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Antibody Engineering to Enhancement of Ranibizumab Binding Affinity for the Prevention and Treatment of Diabetic Retinopathy

Fateme Sefid[1], Kimia Monshizadeh[1], Ghasem Azamirad[2], Mohammad Yahya Vahidi Mehrjardi[3*]

[1]Department of Medical Genetics, Shahid Sadoughi University of Medical Science, Yazd, Iran
[2]Department of Mechanical Engineering, Yazd University, Yazd, Iran
[3]Diabetes Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Abstract

Objective: The VEGF function blockage effectively reduces the progression of diabetic retinopathy. Ranibizumab and bevacizumab are some anti-VEGF monoclonal antibodies (mAb). Considering the importance of affinity maturation of ranibizumab, we aimed to find the essential amino acids of the ranibizumab antibody (Ab).

Materials and Methods: We tried to find the important amino acids of this antibody via Paratome, Meta-PPISP, and the WESA web server. Subsequently, these amino acids were mutated to improve the binding affinity of the Ab variants to antigen (Ag). In this regard, the ranibizumab anti-VEGF-A was mutated. The structural docking prediction of the ranibizumab-VEGF-A complex was used for the design and validation of ranibizumab with a higher affinity for binding to VEGF-A. Finally, we measured the binding affinity of Ab variants to Ag by computational docking.

Results: Bioinformatic analyzes such as molecular docking and dynamics showed that several mutant variants successfully improved the properties of Ab binding compared to the wild-type Ab.

Conclusion: Consistent with the use of anti-VEGF monoclonal antibodies in the treatment of diabetic retinopathy, the mutant variants of ranibizumab may be potential candidates for stronger affinity binding to VEGF, which may affect the specificity and sensitivity of the antibody.

Keywords: Antibody engineering, Anti-VEGF-A, Bioinformatic, Binding affinity, Ranibizumab

QR Code:

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Corresponding Author:
Mohammad Yahya Vahidi Mehrjardi, Diabetes Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
Tel: (98) 913 351 4629
Email: mmvahidi@gmail.com
Orcid ID: 0000-0003-0535-1590

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Introduction

Diabetes mellitus is widespread, so its prevalence and complications threaten global health (1,2). Prolonged uncontrolled blood sugar develops microvascular and macrovascular complications (3). One of the most common microvascular complications is diabetic retinopathy (DR). DR is the main cause of vision loss in diabetics (4). High glucose levels and the accumulation of defective proteins lead to wall thickening and endothelial damage of the small retinal vessels. These vascular transformations lead to reduced blood supply of the retinal tissue, destruction of the retinal blood barrier (BRB), and increased permeability of the endothelium, which eventually causes leakage of fluid and proteins. Meanwhile, an inflammatory angiogenic cascade progresses to regenerate pathological small vessels through the upregulation of vascular endothelial growth factor (VEGF) and inflammatory cytokines (5). The small vessels cause the formation of fibrous tissue, which worsens retinopathy and eventually extends vision loss. This state of disease is called proliferative diabetic retinopathy (PDR) (6).

VEGF is a potent mitogen that stimulates cohesive and sufficient vasculogenesis and the angiogenesis process in retinal tissue. In patients with DR, VEGF plays a pathological role in the formation of new vessels, which are irregular and prone to fluid leakage. VEGF targets the formation of tight junctions (TJ) within the BRB, leading to endocytosis of key TJ proteins and a subsequent increase in permeability (7). Therefore, blocking the function of VEGF effectively reduces the progression of DR. A study has shown that anti-VEGF injections can replace the only conventional treatment for DR, pan-retinal photocoagulation (PRP). Although PRP improves vision in DR patients, it has some complications such as nyctalopia, worsening of macular edema, and pain during treatment, so new methods such as anti-VEGF monoclonal antibodies (mAb) are needed (8). Anti-VEGF therapy is a new method for the treatment of DR. Ranibizumab, bevacizumab and faricimab are some anti-VEGF mAbs that have been approved by the Food and Drug Administration (FDA) for the treatment of DR (9).

Ranibizumab is a human mAb fragment that binds to all different isomers of VEGF-A and prevents its binding to VEGF-A receptors. Thus, ranibizumab inhibits angiogenesis and vision loss in DR. The molecular weight of ranibizumab is 48 kDa, which allows it to penetrate the inner layers. Although ranibizumab has only one binding site for VEGF-A, its binding affinity is 5-20 times higher than bevacizumab. The serum and intravitreal half-life of ranibizumab is 2 hours and 7-9 days, respectively (10). Human mAbs have revolutionized the treatment of human diseases. The broad spectrum of administration of mAbs is due to their lower off-target adverse effects, fewer drug-drug interactions, good solubility and stability, low immunogenicity, higher specificity, and potentially higher efficacy through targeted therapy (11,12).

In addition, the particular structural and functional organization of mAbs offers the possibility of genetic manipulation. Optimizing the affinity of mAbs by antibody engineering methods increases efficiency, reduces production costs, and the required dosage (13). A variety of different affinity maturation strategies increase the affinity of mAbs, replacing specific or random residues in the loops of the complement-determining region (CDR) along the entire variable fragment (Fv). Affinity maturation strategies have focused on the CDR L3 and in particular the CDR H3 residues associated with the paratope region, which are usually responsible for most stabilizing contacts (14). Affinity maturation includes error-prone PCR, random

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mutagenesis, and site-directed mutations. Site-directed mutagenesis is an in vitro gene modification that targets a specific segment of the DNA sequence to study the function of the sequence structure (15).

Optimizing the affinity of mAbs by site-directed mutagenesis involves several steps, such as 3D structural data and interactions of the Ab-Ag complex, sequencing of CDRs, selection of optimal residues, site-directed mutation, Ab production, and finally measurement of performance using techniques such as molecular docking and dynamics (16). It should be noted that both the variable regions of the light and the heavy chain are involved in the binding of the paratope to the Ag. Therefore, mutations and changes in the antibody sequence give rise to new types that need to be structurally analyzed (17).

Considering the suitability of the anti-VEGF-A Ab for the treatment and prevention of DR and the need to improve the performance of Abs, including increasing the affinity of the Ab to the desired target, the aim of this research is to improve the treatment of DR through the use of mAbs. In this context, it is necessary to improve the binding affinity of the Ab to the Ag using targeted mutagenesis methods in the amino acids of the variable part of the ranibizumab mAb.

Material and methods

Figure 1 shows the overall bioinformatics path of this study. Each of the steps will be explained in detail below.

Ranibizumab Complementarity-Determining Region Prediction

The binding action of the antibody is primarily intervened through the complementarity-determining locale (CDR). The Paratome web server, available at http://ofranservices.biu.ac.il/site/services/paratome, predicts an antibody's antigen-binding regions (ABRs) according to antibody structure or its sequence. Paratome was built by fundamentally adjusting a non-redundant set of all known antibody-antigen complexes within the PDB, from which basic consensus components that are commonly included in

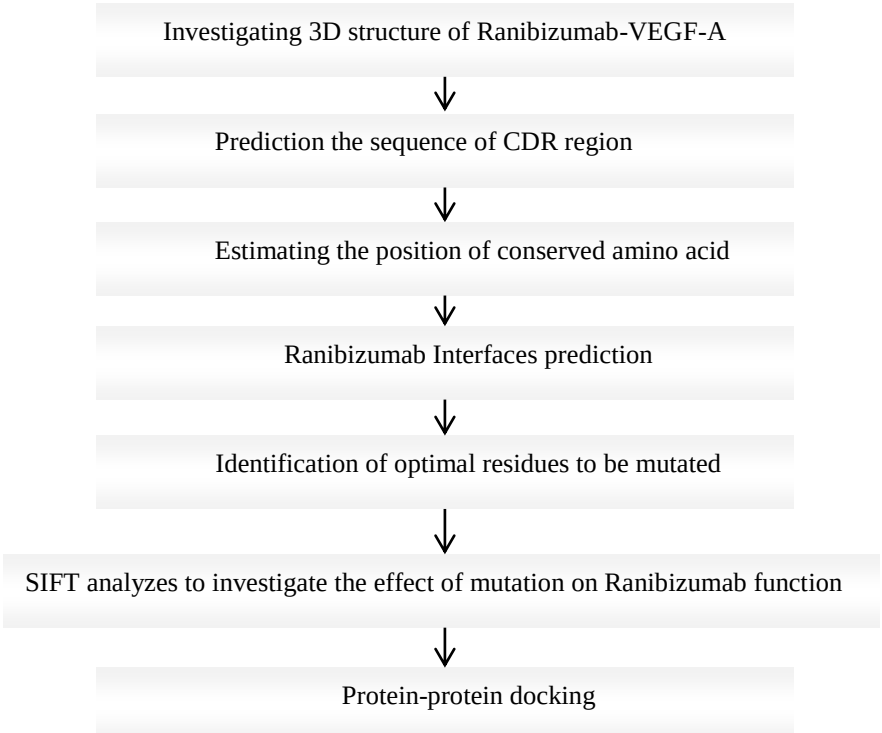

Figure 1. Scheme of the full procedure. The methodology used to perform affinity maturation through in silico modeling is

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antigen authoritative over antibodies were distinguished.

Ranibizumab conservation of amino acid positions evolution

The ConSurf server at https://consurf.tau.ac.il/ is a bioinformatics tool for estimating the evolutionary conservation of amino acid positions in a protein molecule based on the phylogenetic relations between homologous sequences. The degree to which an amino corrosive position is developmentally preserved (i.e., its developmental rate) is significantly dependent on its basic and useful significance. Hence, preservation examination of positions among individuals from the same family can regularly uncover the significance of each position for the protein's structure or work. In ConSurf, the developmental rate is evaluated based on the developmental relatedness between the protein and its homologs and considering the similitude between amino acids as refl ected within the substitutions framework.

Ranibizumab Interfaces prediction

xgBoost-based Interface Prediction of Specific Partner Interactions (BIPSPI) at http://bipspi.cnb.csic.es/xgbPredApp/ is a new method for the prediction of partner-specific protein interfaces from PDB files or input sequences. BIPSPI utilizes Extraordinary Slope Boosting (XGBoost) models prepared on the buildup sets of the protein complexes compiled in Protein-Protein Docking Benchmark adaptation 5 and a scoring work that changes over combined forecast to interface buildup expectations.

Significant residues selection

Some amino acids were chosen as noteworthy residues within the Ranibizumab structure by utilizing the about of a distinctive program. These residues are found in one of three CDR locales anticipated by the Paratome server. Chosen residues have a score over 0.5 in the BIPSPI computer program, a score over 0.00 in the Cons PPISP program, and a score over 4 in the GHecom computer program. In this respect, PredUS, Meta-PPISP, and WESA predicted residue investigations to choose the critical amino acids.

SIFT analyses

The SIFT server, which is accessed at http://sift.jcvi.org/, predicts if an amino acid substitution would influence protein function. The degree of preservation of amino corrosive residues in grouping arrangements gotten from closely comparative arrangements procured utilizing PSI-BLAST is utilized to foresee Filter. The filter can be utilized to identify actually happening nonsynonymous polymorphisms as well as lab-induced missense transformations.

Ranibizumab variants sketching

Twenty variants were created, including mutations in at least one of the three CDRs. The 3D structure of all offered variants is determined by SAbPred at http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/WelcomeSAbPred.php (The Oxford Protein Informatics Group (OPIG) created an antibody modeling and prediction software tool.)

Antigen-antibody docking

HADDOCK at http://haddock.science.uu.nl/services/HADDOCK/haddock.php uses the 3D structures of each variation and VEGF-A subunit as input. HADDOCK (High Ambiguity Driven Protein-Protein Docking) is an information-driven adaptable docking strategy for modeling biomolecular complexes.

Ethical considerations

The ethics committee of Shahid Sadoughi University of Medical approved the study proposal (Code: IR.SSU.MEDICINE. REC. 1401.101).

Results

Ranibizumab CDR Prediction

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Paratome is a browser server for identifying Antigen Binding Regions (ABRs) in antibodies. This server predicted three ABRs in the Ranibizumab heavy chain and three ABRs in the Ranibizumab light chain. These regions are GYDFTHYGMNW (26-36) as ABR1, VGWINTYTGEPTYAADF (48-64) and AVYYCAKYPYYY (92-103) as ABR2 and ABR3 in Ranibizumab heavy chain, and DISNYLNWYQQ (28-38), VLIYFTSSLHSGVPSRFSGSGSGT (46-69), and CQQYSTVPWT (88-97) as ABR1, ABR2, and ABR3 in Ranibizumab light chain. Paratome results are shown in Figure 2.

Ranibizumab conservation of amino acid positions evolution

The consurf server was utilized for the recognizable proof of utilitarian districts in proteins. The nine-color preservation scores

>QWX93389.1 ranibizumab light chain antibody fragment [synthetic construct]
DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYST
VPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY
EKHKVYACEVTHQGLSSPVTKSFNRGEC

>QWX93388.1 ranibizumab heavy chain antibody fragment [synthetic construct]
EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAV
YYCAKYPYYYGTSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
SLSSVVTVPSSSLGTQTY
ICNVNHKPSNTKVDKKVEPKSCDKTHL

Figure 2. Ranibizumab CDR Prediction. Paratome predicts 3 regions as ABRs in the Ranibizumab heavy chain and 3 regions as ABRs in the Ranibizumab light chain

Figure 3. Ranibizumabconservation of amino acid positions evolution by Consurf server. The nine-color conservation scores are projected onto the 3D structure of the antibody and the colored protein structure is shown by FirstGlance in Jmol.

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are anticipated onto the counteracting agent and antigen sequences, and the colored protein structure is outlined by FirstGlance in Jmol by the consurf server and appears in Figure 3.

Ranibizumab interfaces prediction

BIPSPI could be a device for foreseeing partner-specific protein-protein interfacing from the protein sequence or its structure. BIPSPI expectations from two input structural models were performed utilizing Anticipate from the structural information choice. It is conceivable to characterize an isolated edge for antigen and antibody expectations. These limits can be utilized to explore different anticipated precision/recall values. An estimation of the anticipated accuracy at the set edges is given as the edge. Buildups whose score has an anticipated exactness more prominent than or rise to the accuracy limit (0.500) are highlighted in red. 28, 29, 30, 49, 50, 91, 92, 93, 94, 213, and 214 in the light chain and 31, 32, 33, 50, 52, 54, 55, 101, 102, 103, 104, 106, 107, 108 and 109 in the heavy chain have an expected precision greater than the precision threshold (0.500) respectively. Figure 4 represents an interactive visualization of predicted antibody residues.

Significant residues selection

We select 28, 29, 30, 49, 50, 91, 92, 93, and 94 residues in the light chain and 31, 32, 33, 50, 52, 54, 55, 101, 102, and 103 residues in the heavy chain by employing the results of different software. These residues are located in one of three ABR Paratome predicted regions. The specially selected residues were confirmed by at least one other software. The BIPSPI score above 0.5 was considered the threshold. In this regard, ConSurf predicted residues for research to select the significant amino acids.

SIFT analyses

SIFT is a sequence homology-based tool that sorts intolerable amino acid substitutions and predicts whether a protein amino acid substitution has a phenotypic effect. SIFT is based on the assumption that protein evolution correlates with protein function. Functionally important sites should be conserved in the protein family alignment, while irrelevant sites should change in alignment. A protein of interest that requires mutations is subjected to SIFT. Regions that do not tolerate many substitutions are highlighted in red in the Score output file and we can target these regions for mutations. SIFT results for selected residues are shown in Table 1.

Ranibizumab variants sketching

20 variations counting changes in at slightest one of 3 ABRs advertised. Buildups which were affirmed by distinctive computer programs transformed in recommended variations arbitrarily.

Antigen-antibody docking

HADDOCK analyzes ligand and receptor joining based on biochemical and/or biophysical data. Table 2 shows the data on variations in which the HADDOCK score is more than the control one. The positioning of complex structures is based on HADDOCK

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>QWX93389.1 ranibizumab light chain antibody fragment [synthetic construct]
DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSG
SGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVC
LLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC

>QWX93388.1 ranibizumab heavy chain antibody fragment [synthetic construct]
EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADF
KRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTLVTVSSASTKGPSVFPL
APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY
ICNVNHKPSNTKVDKKVEPKSCDKTHL
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Figure 4. BIPSPI visualization of predicted residues in the antibody sequence. Residues whose score has an expected precision greater or equal to the precision threshold (0.500) are highlighted in red.

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Table 1. SIFT results for selected residues

Predict Not ToHerated	Position	Seq Rep	Predict ToHerated
Light chain:			
w f y m c h g e d v p a I Q H R K N	28T	1.00	T S
y w v t s r q p n m H k i h g f e d a	29C	1.00	C
w	30S	1.00	c y m h i F p V H G n d T Q R A S E K
w h y f m i q n d e k R C V H	49A	1.00	T P S G A
g d w h y r e q k s c t m N I A F V H	50P	0.99	P
	91T	0.99	c w p M q k D N E g r S I A H V f T H Y
w v t s r q p n m H k i h g f e d c a	92Y	0.99	Y
d c g e n w s k r p q t a v m H i	93Y	0.99	F H Y
y w v t s r q p n m H k i h g f e d a	94C	1.00	C
Heavy chain:			
Predict Not ToHerated	**Position**	**Seq Rep**	**Predict ToHerated**
w h y m c r e i H q k d v p g n F A	31S	0.91	T S
m w i f v H c y r p h k e A T D N S Q	32G	0.91	G
c w p m e k q n r	33Y	0.90	t D v a I H H G S Y F
w h y f m i d c n q H e v T P K S	50G	0.91	R A G
c w f m y i h v H g p s a N D T	52E	0.88	K R Q E
h w d q p n e c r g k y a t f S	54V	0.91	M I H V
w h m f y c q n r d k p E V	55G	0.86	T H I S A G
d c w p e n g k q r s t a m v i H d	101Y	0.91	F H Y
y w v t s r q p n m H k i h g f e d a	102C	0.91	C
w h y f m i r n d H k p E G V S C	103A	0.91	T Q A

Amino acid color code: nonpolar, uncharged polar, basic, acidic.
Capital letters indicate amino acids appearing in the alignment, lowercase letters result from prediction.

Table 2. Docking between the normal and 5 best mutated Ranibizumab human antibody variants with VEGF-A antigen

Variable	Control	Variant 5	Variant 11	Variant 16
HADDOCK score	-139.7 (±9.6)	-148.0 (±1.2)	-148.8 (±8.7)	-157.1 (±4.8)
CHuster size	43	76	78	21
RMSD from the overaHH Howest-energy structure	0.9 (±0.5)	0.5 (±0.3)	0.8 (±0.5)	0.6 (±0.4)
Van der WaaHs energy	-63.4 (±6.9)	-68.0 (±4.9)	-69.7 (±3.5)	-62.4 (±2.6)
EHectrostatic energy	-370.7 (±41.2)	-401.6 (±28.8)	-387.2 (±52.7)	-557.6 (±41.2)
DesoHvation energy	-9.6 (±9.3)	-7.1 (±3.1)	-12.8 (±7.4)	8.7 (±5.2)
Restraints vioHation energy	74.1 (±17.52)	74.4 (±30.52)	111.5 (±27.88)	81.4 (±30.55)
Buried Surface Area	2036.9 (±106.9)	2202.8 (±34.5)	2123.4 (±85.1)	2139.9 (±37.0)
Z-Score	-1.8	-2.4	-1.9	-2.2

scores. HADDOCK offers a completely adaptable scoring plot since the weight of the different vitality terms can be characterized independently for each stage of the docking.

Discussion

The present study aimed to produce and characterize an optimized monoclonal antibody against VEGF-A. Previous studies have shown that VEGF plays an essential role in developmental vasculogenesis and angiogenesis in retinal tissue (18). VEGA-targeted therapies have been shown to be beneficial in inhibiting angiogenesis and preventing fluid leakage in patients with DR (9,19). Several VEGF inhibitors have been approved for the treatment of diabetic retinopathy. Ranibizumab, a humanized anti-VEGF mAb, has been approved by the FDA for the inhibition of angiogenesis and vision loss in diabetic retinopathy (20). In addition, the affinity of conventional mAb needs to be optimized to improve their functional and pharmacokinetic properties such as efficiency, reduction of production costs, and required dosage (13,21). To improve the affinity of conventional monoclonal antibodies, their

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three-dimensional structure must be predicted, their critical amino acids identified and mutations made in the functional amino acids. Finally, the mutated variants are studied by in silico evaluation and modeling techniques to find the optimized variants (22).

Alternatively, mAbs have become an important part of the therapeutic field of medicine due to their potent therapeutic properties. It is estimated that approximately 80 mAbs have been approved for commercial use to date. In 2018, the FDA approved 12 new mAbs, accounting for 20% of all approved drugs. Oncologic and immunologic/infectious diseases are the main targets of most mAb therapies, but they are also used to treat many other diseases (23). MAbs have numerous important advantages as therapeutic agents, not least their targeted specificity, which is their primary mode of action. As a result, they have lower toxicity and a lower side effect profile, as is common with many small molecule drugs (24).

We employed a Paratome web server to predict the ABRs of the ranibizumab Ab. The alignment technique used by this server is based on a non-redundant set of all known antibody-antigen complexes from the PDB database.

For each chain, the Paratome web server predicted three ABRs. GYDFTHYGMNW (26-36) as ABR1, VGWINTYTGEPTYAADF (48-64) as ABR2, and AVYYCAKYPYYY (92-103) as ABR3 for the ranibizumab heavy chain. The Paratome predicted DISNYLN WYQQ (28-38), VLIYFTSSLHSGVPSRFSG SGSGT (46-69) and CQQYSTVPWT (88-97) as ABR1, ABR2 and ABR3, respectively, for the light chain of this antibody. Using BIPSPI and ConSurf, we selected 28, 29, 30, 49, 50, 91, 92, 93, 94 light chain residues and 31, 32, 33, 50, 52, 54, 55, 101, 102, 103 heavy chain residues based on the threshold considered. These residues were detected in Ag-Ab interactions using the previously mentioned software program.

These amino acids can be replaced by others that have a higher affinity for the target Ag. SIFT predicts whether a change in the amino acid will have a phenotypic effect or not.

After identifying the functional and conserved amino acids in the CDRs that can be replaced by another amino acid, the affinity of the Ab is increased. On this basis, we have developed 20 variants of the aforementioned Ab. The affinity of these developed variants with the target antigen was calculated using HADDOCK.

The stronger the affinity of the Ab for the Ag, the higher the negative score of the planned modifications. We compare the binding values of the new variants with the binding value of the primary Ab as a control. The results of the HADDOCK software binding experiments for wild-type and mutant Ab show that four of these variants have a lower score than the original Ab, indicating a greater binding affinity to the Ag.

Conclusion

According to the use of anti-VEGF mAbs in the treatment of DR, the mutant variants of ranibizumab may be potential candidates for stronger binding to VEGF, which can affect the specificity and sensitivity of the antibody.

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No funding has been received for this experiment.

Conflict of Interest

The authors pronounced that they have no dispute interest.

Authors' contributions

F. S: performed the bioinformatics section and wrote the manuscript in conclusion.

K. M: contributed data and performed the analysis.

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Gh. A: performed the analysis and wrote initial draft of the manuscript.

All authors agree to be fully accountable for ensuring the study's accuracy and have read and approved the final manuscript.

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