.uniprot.org). The M22 Fab antibody structure was obtained from the crystallography data available in the RCSB database (PDB ID 3G04; www.rcsb.org/pdb).

Analysis of the conserved region

Several variations of the human TSHR mRNA sequence were obtained from the UniProt database and alignment of these sequences was analyzed using ClustalW in BioEdit software [5]. Subsequently, the non-polymorphic regions (conserved regions) within the 13 variants of the sequences were determined. The purpose of this analysis was to obtain a fragment of the TSHR antigen that could be recognized by the antibodies of all individuals.

Epitope mapping analysis

Epitope mapping was performed to predict the conserved region capable of binding to antibodies. This analysis was performed with BepiPred-2.0 server (http://www.cbs.dtu.dk/services/BepiPred/ or www.iedb.org) [8]. The combination of conserved and epitope areas was considered when dividing peptide fragments into TSHR56 (exon 1) and TSHR169 (exons 1-6).

Modeling of TSHR56 and TSHR169

Both peptide targets were modeled through a homology model approach using the Phyre server (version 2.0; http://www.sbg.bio.ic.ac.uk/phyre2) [9] for TSHR56 and the SWISSMODEL server (http://swissmodel.expasy.org) [1] for TSHR169. Homology modeling is able to accurately predict protein models because it uses a protein structure template [4, 11]. The second structure of the model was validated by Ramachandran plot assessment, performed using RAMPAGE server (http://www-cryst.bioc.cam.ac.uk/servers) [14]. Structural models with low favorable values and low model quality were improved using MODREFINER server (http://zhanglab.ccmb.med.Umich.edu/ModRefiner/) [22].

Analysis of molecular docking

Molecular docking was used for determining the favorable interaction between receptor and ligands [19, 21]. This study, TSHR56/169 employed as receptors and M22 recognized as ligands. The TSHR56 (amino acid residues 22-56) and TSHR169 (amino acid residues 36-169) models were used to predict their specific docking interactions using HADDOCK web server (http://milou.science.uu.nl/services/HADDOCK2.2). The HADDOCK application is able to accurately predict protein complexes because it uses algorithms for flexible docking as well as more specific parameters [21]. The affinity analyses for both antigen molecules against antibodies were conducted using the PRODIGY server application (http://milou.science.uu.nl/services/PRODIGY) [23].

Analysis of molecular interaction

The complex results then needed to be further analyzed to determine the amino acids involved in the interaction in order to show its specificity. The analysis was performed using DIMPLOT software in LigPlotPlus (version 1.4.5) and PIM SERVER (http://caps.ncbs.res.in/pima/) [13, 15]. The interaction results were then compared with the TSHR260-M22 reference control published by Sanders et al. [20]. All biomolecules were visualized using the PyMol application (version 1.3).

Results and discussion

Design of TSHR56 and TSHR169 antigens

The TSHR antigen is 760 amino acids in length. The antigen design aimed to obtain short fragments that remained functional. The two main analysis used were conserved region and epitope mapping analysis. Conserved region analysis of the 13 variants of human TSHR mRNA

revealed three non-polymorphic amino acid sequences, which were amino acid residues 1-134 (CR1), 136-182 (CR2) and 184-224 (CR3) (Fig. 1).

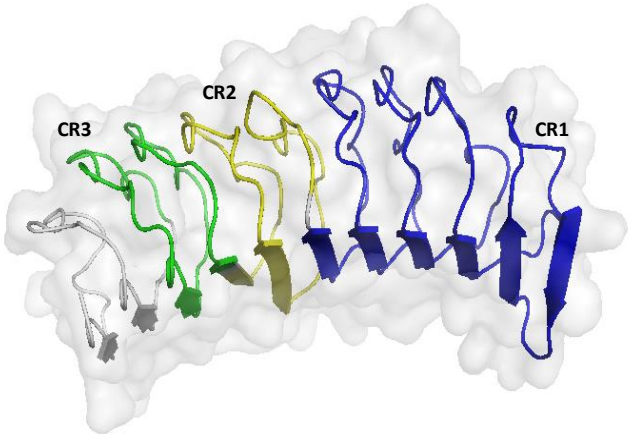


Fig. 1 The conserved regions of the thyroid-stimulating hormone receptor (TSHR) are colored blue (CR1, amino acid residues 30-134), yellow (CR2, amino acid residues 136-182) and green (CR3, amino acid residues 184-224)

Furthermore, the selected antigen areas bound to the antibodies (epitope) based on three conserved region sequences. It is important to obtain a short TSHR antigen sequence while it is still being able to recognize antibodies from all individuals. Epitope mapping prediction revealed that the TSHR epitope region was more dominant in the CR1 and CR2 (TSHR169) regions (Fig. 2). These results are also supported by those of Sanders et al. [20], who reported that the residue sequence 1-260 (TSHR260) bound to human monoclonal autoantibody (M22).

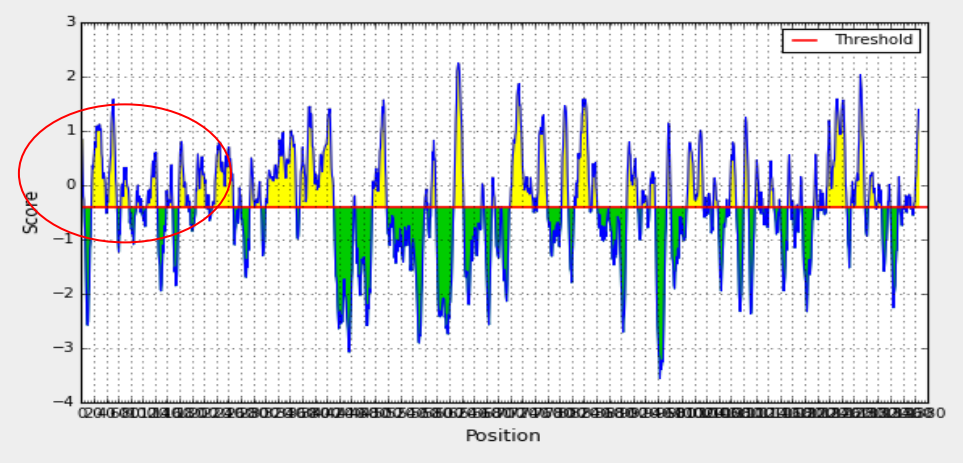
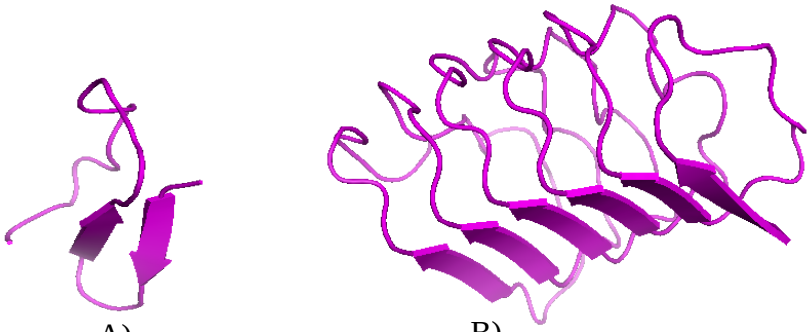


Fig. 2 The thyroid-stimulating hormone receptor (TSHR) protein epitope area predicted using the Bepipred Linear Epitope Prediction method (www.iedb.org). The red circle indicates the epitope region in CR1 and CR2.

Modeling of TSHR56 and TSHR169 antigens was performed based on the conserved region and epitope mapping that had been done previously (Fig. 3). Three-dimensional structural modeling of both antigen peptides was performed through a homology modeling approach that was validated by a Ramachandran score of 100% and 97.7% for TSHR56 and TSHR169, respectively (Table 1, Fig. 4).

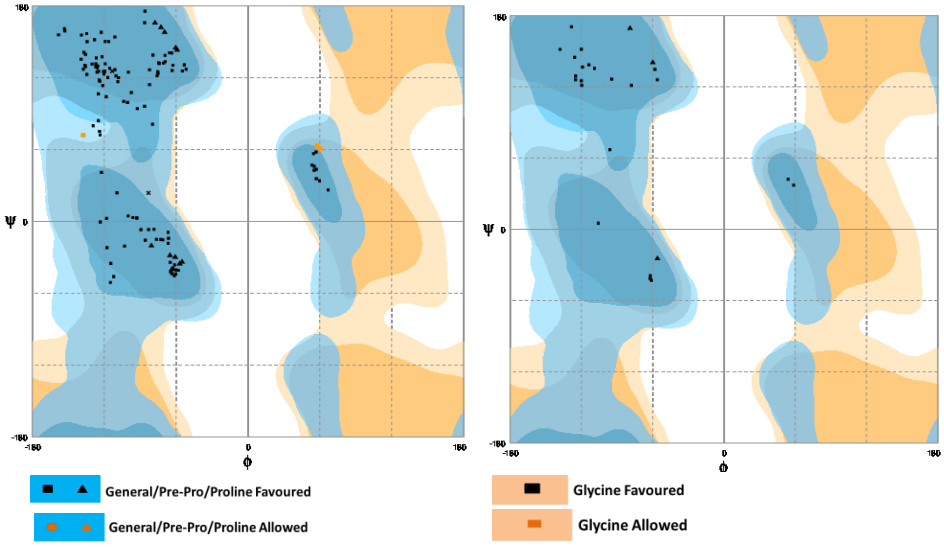


A) B)

Fig. 3 Three-dimensional structural modeling of TSHR56 (A) and TSHR169 (B)

Table 1. Evaluation of 3D model quality based on the Ramachandran score

Antigen model	Ramachandran score (favored region)	
	Before refinement	After refinement
TSHR56	88%	100%
TSHR169	94.7%	97.7%

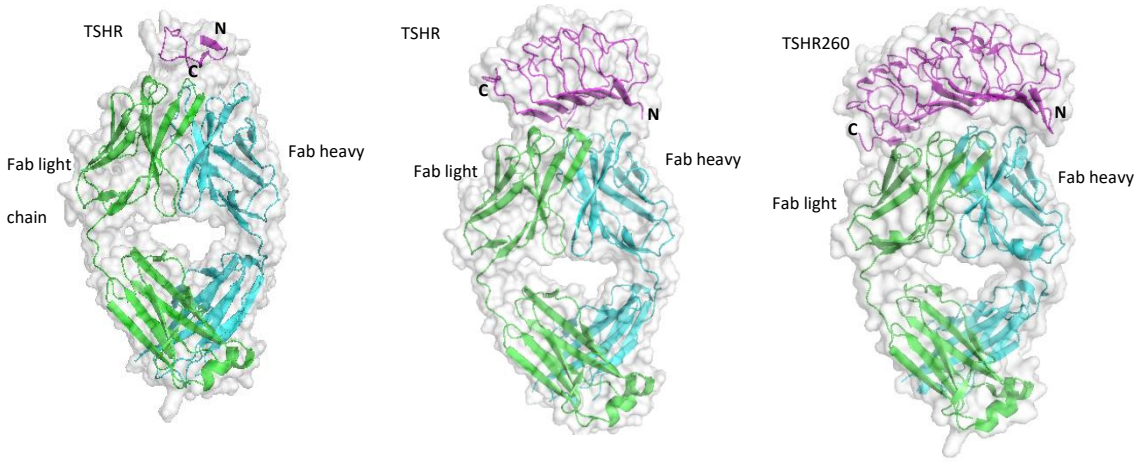


A) B)

Fig. 4 Ramachandran plot of TSHR56 (A) and TSHR169 (B) after refinement. All amino acids were plotted in favored region (Ramachandran quadran).

Comparison of the interaction affinity of TSHR56 and TSHR169 with M22 Fab

Interactions between antigens and antibodies are considered to be good when they have a high affinity and interact in specific regions according to the cellular conditions. In this study, interaction types were compared to the modeled x-ray results of the complex published by Sanders et al. [20]. The TSHR56 and TSHR169 docking results revealed binding to an antibody paratope similar to the control TSHR260-M22 (Fig. 5). TSHR169 was found to have a slightly higher affinity (-10.2 Kcal/mol) compared to TSHR56 (-10.1 Kcal/mol) (Table 2).



A) B)

Fig. 5 Three-dimensional structural modeling results for docking of the TSHR56 and TSHR169 peptides in the M22 Fab complex (A).
The 3D structural model of TSHR260-M22 was used as a control (B).

Table 2. Docking scores and affinity energy of TSHR fragment interactions within the M22 Fab complex

Antigen	Monoclonal antibodies	Sequence of TSHR residues	HADDOCK score	Affinity energy, (Kcal/mol)
TSHR56	M22 Fab (TSAb)	22-56 AA	-53.3 ± 10.1	-10.1
TSHR169		36-169 AA	-144.7 ± 2.4	-10.2
TSHR260		22-260 AA	-204.7 ± 3.7	-11.5

The docking score reveals the favorable complex, but it does not represent the affinity. The binding affinity could be used to better predict the antigen-antibody interaction. The lowest affinity energy score indicates the antigen with the best binding affinity. The results revealed that TSHR260, the control, had the lowest affinity. The comparison of TSHR56 and TSHR169 was slightly different which TSHR169 found to have a higher affinity than TSHR56. These results suggest that this design should be appropriate for further development and that detailed evaluation of the interaction between complexes will reveal the best antigen design. The hydrogen bonding, electrostatic interaction and hydrophobic interactions of both complexes were compared with the TSHR260-M22 model as a reference (Table 3).

Antibody (M22) bound antigen (TSHR) to through weak and noncovalent interactions such as electrostatic interactions, hydrogen bonds, and hydrophobic interactions. The result showed that TSHR169 has similarity with the control group based on the amino acids that involved in the interaction. Evaluating of type of interaction is important to reveal the affinity and specificity of antibody-antigen interaction. The contribution of all interaction types depends on the composition of amino acid from antibody and antigen. In addition, the hydrophobic interaction operate over very short ranges and act to pull together two surfaces that are complementary in shape Meanwhile, electrostatic interactions between charged side chains, and hydrogen bonds bridging oxygen and/or nitrogen atoms, accommodate specific features or reactive groups while strengthening the interaction overall [7, 18]. There were three hydrogen bonds in the TSHR56-

M22 interaction, while the interaction between TSHR169 and the M22 Fab complex was found to have ten hydrogen bonds. Hydrophobic and electrostatic interaction were found in TSHR169 is quite similar with the TSHR260 (control). The similarity indicated with the bold one. In contract, TSHR56 is significantly different with the control group. The molecular interaction analysis suggest that the TSHR169-M22 complex which has a stable structure and low-affinity energy is very promising for further development in vivo and in vitro studies.

Table 3. Amino acid interactions of several TSHR fragments in the M22 Fab complex

Hydrogen bonds[a]	TSHR56 **R38**, D43, T56	TSHR169 **R38, K58, R80**, T104, **E107**, R109, **K129, D151**, **E157**, D160	TSHR260 **R38, K58, R80, E107, K129, D151, E157, K183, Y185, N208, Q235, R255**
	M22 Fab D93(A), **D54(B), Y99(B)**	M22 Fab N31(A), **Y50(A),** R66(A), D93(A), **D95A(A), R28(B), T30(B), D54(B), T57(B), Y99(B),** S100(B)	M22 Fab Ref **Y49(A), Y50(A), D52(A), Q53(A), V58(A), D95A(A), R28(B), T30(B), D52(B), T53(B), D54(B), T57(B), E96(B), G98(B), Y99(B)**
Hydrophobic interaction[a]	TSHR56 E34, D36, V39, T40, K42, I44, R46, I47, Q55	TSHR169 D36, **T56, Y82**, H105, **F130, I152, F153, I155**	TSHR260 **R38, T56, K58, R80, Y82, F130, I152, F153, I155, Y185, K209, R255**
	M22 Fab N30(A), D95A(A), W33(B), D52(B), T54(B), **Y56(B), P97(B), G98(B)**	M22 Fab N30(A), S31(B), Y32(B), W33(B), T54(B), **Y56(B),** E96(B), **P97(B), G98(B), W100C(B)**	M22 Fab Ref **Y49(A), Y50(A), Q53(A), L54(A), R28(B), Y56(B), P97(B), G98(B), Y99(B), W100C(B)**
Electrostatic interaction[b]	TSHR56 E34, E35, D36, R38, K42, D43	TSHR169 **K58, R80,** H105, R109, **K129, D151**, D160	TSHR260 **K58, R80, K129, D151, K183, K209, R255**
	M22 Fab D93(A), **D95A, D52(B), D54(B)**	M22 Fab **D51(A),** R66(A), D93(A), **D95A**, **R28(B), D52(B), D54(B)**	M22 Fab Ref **D51(A), D52(A), D60(A), D95A, R28(B), D52(B), D54(B), E96(B)**

Note: Bold amino acid indicates it has the same binding site with the control
[a]Identified with DIMPLOT software in LigPlotPlus (version 1.4.5)
[b]Identified with PIMA server

In the crystalline structure image, the interaction of the stimulant-type autoantibodies (M22 Fab) appears to occur slightly further away from the TSHR N-terminal and does not interact with the extreme N-terminal portion of the LRD TSHR present in the amino acid sequence 22-34. The initial interaction of the M22 antibody has been reported to occur at Arg38

within the LRD TSHR (distance < 4Å) [7]. Antibody has specificity and the affinity when interacting with antigen and the mechanism due to amino acid side chain or all of the hypervariable loops [7].

Conclusion

TSHR169 represents a TSHR antigen biomarker for Graves' disease as it is both specific and efficient in terms of production cost. TSHR169 is able to bind to M22 Fab with a HADDOCK score of -144.7 ± 2.4 and the affinity energy is equal to -10.2 Kcal/mol, indicating its ability to bind specifically to the thyroid-stimulating autoantibody.

Acknowledgements

The authors wish gratefully to acknowledge the financial assistance received from the Directorate General of Strengthening for Research and Development at the Ministry of Research, Technology and Higher Education through Doctoral Research Grant No 119/UN11.2/PP/SP3/2017.

References

1. Biasini M., S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. G. Cassarino, M. Bertoni, L. Bordoli, T. Schwede (2014). SWISS-MODEL: Modelling Protein Tertiary and Quartenary Structure Using Evolutionary Information, Nucleic Acids Research, 42(Web Server issue), W252-W258.
2. Braverman L. E., D. S. Cooper (2013). Thyroid Diseases: Thyrotoxicosis. In: Werner & Ingbar's The Thyroid: A Fundamental and Clinical Text, 10th Edition, Philadelphia: Lippincott William and Wilkins, 354-360.
3. Chen C. R., P. A. Hubbard, L. M. Salazar, S. M. McLachlan, R. Murali, B. Rapoport (2015). Crystal Structure of a TSH Receptor Monoclonal Antibody: Insight into Graves' Disease Pathogenesis, Molecular Endocrinology, 29(1), 99-107.
4. Fiser A. (2010). Template-based Protein Structure Modelling, Methods in Molecular Biology, 673, 73-94.
5. Hall T. (2011). BioEdit: An Important Software for Molecular Biology, GERF Bulletin of Biosciences, 2(1), 60-61.
6. Heuck C. C., A. Kallner, A. S. Kanagasabapathy, W. Riesen (2000). Diagnosis and Monitoring of Diseases of the Thyroid, WHO Document, 4-5.
7. Janeway C. A., P. Travers, M. Walport, M. J. Shlomchik (2001). Immunobiology: The Immune System in Health and Disease (5 Ed.), New York, Garland Science.
8. Jespersen M. C., B. Peters, M. Nielsen, P. Marcatili (2017). BepiPred-2.0: Improving Sequence-based B-cell Epitope Prediction Using Conformational Epitopes, Nucleic Acids Research, 45(Web Server Issue), W24-W29.
9. Kelley L. A., S. Mezulis, C. M. Yates, M. N. Wass, M. J. E. Sternberg (2015). The Phyre2 Web Portal for Protein Modelling, Prediction and Analysis, Nature Protocols, 10(6), 845-858.
10. Kleinau G., S. Neumann, A. Grüters, H. Krude, H. Biebermann (2013). Novel Insights on Thyroid-stimulating Hormone Receptor Signal Transduction, Endocrine Reviews, 34(5), 691-724.
11. Krishnan S., S. Ravi, S. Ponmalai, P. Rani (2015). A Molecular Dynamics Study on RAGE-Aβ42 Interaction and the Influence of G82S RAGE Polymorphism on Aβ Interaction, International Journal Bioautomation, 19(4), 433-446.
12. Kumar S., L. Jena, S. Galande, S. Daf, A. K. Varma (2014). Molecular Docking Explains Atomic Interaction between Plant-originated Ligands and Oncogenic E7 Protein of High Risk Human Papillomavirus Type 16, International Journal Bioautomation, 18(4), 315-324.

13. Laskowski R. A., M. B. Swindells (2011). LigPlot+: Multiple Ligand-protein Interaction Diagrams for Drug Discovery, Journal of Chemical Information and Modeling, 51(10), 2778-2786.
14. Lovell S. C., I. W. Davis, W. B. Arendall III, P. I. W. de Bakker, J. M. Word, M. G. Prisant, J. S. Richardson, D. C. Richardson (2003). Structure Validation by Calpha Geometry: Phi, Psi and Cbeta Deviation, Proteins, 50(3), 437-450.
15. Mathew O. K., R. Sowdhamini (2016). PIMA: Protein-protein Interaction in Macromolecular Assembly – A Web Server for its Analysis and Visualization, Bioinformation, 12(1), 9-11.
16. McLachlan S. M., B. Rapoport (2013). Thyrotropin-blocking Autoantibodies and Thyroid-stimulating Autoantibodies: Potential Mechanisms Involved in the Pendulum Swinging from Hypothyroidism to Hyperthyroidism or vice versa, Thyroid, 23(1), 14-24.
17. National Institute of Health Research and Development (NIHRD), Ministry of Health (Indonesia) (2013), Report on Result of National Basic Health Research, 83-90.
18. Patil R., S. Das, A. Stanley, L. Yadav, A. Sudhakar, A. K. Varma (2010). Optimized Hydrophobic Interaction and Hydrogen Bonding at the Target-ligand Interface Leads the Pathway of Drug-designing, PLoS One, 5(8), e12029.
19. Pedotti M., L. Simonelli, E. Livoti, L. Varani (2011). Computational Docking of Antibody-antigen Complexes, Opportunities, and Pitfalls Illustrated by Influenza Hemagglutinin, International Journal of Molecular Sciences, 12(1), 226-251.
20. Sanders J., D. Y. Chirgadze, P. Sanders, S. Baker, A. Sullivan, A. Bhardwaja, J. Bolton, M. Reeve, N. Nakatake, M. Evans, T. Richards, M. Powell, R. N. Miguel, T. L. Blundell, J. Furmaniak, B. R. Smith (2007). Crystal Structure of the TSH Receptor in Complex with a Thyroid-stimulating Autoantibody, Thyroid, 17(5), 395-410.
21. Van Zundert G. C., A. M. Bonvin (2014). Modelling Protein-protein Complexes Using the HADDOCK Web Server "Modeling Protein Complexes with HADDOCK", Methods in Molecular Biology, 1137, 163-179.
22. Xu D., Y. Zhang (2011). Improving the Physical Realism and Structural Accuracy of Protein Models by A Two-step Atomic-level Energy Minimization, Biophysical Journal, 101, 2525-2534.
23. Xue L. C., J. P. Rodrigues, P. L. Kastritis, A. M. Bonvin, A. Vangone (2016). PRODIGY: A Web Server for Predicting the Binding Affinity of Protein-protein Complexes, Bioinformatics, 32(23), 3676-3678.

Zulkarnain Zulkarnain, Ph.D. Student
E-mail: zulkarnain_md@unsyiah.ac.id

Zulkarnain worked as a lecturer and researcher at the Physiology Department of Medical Faculty, Syiah Kuala University. He has completed M.Sc. degree in Basic Medical and Biomedical Science from the Gadjah Mada University, Yogyakarta, Indonesia. His research activities are focused on endocrine and metabolic disorders and male infertility. Currently, he is a Ph.D. candidate for the Medical Science Program at the Brawijaya University.

Hidayat Sujuti, Ph.D.
E-mail: hidayatsujuti@yahoo.com

Hidayat Sujuti is an Ophthalmologist at Saiful Anwar Hospital, Malang, Indonesia. He is also a lecturer in Biochemistry Department, Faculty of Medicine, Brawijaya University. His research interests are in cell and molecular biology, cancer research, biotechnology and immunology.

Prof. Djoko Wahono Soeatmadji
E-mail: djokowsoeatmadji@yahoo.com

Djoko Wahono Soeatmadji is a Professor at the Internal Medicine Department, Brawijaya University. He is an endocrinologist at Saiful Anwar Hospital, Malang, Indonesia. His research interests are in endocrinology, diabetes and metabolism.

Didik Huswo Utomo
E-mail: didik.huswo@gmail.com

Didik Huswo Utomo is a bioinformatician. He received his B.Sc. degree at the Biology Department of the Brawijaya University, and M.Sc. degree at the Bandung Institute of Technology, Indonesia, with concern in Bioinformatics. Currently, he is a Senior Bioinformatics Trainer in Indonesian Institute of Bioinformatics and works as a Guest Lecturer at the Biology Department, Brawijaya University.

Prof. Aulanni'am Aulanni'am
E-mail: aulani@ub.ac.id

Aulanni'am is a Professor in Biochemistry, Faculty of Sciences and Faculty of Veterinary Medicine, Brawijaya University. Her research interests are in molecular markers for metabolic disorders and autoimmune diseases, biosimilar materials, medical devices and immunocontraception. She has also developed animal model for diabetic, autoimmune thyroiditis, hypercholesterolemia, inflammatory bowel disease, rheumatoid arthritis, and cancer rats that has led to the discovery of its molecular marker.