

Functional Recombinant Extra Membrane Loop of Human CD20, an Alternative of the Full Length CD20 Antigen

Mahdi Habibi Anbouhi¹, Aida Feiz Barazandeh¹, Saeid Bouzari², Mohsen Abolhassani³, Hossein Khanahmad⁴, Majid Golkar⁵, Mohammad Reza Aghasadeghi⁶, Mahdi Behdani¹, Ali Jahanian-Najafabadi² and Mohammad Ali Shokrgozar^{*1}

¹National Cell Bank of Iran, Pasteur Institute of Iran, Tehran; ²Molecular Biology Unit, Pasteur Institute of Iran, Tehran; ³Hybridoma Laboratory, Dept. of Immunology, Pasteur Institute of Iran, Tehran; ⁴Production and Research Complex, Pasteur Institute of Iran, Karaj; ⁵Molecular Parasitology Laboratory, Dept. of Parasitology, Pasteur Institute of Iran, Tehran; ⁶Dept. of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

Received 28 March 2012; revised 23 June 2012; accepted 25 June 2012

ABSTRACT

Background: Targeting of CD20 antigen with monoclonal antibodies has become the mainstay in the treatment of non-Hodgkin's lymphomas and immunotherapeutic depletion of malignant B cells. Accessibility of antigen is one of the crucial factors in development of monoclonal antibodies against this antigen. One major problem in expression of full length CD20 is aggregation and misfolding. Therefore, production of an alternative polypeptide is easier and favorable comparing to that of a full length transmembrane protein CD20. **Methods:** In this study, we expressed the extra membrane loop of hCD20 (exCD20) consisting of a non-glycosylated 47-amino acids region. The exCD20 coding sequence was amplified by PCR and cloned in pET32a(+) expression vector. The desired protein was expressed in fusion with thioredoxin and 6× His tag in *E. coli* Origami strain. ELISA and Western-blotting data were performed to indicate the functionality of this protein. **Results:** We have obtained the exCD20 recombinant protein which can be detected in ELISA and Western-blot experiments. This recombinant fusion protein was soluble and stable without aggregation and misfolding problems. **Conclusion:** The recombinant extra membrane loop of human CD20 protein in fusion with thioredoxin (exCD20) can be used in function assays and some applications such as ELISA, immunoblotting, affinity purification, immunization, screening, and development of anti-CD20 antibodies. *Iran. Biomed. J. 16 (3): 121-126, 2012*

Keywords: *E. Coli*, CD20, Thioredoxin

INTRODUCTION

World Health Organization has reported that 7.6 million people worldwide died from cancer in 2008. The Non-Hodgkin's lymphoma is expected to be the fifth most common cancer in American men and women [1]. It is a disease in which malignant (cancer) cells are formed in the lymph system. For lymphomas, chemotherapy and radiation therapy have been the mainstay of treatment. On the contrary of immunotherapy, both of these modalities suffer from a lack of specificity [2].

All the currently available anti-CD20 antibodies have been selected and produced against the extra membrane loop of hCD20 via different immunization methods [3]. The human B-lymphocyte-restricted differentiation antigen Bp35 (CD20, MS4A1), a 35-kDa hydrophobic phosphor-protein, is expressed as a

cell surface, non-glycosylated protein during early pre-B-cell development [4] and predicted to span the plasma membrane four times. It is highly expressed on the plasma membrane of almost all plasma B cells, but not on hematological stem cells [5] and plasma cells [6]. This 297 amino acid length protein is consisted of cytoplasmic N- and C-termini and four hydrophobic regions for anchoring the molecule in the membrane. Although the biological function of CD20 remains unclear, some evidence indicated that it might function as a calcium ion channel [7-11]. The importance of CD20 as a target for immunotherapeutic depletion of B cells is irrefutable and anti-CD20 monoclonal antibodies appear to be ideal for the treatment of B-cell malignancies [12].

While for many soluble proteins suitable overexpression systems are used routinely, high-level production of membrane proteins is still challenging.

*Corresponding author; Tel. & Fax: (+98-21) 6649 2595; E-mail: mashokrgozar@pasteur.ac.ir

One phenomenon frequently observed in *E. coli* is that many heterologous proteins become incorrectly folded and accumulate in the cytoplasm as insoluble aggregates, called inclusion bodies [13, 14]. Therefore, expression of full length hCD20 protein produces a recombinant protein with incorrect folding and/or probably aggregated forms and using some techniques like liposome technology for hCD20 antigen (Abnova, Taiwan) has not proved yet.

Up to now, a few other studies have focused on expression of full length hCD20 protein in different forms such as purified recombinant protein on the surface of a mammalian cell membrane or transmembrane domain of CD20 in fusion with gIII protein of a phage [15-18]. Misfolding, aggregation and hyper immunogenicity and complexity of fused protein/particle to the recombinant CD20 are the drawbacks that should be avoided.

In this study, we selected the 47 amino acid-extra membrane loops of hCD20 between the third and fourth transmembrane regions [19] to express it in fusion with thioredoxin of pET32a(+) prokaryotic expression vector. After expression, it would be possible to assess the functionality of recombinant exCD20 by ELISA and Western-blotting. The functional exCD20 may be used in some functional assays and applications, such as ELISA, immunoblotting, affinity purification, immunization, screening, and development of anti-CD20 antibodies.

MATERIALS AND METHODS

Cloning and construction of exCD20 expression cassette. Amplification of exCD20 coding sequence was performed by PCR with 53°C of annealing temperature and using specific primers and the pORF9-hCD20a eukaryotic plasmid (InvivoGen, USA) as template. This vector is an expression vector containing the full length of human CD20a (MS4A1) isoform 1 gene. Cloning was facilitated by *Bam*HI and *Xho*I restriction enzyme sites engineered in the primers used for amplification (underlined): tCD20F: TGGATCCAATATTTAAAATTTCCCAT and tCD20R: AATCTCGAGTTATATGCTGTAACAGTA TTGG. The purified PCR product was ligated to pTZ57R/T plasmid (Thermo Scientific, USA) and used to Transform *E. coli* TOP10 strain competent cells by InsTAclone™ PCR Cloning Kit (Thermo Scientific, USA). Accuracy of PCR amplification and insertion was confirmed by colony PCR using T7 promoter and tCD20R primers and finally by sequencing. The authentic sequence was finally subcloned into the pET32a(+) prokaryotic expression vector (Novagen, USA) for high-level expression of peptide sequence

fused to the 109aa TRX•Tag™ thioredoxin protein and 6× His tag.

Expression and purification of exCD20. Since the extra membrane loop of hCD20 has a disulfide bond between C₁₆₇ and C₁₈₃, the recombinant pET32a(+) plasmid was transformed into *E. coli* Origami™ strain (Novagen, USA) as expression host strain to enhance disulfide bond formation in the cytoplasm. One ml of overnight cultured single colony of the transformed *E. coli* Origami™ strain was used to inoculate 300 ml TB medium supplemented with 300 µl 1,000× ampicillin stock (100 µg/ml ampicillin final concentration) in a baffled shaker flask. The flask was incubated at 37°C at 200 rpm reaching optical density (OD₆₀₀) of 0.6 to 0.9 in an incubator shaker. Expression was induced by adding 0.5 mM isopropyl-beta-D-thiogalactopyranoside (Applichem, Germany). Induction was followed by 5 hours of incubation at 30°C and 200 rpm. *E. coli* cells were harvested by centrifugation at 5,000 ×g at 4°C for 8 min. The cell pellet was resuspended in 10 ml lysis buffer (50 mM Tris-HCl, pH 8.0), 25% w/v sucrose (Merck, Germany), 1 mM EDTA (Sigma, Germany), 100 µg/ml lysozyme (Sigma, Germany), and 1 ml complete protease inhibitor cocktail (Roche, Germany) in a 50 ml falcon tube and incubated on ice for 30 min. Then, it was frozen at -20°C and thawed by immersing the tube in 37°C water. Ten ml of the lysis buffer was added to the lysate and centrifuged at 8,000 ×g at 4°C for 8 min. Supernatant was collected and the expressed protein was purified by HisPur™ Ni-NTA Resin (Thermo Scientific, USA) via its 6× His tag. Imidazole removal and complementary purification of exCD20 protein was performed by size exclusion chromatography (SEC) by ÄKTA Explorer FPLC system (GE Healthcare Life Sciences, UK) and two separated peaks were fractionated.

Characterization of exCD20:

ELISA tests. ELISA plates (Nunc, Germany) were coated with 1 µg per well of exCD20 in PBS at 4°C overnight. Then, they were blocked with 2.5% BSA at 37°C for 2 h. After 3 washes, 100 µl of anti-CD20 peptide antibody in PBS (10 µg/ml) [20] was added to the wells and the plates were incubated at 37°C for 1.5 h. The wells were washed, and 100 µl of 1:4,000 diluted horseradish peroxidase-conjugated rabbit anti-mouse Ig (Sigma, Germany) was added, then incubated for 1.5 h at 37°C. After 5 washes, 100 µl of tetramethylbenzidine solution (Sigma, Germany) was added, the plates were incubated at room temperature in dark, then the reaction was stopped after 1 min. P5 peptide (the complete extra membrane loop of hCD20 with a disulfide bond between C₁₆₇ and C₁₈₃) was

Table 1. Nucleotide sequence of a gene coding for extra-membrane loop of human CD20 and its amino acid sequence. A 225-amino acid sequence was produced by expression of exCD20 pET32a recombinant vector.

Name		Size	Sequence
extra-membrane loop of hCD20	Nucleotide	141 bp	AATATTA AAAATTTCCCATTTTTTAAAAATGGAGAGTCTGAATTTTATTA GAGCTCACACACCATATATTAACATATACAACGTGAACCAGCTAATC CCTCTGAGAAAACTCCCCATCTACCCAATACTGTTACAGCATA
	Amino acid	47 aa	NIKISHFLKMESLNFIRAHTPYINIYNCEPANPSEKNSPSTQYCYSI
Recombinant exCD20	Amino acid	225 aa	EGDIHMSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEI ADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLKNGEVAATKVGALS KGQLKEFLDANLAGSGSGMHMHHHHHSSGLVPRGSGMKETAAAKFERQ HMDSPDLGTDDDDKAMADIGSNIKISHFLKMESLNFIRAHTPYINIYNCEP ANPSEKNSPSTQYCYSILEHHHHHH

synthesized (Biomatik, Canada) and used as positive control. Lysate of *E. coli* Origami strain transformed by empty vector pET32a(+) (TRX), as well as PBS were used as negative controls. OD was measured at 450 nm by ELISA reader (State Fax 2100, USA). All procedures were performed in triplicate.

SDS-PAGE and Western-blot analysis. One microgram of each two peaks of SEC experiment (Fig. 2) was run on 10% NuPAGE[®]Novex Bis-Tris Mini Gels (Invitrogen, USA). The separated proteins were transferred to Hybond-C nitrocellulose membrane (GE Healthcare, UK) by using a Bio-Rad protein transfer apparatus (100 v, 250 mA, 90 min). PageRuler[™] Prestained Protein Ladder (Thermo Scientific, USA) was used in both experiments. Following protein transfer, the nitrocellulose membrane was blocked by blocking buffer containing 5% skimmed milk plus 0.25% Tween. The exCD20 band was detected by the anti-CD20 peptide antibody (10 µg/ml in the blocking buffer, 1 h, 25°C), and alkaline phosphatase-conjugated anti-mouse antibody (Sigma, Germany) (1:2,000 dilution in the blocking buffer, 25°C, 1 h) as primary and secondary antibodies, respectively. Alkaline phosphatase enzyme conjugate was detected by nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt substrates (Thermo Scientific Pierce, USA).

RESULTS

Cloning and construction of exCD20 expression cassette. In order to construct the exCD20 expression cassette, the 141-nucleotide sequence (47 amino acids) of the extra membrane loop of hCD20 (Table 1) was amplified by PCR and using tCD20F and tCD20R amplification primers. Amplified region gave a band of the expected size (around 150 bp) on a 1.5% agarose gel (Fig. 1A). The PCR product was cleaned up and after digestion with restriction enzymes, cloned into

the pTZ57R/T and then subcloned into the pET32a(+) expression vector. The band of around 720 bp was corresponding to the PCR product of colony PCR with T7 Promoter and tCD20R primers (Fig. 1B). The positive clone was sequenced (Genfanavaran, Iran) using tCD20F and tCD20R primers. The sequencing data was compared with sequence of hCD20 gene in the NCBI website (Gene ID: 931) and the sequence of inserted exCD20 was confirmed.

Expression, purification and characterization of exCD20. After Transformation of recombinant vector into *E. coli* Origami[™], it was cultivated and induced with isopropyl-beta-D-thiogalactopyranoside. The expressed protein was a 225-amino acid sequence including the extra membrane loop of hCD20 peptide

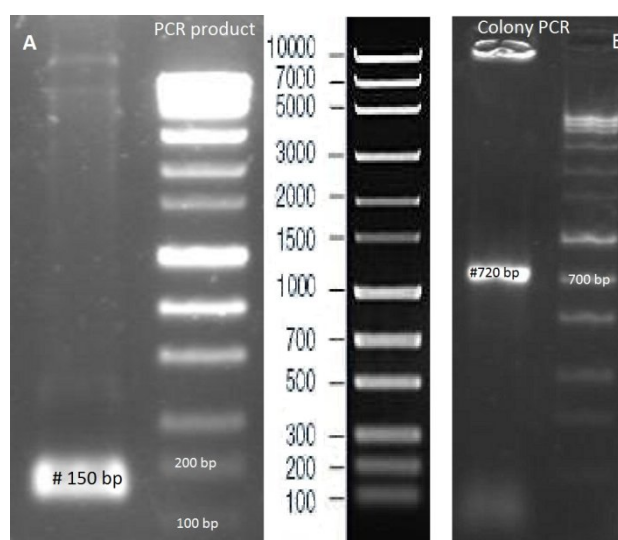


Fig. 1. Gel electrophoresis of PCR product and colony PCR. The 141-bp exCD20 coding sequence was amplified by PCR and 2 µl sample of PCR product was run on a 1.5% agarose gel (A). This sequence was cloned into pET32a(+) expression vector and the cloning was confirmed by colony PCR. The amplified product was visualized around 720 bp on a 1% agarose gel (582 bp, from T7 promoter binding site to *Bam*HI cut site + 141 bp, the size of insert) (B).

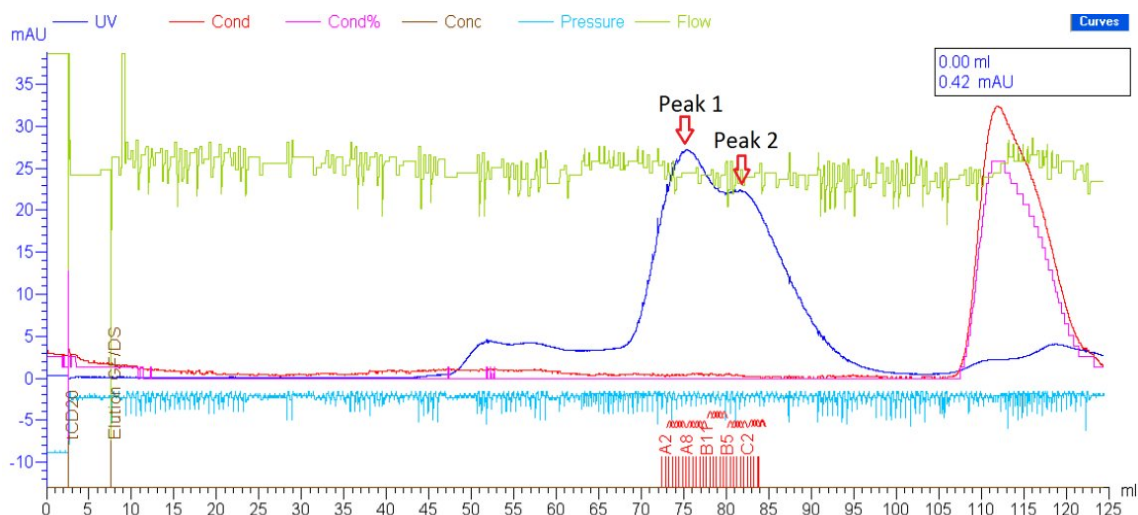


Fig. 2. Chromatogram of size exclusion chromatography. The sample was loaded onto a Superdex 75 column. Two overlapped main protein peaks in addition to some small protein peaks (peaks 1 and 2) were separated. A conductivity peak corresponding to the imidazole was obtained at the end of size exclusion chromatography experiment.

(bolded and underlined), a 109aa-TRX•TagTM thioredoxin protein and two 6× His tags (Table 1). The expressed protein was purified by Ni-NTA column according to the manufacturer's instruction and the exCD20 protein was eluted and collected in volume of 1 ml. The Ni-NTA purified protein was loaded on SEC column and fractionated and two main peaks of protein were obtained. Also, there were some small protein peaks before the main peaks. A conductivity peak corresponding to the imidazole was obtained at the end of SEC experiment (Fig. 2). The ELISA results for exCD20 and P5 peptide were strongly positive comparing to the negative controls. In comparison with the negative control [TRX, the lysate of *E. coli* Origami which was transfected with empty pET32a(+) plasmid], the OD_{450nm} values for exCD20 and P5 peptide were significantly higher (Table 2). The two main peaks were analyzed by SDS-PAGE (10% gel). Silver staining showed the expected molecular mass of near 30 kDa fusion recombinant protein in both main peaks (Fig. 3A). Western-blot analysis showed the reactivity of mouse anti-CD20 peptide antibody with these two bands (Fig. 3B).

DISCUSSION

In the present study, we obtained a novel recombinant extra membrane loop of hCD20 in fusion with thioredoxin and its native disulfide bridge (exCD20).

Since 1988, a few studies have attempted to express recombinant hCD20 antigen for different purposes [15-18]. In one study, the full length of hCD20 was cloned and expressed [18]. However, aggregation, misfolding and inclusion body formation are common phenomena

in expression of full length transmembrane proteins because of hydrophobic regions (membrane-spanning domains) within these types of proteins. In another study [17], a recombinant vector with full length of hCD20 was transfected into a mammalian cell line and CD20 was expressed on the surface of the cells in order to study the structure and function of CD20. Also, in a study [16], the extra-membrane loop of hCD20 has been displayed on the surface of M13K07 phage and this recombinant phage was used to develop immune response in animal. In this approach, most of the antibodies were developed against the phage but not the fused peptide, because the phage is a complex and strong immunogenic carrier. In addition, the native disulfide bond of hCD20 does not construct in the normal cytosolic condition in the bacteria as good as engineered strain like *E. coli* Origami host strain [21].

Since all the currently available anti-CD20 antibodies have been selected and produced against the extra membrane loop of hCD20 [3], in our study, we

Table 2. ELISA results. One µg of the exCD20, P5 peptide or lysate of *E. coli* Origami strain which transformed by empty vector pET32a(+) (TRX), were coated in each well. The anti-CD20 peptide antibody and anti-mouse alkaline phosphatase-conjugated antibody were used as primary and secondary antibodies, respectively. ELISA test was performed in triplicate.

Sample	Mean of optical density (OD _{450nm})	
	With antigen	Without antigen
exCD20	3.40	0.10
P5 peptide	2.98	0.09
TRX	0.17	0.11
PBS	0.06	0.06

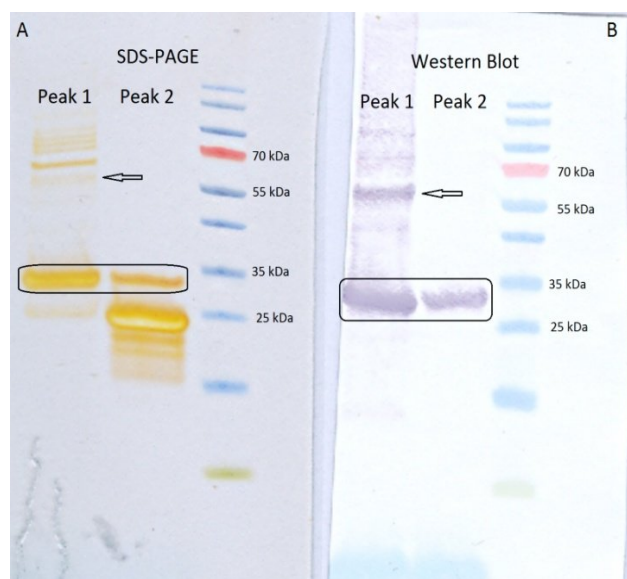


Fig. 3. SDS-PAGE and Western-blot analyses of size exclusion chromatography (SEC) column purified samples. One μg from peaks 1 and 2 of chromatography-purified SEC fraction was subjected to SDS-PAGE (10% acrylamide gel) under reducing conditions (A). Separated proteins were then transferred to the nitrocellulose membrane and stained by a mouse anti-CD20 peptide antibody and anti-mouse alkaline phosphatase-conjugated antibody as primary and secondary antibodies, respectively (B).

used the extra membrane loop of hCD20 instead of its full length. This region was expressed in a Thioredoxin fusion system in the *E. coli* Origami strain to force the formation of disulfide bridge [21] between its two cysteines in the cytosol as native form on the surface of B-cells [22]. This strategy resulted in a stable, soluble and functional protein [23, 24].

As Table 2 shows, in ELISA a high $\text{OD}_{450\text{nm}}$ value for exCD20 as well as P5 peptide were obtained. This result confirmed that exCD20 had an epitope with appropriate conformation to interact with the anti-CD20 peptide antibody. Western-blotting also confirmed this conclusion (Fig. 3).

Two bands of peak 1 and one band of peak 2 were detected in Western-blot experiment. The bands of about 30 kDa are corresponding to the exCD20 fused with the thioredoxin and His tags and the band of about 60 kDa seems to correspond to its dimer form (Fig. 3).

Purification steps show the necessity of optimization of conditions for purification of exCD20 with Ni-NTA column. Some changes in concentration of imidazole in the washing step, the volume of washing buffer and the time of binding incubation may reduce the contaminations and increase the xCD20purity.

The anti-CD20 peptide antibody which was developed against P5 peptide [20], detected the 30 kDa-band exCD20 in the Western-blot. The 60 kDa-band seems to be the exCD20 dimer (Fig. 3).

In conclusion, the functional exCD20 protein can be expressed in *E. coli* Origami host strain. This recombinant extra membrane loop of hCD20 can be used instead of its full length protein without aggregation and misfolding problems in development of poly- and/or monoclonal anti-hCD20 antibodies. Also, it may be used in ELISA, immunoblotting, affinity purification, immunization and screening.

ACKNOWLEDGEMENTS

We are very grateful to Prof. Dr. Serge Muyldermans (Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussel), Dr. Amir Amanzadeh and Mr. Hassan Sanati for their outstanding technical assistances. Financial support of this study has been provided by Pasteur Institute of Iran, Postgraduate Office.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*.2010 Dec;127(12):2893-917.
2. Furman RR, Coleman M, Muss D, Leonard JP. Monoclonal antibodies in the treatment of non-Hodgkin's lymphoma. *Cancer Treat Res*.2006;131:221-50.
3. Teeling JL, Mackus WJ, Wiegman LJ, van den Brakel JH, Beers SA, French RR, et al. The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. *J Immunol*.2006 Jul;177(1):362-71.
4. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, et al. Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. *Blood*.1994 Jan;83(2):435-45.
5. Nishida M, Usuda S, Okabe M, Miyakoda H, Komatsu M, Hanaoka H, et al. Characterization of novel murine anti-CD20 monoclonal antibodies and their comparison to 2B8 and c2B8 (rituximab). *Int J Oncol*. 2007 Jul;31(1): 29-40.
6. Perosa F, Favoino E, Caragnano MA, Prete M, Dammacco F. CD20: a target antigen for immunotherapy of autoimmune diseases. *Autoimmun Rev*.2005 Nov;4(8):526-31.
7. Robak T, Robak E. New anti-CD20 monoclonal antibodies for the treatment of B-cell lymphoid malignancies. *BioDrugs*.2011 Feb;25(1):13-25.
8. Cragg MS, Walshe CA, Ivanov AO, Glennie MJ. The biology of CD20 and its potential as a target for mAb therapy. *Curr Dir Autoimmun*.2005;8:140-74.
9. Einfeld DA, Brown JP, Valentine MA, Clark EA, Ledbetter JA. Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. *EMBO J*. 1988

- Mar;7(3):711-7.
10. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Expression of cell surface markers after human B lymphocyte activation. *Proc Natl Acad Sci USA*.1981 Jun;78(6):3848-52.
 11. Beers SA, Chan CH, French RR, Cragg MS, Glennie MJ. CD20 as a target for therapeutic type I and II monoclonal antibodies. *Semin Hematol*. 2010 Apr;47(2):107-14.
 12. Deans JP, Li H, Polyak MJ. CD20-mediated apoptosis: signalling through lipid rafts. *Immunology*.2002 Oct;107(2):176-82.
 13. Hunte EPC. Production and Purification of Recombinant Membrane Proteins. In: Hunte C, editor. Membrane Protein Purification and Crystallization; A Practical Guide. 2nd ed. Academic Press; 2003.p. 55-83.
 14. Mus-Veteau I. Heterologous Expression of Membrane Proteins for Structural Analysis. In: Mus-Veteau I, editor. Heterologous Expression of Membrane Proteins; Methods and Protocols. 1st ed. Humana Press; 2010. p. 1-16.
 15. Hong HY, Sun YX, Guo YX, Wang JN, Lai CN, Qi ZT, et al. Cloning and Expression of Human CD20 Gene on NIH-3T3 Cell Membrane. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*. 2000;32(4):430-3.
 16. Zhang XY, Sun ZW, Yu WY, Cheng JZ. Cloning and expression of fusion gene of transmembrane domain of human CD20 and g3pN in *Escherichia coli*. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi*.2004 Jul;20(4):481-3.
 17. Bubien JK, Zhou LJ, Bell PD, Frizzell RA, Tedder TE. Transfection of the CD20 Cell Surface Molecule into Ectopic Cell Types Generates a Ca Conductance Found Constitutively in B Lymphocytes. *Cell Biol*.1993;121(5):1121-32.
 18. Ernst JA, Li H, Kim HS, Nakamura GR, Yansura DG, Vandlen RL. Isolation and Characterization of the B-Cell Marker CD20. *Biochemistry*.2005 Nov;44(46):15150-8.
 19. Ernst JA, Li H, Kim HS, Nakamura GR, Yansura DG, Vandlen RL. Isolation and characterization of the B-cell marker CD20. *Biochemistry*.2005;44(46):15150-8.
 20. Habibi Anbouhi M, Abolhassani M, Bouzari S, Khanahmad S, Aghasadeghi M, Madadkar S.Immunological evaluation of predicted linear B-cell epitopes of human CD20 antigen. *Biotechnol Appl Biochem*.2012;X:1-6.
 21. de Marco A. Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*. *Microbial Cell Fact*.2009 May;8:26.
 22. Oscherwitz J, Gribbin TE, Cease KB. A CD20 tandem-epitope immunogen elicits antibody in mice that binds murine cell surface CD20 and depletes splenic B cells *in vivo*. *Mol Immunol*.2010 Apr;47(7-8):1484-91.
 23. Nikitina J, Shutova T, Melnik B, Chernyshov S, Marchenkov V, Semisotnov G, et al. Importance of a single disulfide bond for the PsbO protein of photosystem II: protein structure stability and soluble overexpression in *Escherichia coli*. *Photosynth Res*.2008 Oct-Dec;98(1-3):391-403.
 24. Xiong S, Wang YF, Ren XR, Li B, Zhang MY, Luo Y, et al. Solubility of disulfide-bonded proteins in the cytoplasm of *Escherichia coli* and its "oxidizing" mutant. *World J Gastroenterol*.2005 Feb;11(7):1077-82.