Construction and cloning of a recombinant expression vector containing human Cd20 Gene for antibody therapy in Non-Hodgkin Lymphoma

Seyedeh Arezoo Hosseini [1] Mojtaba Tahmoorespur [2] Mohammad Hadi Sekhavati [3] Mohammad Reza Nassiri [2]

PhD in Department of Animal Science [1] , Ferdowsi Univeristy , Mashhad Iran. Professor Department of Animal science [2] , Ferdowsi Univeristy , Mashhad Iran. Assistant Professor Department of Animal Science [3] , Ferdowsi University, Mashhad, Iran.

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Original Article

Abstract

Introduction: Non-Hodgkin lymphoma (NHL) is a cancer that starts in lymphocytes. The main treatment for NHL is chemotherapy and radiation. Today immunotherapy is a promising therapeutic approach in the treatment of a variety cancers which is high specific unlike previous methods. Antibodies do not penetrate effectively into tumore tissues because of their large size. Whereas the small size of nanobodies (Camelid single-domain antibodies) allow them to efficiently enter into tissues and bind to the epitopes. Human CD20 over expression in the B-cell lymphoma makes this antigen as a validated target for immunotherapy. One major problem in production of full length CD20 is aggregation and misfolding. Therefore, production of a polypeptide is easer and favorable comparing to that of a full length transmembrane protein CD20.

Methods: The fragment consisting of human CD20 extra membrane loop and hinge and Fc of camelid IgG was constructed. Then it was facilitated by *HindIII* and *XhoI* restriction enzyme sites and fused to the 6× His tag for purifiction. The stop codon was engineered in the terminal sequence. The engineered coding sequence was synthesized by Generay Company and then inserted into the pcDNA3.1 (+) vector to obtain recombinant expression vector. The accuracy of ligation reaction was confirmed by colony PCR, sequencing and digestion.

Results: The colony PCR result showed 1132 kb fragment. The results of digestion and sequencing showed that the protein was what we had hoped to acquire.

Conclusion: We have obtained the recombinant expression vector inorder to express in mammalian cell which can be used to produce novel anti-human CD20 monoclonal antibody in future.

Correspondence:
Mojtaba Tahmoorespur, PhD.
Department of Animal Science, Ferdowsi University of Mashhad.
Mashhad, Iran
Tel:+98 9151159911
Email:
m_tahmoorespur@yahoo.com

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Introduction:

The number of global cancer deaths continues to increase largely because of the several factors such as aging, growth of the world population and an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries (1). Lymphoma is cancer that begins in cells of the lymph system. The two main types of lymphoma are Hodgkin lymphoma and non-

Hodgkin lymphoma. Non-Hodgkin lymphoma (also known as non-Hodgkin's lymphoma, NHL, or sometimes just lymphoma) form from different types of white blood cells (B-cells, T-cells, NK cells) (2). The American Cancer Society's estimates for NHL in 2015 is about 71,850 new cases and 19,790 deaths due to NHL (3). Globocan 2012 has reported that the Non-Hodgkin's lymphoma is expected to be the fifth most common cancer in American men and women (2). NHL is more common in developed areas (1). The main types of treatment for lymphomas are chemotherapy and radiation therapy. On the contrary of immunotherapy, the limited applicability of these treatments resides in the lack of specificity (4,5).

Consequently, the effective treatment of NHL is the main aspects in this area (4).

Nowadays CD20 (B-lymphocyte-restricted differentiation antigen Bp35, MS4A1) is considered as a antigen for routine monoclonal antibody (mAb) immunotherapy. CD20 is expressed on the surface of almost all normal and malignant B cells and its extracellular epitopes are recognized by the various kinds of mAbs are close to the cell surface and thus permit efficient engagement of Fc-dependent effector mechanisms. This characteristic makes CD20 as a good candidate for mAb immunotherapy (5,6). CD20 also was considered as a target for rituximab, the first and chimeric anti-CD20 mAb that approved by Food and Drug Administration (FDA) and developed to date for lymphoma. Rituximab was originally used for treating low-grade non-Hodgkin lymphoma and be also effective against other types of lymphomas (7-9) and some autoimmune diseases (10-13). The CD20 antigen (B-lymphocyte-restricted differentiation antigen Bp35, MS4A1) is nonglycosylated hydrophobic phosphor-protein with a molecular weight of 35 or 37 kilodaltons (kDa), depending on the degree of phosphorylation (6). This antigen is encoded by membrane-spanning 4-A (MS4A) gene and expressed on B lymphocytes with the expression of surface IgM at the same time (14-15). The CD20 antigen is present on the plasma membrane of almost all plasma B cells, but not on hematological stem cells (15,16) and plasma cells (14). The CD20 with 297 amino acid length predicted to span the plasma membrane four times and form tetraspan transmembrane molecule (16,17). This protein is consisted of three hydrophobic regions of approximately 53, 25 and 20 amino acids with a single extracellular loop and cytoplasmic N- and C-termini (6, 18-21). The CD20 antigen is expressed in both mantle-zone and germinal-center areas of secondary follicles of lymphoid tissue and can be expressed on follicular dendritic cells (FDCs) in germinal centers too. CD20 antigen has been detected in low-level on a subpopulation of T lymphocytes (6). The function of CD20 remained obscure, various biochemical and cell biological studies have indicated that it might form or regulate a voltage-independent calcium channel calcium ion channel (5,6,18,20-24). Several evidence have clearly demonstrated that CD20 is an ideal target for immunotherapy of B-cell lymphoma because it is highly expressed in more than 80% of the B-cell lymphoma (26) and it remains on the cell surface after cross-linking with antibodies; and this protein is not shed to the circulation to inhibit the antibody therapy (6,26,27). All the currently available anti-CD20 antibodies have been produced against the extra membrane loop of human CD20 (28). This loop of approximately 40 amino acids located between the proposed third and fourth transmembrane helical domains (5) and the length of this accepted small loop is highly conserved between members of the MS4A family (29). One major problem in production of full length CD20 is aggregation and misfolding. Therefore, production of a polypeptide is easer and favorable comparing to that of a full length transmembrane protein CD20. Before our research, Anbouhi *et al* in 2012 have produced recombinat human CD20 in fusion with thioredoxin and shown that the extra membrane loop of hCD20 can be used as an alternative of the full length CD20 antigen, without aggregation and misfolding problems. They confirmed that extra membrane loop of hCD20 had an epitope with appropriate conformation to interact with the anti CD20 peptide antibody (2). Fasihi-Ramandi *et al* in 2015 have designed and expressed the new chimeric hCD20 extra-loops consists of 135 amino acids which is less than that of native antigen that duplicated with the appropriate linkers (30). The efficacy of antibody-based therapy is still limited and one of the promising novel developments that may overcome the drawbacks of monoclonal antibody-based therapies is the

employment of nanobodies. So, the aim of this research was to construct and clone recombinant human CD20 gene in order to produce novel monoclonal antibody in lymphoma therapy.

Methods:

To achieve our insert (recombinant human CD20), sequences of extracellular loop of CD20 and hinge and FC of camelid IgG obtained from NCBI. Then recombinant human CD20 (hCD20) was facilitated by *HindIII* and *XhoI* restriction enzyme sites and fused to the 6× His tag for purifiction. The stop codon was engineered in the terminal sequence (Fig 1). Pymol v1.7.6 (31) and clustalw v.2.1 (32) programs were used to predict and study the 3D structure and amino acid sequence alignment of recombinant human CD20. The pUC57 (Generay, china, cat# GV0205) was selected as an intermediate vector. Our engineered construct was optimised by online genscript site and then synthetized by Generay Company (China) (Fig 2).

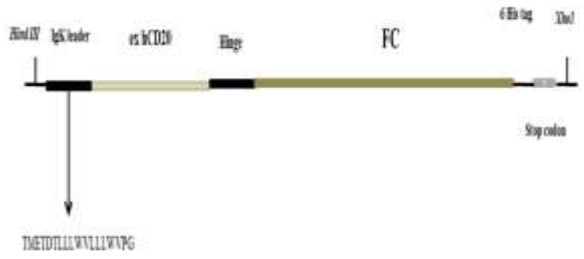

Figure 1. Diagram of the recombinant hCD20 coding sequence. The extera-cellular loop of hCD20 linked to hinge and FC of camelid IgG

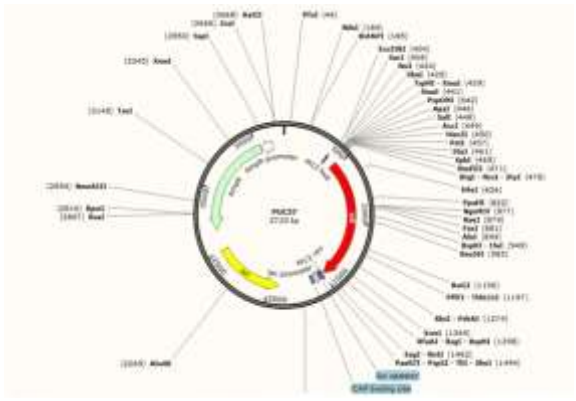

Figure 2. Puc57 plasmid containing recombinant hcD20

To prepare the recombinant vector, the sintetice pUC57 was digested with *XhoI* and *HindIII* (Thermo, USA) to get the insert out. Then recombinant hCD20 fragment was purified from the agarose gel by Ron`s Agarose Gel Mini prep Kit (BioRon, Germany) according to the manufacturer's instruction. A pcDNA version 3.1+ (Invitrogen, USA) was used for cloning and sequencing of the recombinant hCD20. The purified recombinant hCD20 was inserted into the pcDNA version 3.1+ vector using Rapid DNA Ligation Kit (Thermo Fisher Scientific, USA) according to manufacturer's protocol.

In current study, *E. coli* strain Top10 was used as host for cloning, sequencing and maintenance of insert. Competent cell preparation was followed as described by Sambrook and Russell (2001) (33).

The recombinant vector was transformed into the competent *E.coli* Top10. Transformation was performed by the heat shock method (33). Briefly, we first mixed pcDNA 3.1+ containing recombinant hCD20 in a 10 µL volume of pure water. This amount was transferred into a microcentrifuge tube containing 50µL of thawed competent cells. The mixture was incubated for 20 min on ice. Heat shock was carried out by placing the tubes in a water bath for 1 min at 42°C. Readily after the heat treatment, cells were placed on ice for 5 min. In order to grow bacteria better, 250 µL of liquid LB medium was added to competent cell containing recombinant pcDNA3.1+, Then 300 µL of cells were plated on solid LB medium containing ampicillin (100µg/ml) as selective antibiotic. Cells grew overnight at 37°C. To test for positive clones, *E.coli* Top10 transformants were verified by PCR reaction using by universal primer of T7 (F: (TAG-AAG-GCA-CAG-TCG-AGG-C) and BGH (R: TAA-TAC-GAC-TCA-CTA-TAG-GG)). Plasmidial DNA was purified from selected positive using the Ron`s Plasmid Mini Kit (BioRon, Germany) and confirmed by restriction sites enzyme digestion. We used single (*Hind III*) and double (*Hind III* and *Xho1*) digestion to verify ligation reaction. Colony PCR and digestion products were checked through electrophoresis on 1.5% agarose gel and undigested plasmid was used as control. The purified plasmids were subjected to sequencing (Macrogene, South Korea). The nucleotide sequences obtained were analyzed by

CLC Main workbench 5.5 software (CLC bio, Tokyo, Japan).

Results:

In order to construct the recombinant hCD20, the 141-nucleotide sequence (47 amino acids) of the extracellular loop of human CD20 (ex hCD20) and hinge and FC of camel IgG were fused to *Hind III* and *XhoI* restriction enzyme sites. The camelid hinge and FC regions were added to the ex hCD20 because of using the recombinant hCD20 as injected protein for camel in the future. Also camelid IgG regions lead to increase the ex-hCD20 length and half life in camel blood circulation. The protein 3D-structure determination by PyMOL software showed that there was not any interaction between ex- hCD20 and camelid hinge and FC regions. Moreover, the ex-hCD20 was easily recognized as an epitope region for Ab binding.

In this approach we used pcDNA3.1+ as vector because we decide to express recombinant hCD20 in mammalian cells. The pcDNA3.1+ is suitable for this goal because it is containing human cytomegalovirus immediate-early (CMV) promoter for high-level expression and SV40 early promoter that express the SV40 large T antigen. After cleavage by *Hind III/Xho1*, the recombinant hCD20 fragment was inserted into a multiple cloning site downstream of the CMV promoter in pcDNA 3.1+/CMV (Fig 3).

After ligation reaction, *E.Coli*. Top10 cell was transformed with the recombinant vector and cultured in LB containing ampicillin. We have found many single white colonies on the plate which was representing the success rate of cloning was over 90%. Using the two universal primers of T7 and BGH, PCR was performed on colonies PCR and 1132 kb fragment was obtained. Also the accuracy of ligation reaction was confirmed by sequencing and digestion too (Fig 4,5).

The products of colony PCR reaction and digestion were checked through electrophoresis on 1.5% agarose gel. Based on agarose gel analysis, DNA fragments had acceptable quality and yield.

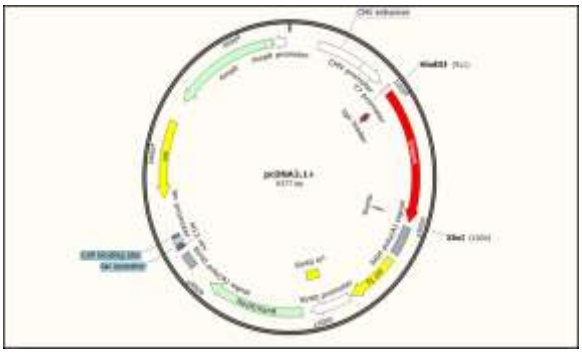

Figure 3. Diagrammatic representation of the construction of the recombinant vector. The recombinant hCD20 inserted in to the pcDNA version 3.1+

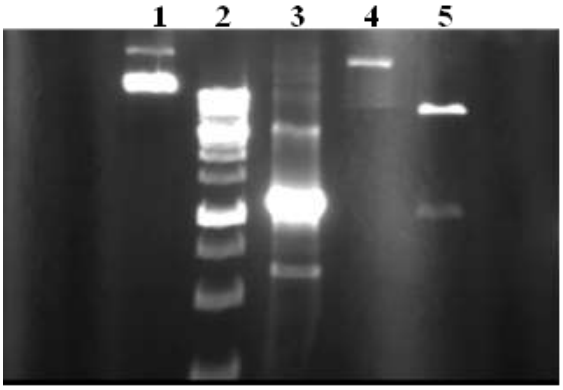

Figure 4. Agarose gel electrophoresis. The colony PCR and digestion products were ran on 1.5% agarose gel. Lane 1: undigested plasmid with 5.4 kb as control; Lane 2: 1 kb DNA ladder, Lane 3: colony PCR product with 1132 bp, Lane4: single digestion by *Hind III*, Lane 5: double digestion by *Hind III* and *Xho 1*

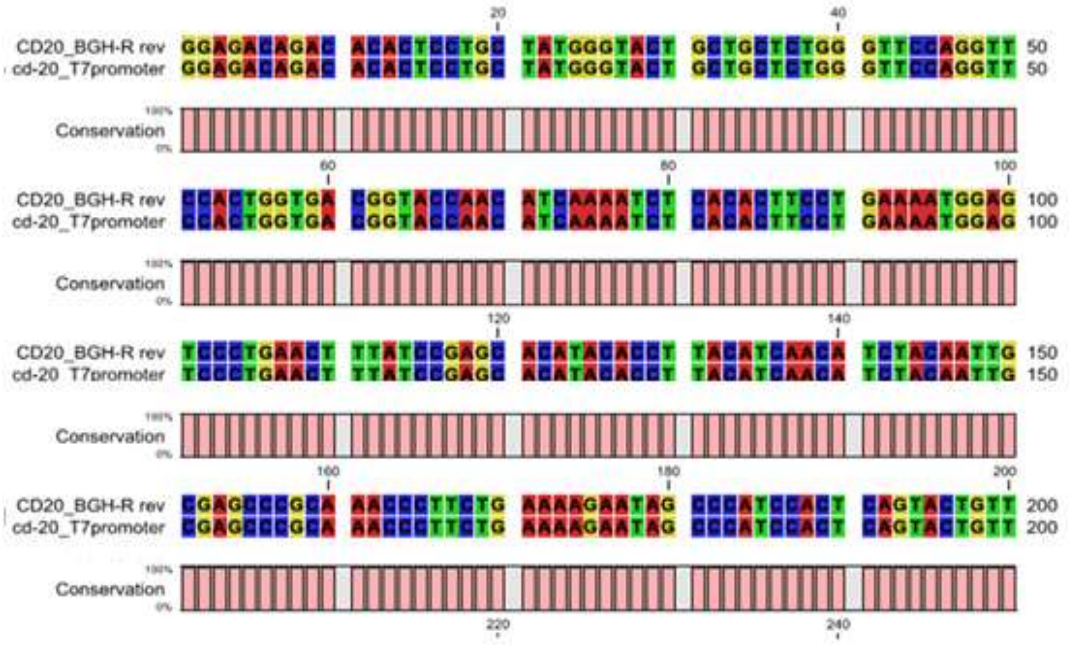

Figure 5. Alignment of the hCD20 nucleotide sequences in the expression vector with the reference sequence from Genbank by CLC program v7.5

Conclusion:

In this study, we obtained a recombinant pcDNA 3.1+ vector containing extra membrane loop of hCD20 infusion with camelid hinge and Fc. Since 1988, several recombinant hCD20 antigens were generated for different purposes in a few studies (2,23,31,34,35). Ernst *et al*. (2005) cloned and expressed the full length of human CD20 antigen in *E.coli*. They showed due to hydrophobic regions (membrane-spanning domains) within this protein, aggregation, misfolding and inclusion body formation was appeared (34). In another work, the structure and function of hCD20 were studied by a recombinant vector with full length hCD20 which was expressed on the surface of the mammalian cells (23). Also, the new recombinant hCD20 consists of an extra-membrane loop of hCD20 and M13K07 phage was used to develop an immune response in animals. In this study, due to the phage is complex and strong immunogenic carrier, most of the antibodies were developed against the phage (35). Anbouhi, *et al*. (2012) have obtained the soluble and stable recombinant hCD20 in *E. coli*.

They have stated that the extra membrane loop of hCD20 can be used as an alternative of the full length CD20 antigen, without aggregation and misfolding problems. Also, they confirmed that extra membrane loop of hCD20 had an epitope with appropriate conformation to interact with the anti CD20 peptide antibody (2). In another study, in order to produce and characterize a new anti human CD20 monoclonal antibody, a chimeric hCD20 extra-loops consists of 135 amino acids (less than that of native antigen) that duplicated with the appropriate linkers was designed and expressed (30). CD20, B-lymphocyte antigen, is highly expressed on most lymphoma and remains a validated target for antibody-based therapy in mature B-cell malignancies (34). Many mAbs for hCD20 have been reported to eradicate malignant B cells. Because of all the currently available anti-CD20 antibodies have been selected and produced against the extra membrane loop of hCD20 (28), in our study, we used the extra membrane loop of hCD20 instead of its full length.

As Figure 4 shows, in agarose gel electrophoresis a sharp band with 1132 bp was obtained. Also one band and two bands were detected in a single and double digestions. These results confirmed that recombinant human CD20 was inserted into the pcDNA 3.1+ correctly. The sequencing also confirmed this conclusion (Fig. 5).

In conclusion, the recombinant expression vector containing extra-cellular loop of human CD20 gene, which can be expressed in mammalian

cells and used for antibody therapy in non-Hodgkin lymphoma. 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. Since major issue with murine mAbs like rituximab is their low half-life, immunogenicity and resistance to mAbs, which is resulting in no-response and early relapse of disease. The large size and low-tissue penetrating of mAbs are important problems in antibody therapy. To overcome the limitations of full-length mAbs, smaller sizes have been generated, such as the naturally derived variable fragments of camelid heavy chain-only antibodies (35). We used the extra membrane loop of hCD20 infusion with hinge and Fc of camelid IgG to produce a novel anti-hCD20 monoclonal antibody in the future. We suggest to transform recombinant vector (pcDNA3.1+ with recombinant hCD20) into the hek293 cell and purifies it and then immune a camel with recombinant hCD20 to obtain anti-hCD20 monoclonal antibody in another study.

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ساخت و کلونینگ وکتور بیانی نوترکیب حاوی ژن CD20 انسانی با هدف ایمونوتراپی برای لنفومای غیر هوجکین

سیده آرزو حسینی [۱] مجتبی طهمورث‌پور [۲] محمد هادی سخاوتی [۳] محمدرضا نصیری [۲]

[۱] دکتری، گروه علوم دامی، دانشگاه فردوسی مشهد، مشهد، ایران. [۲] استاد، گروه علوم دامی، دانشگاه فردوسی مشهد، مشهد، ایران. [۳] استادیار، گروه علوم دامی، دانشگاه فردوسی مشهد، مشهد، ایران.

مجله پزشکی هرمزگان سال بیستم شماره سوم ۹۵ صفحات ۱۸۴-۱۷۷

چکیده

مقدمه: *لنفومای غیرهاجکینی (NHL) سرطانی است که در لنفوسیت‌ها رخ می‌دهد. درمان اصلی NHL شیمی درمانی و پرتودرمانی می‌باشد. امروزه ایمونوتراپی یک روش درمانی امیدوارکننده در درمان انواعی از سرطان‌هاست که برخلاف روش‌های قبلی از اختصاصیت بالایی برخوردار است. اندازه بزرگ آنتی‌بادی‌ها مانع از نفوذ مؤثر آن‌ها در بافت توموری می‌شود. در حالی که نانوبادی‌ها به دلیل سایز کوچک شان وارد بافت و به اپی توپ‌ها متصل می‌شوند. CD20 یک پروتئین در سطح سلول‌های B می‌باشد. بیان بالای CD20 در لمفومای غیرهاجکینی و داشتن اپی توپ خارج سلولی تشخیص داده شده با آنتی بادی‌های مختلف این پروتئین را هدف مناسبی برای ایمونوتراپی با آنتی‌بادی مونوکلونال ساخته است. هدف از این مطالعه، طراحی و کلونینگ وکتور بیانی نوترکیب حاوی لوپ خارج سلولی CD20 انسانی، ناحیه لولا و Fc ایمونوگلوبین شتر می‌باشد.*

روش کار: *در این مطالعه، ما قطعه‌ای شامل لوپ خارج سلولی CD20 انسانی و نواحی لولا و Fc ایمونوگلوبین G شتری را ساختیم. سپس دو ناحیه برشی آنزیم‌های محدودالاثر HindIII و Xho1 و ۶ اسید آمینه هیستیدین جهت خالص سازی به قطعه اضافه شد. کدون پایان در انتهای توالی قرار گرفت. توالی کد کننده‌ی مهندسی شده توسط شرکت Genray چین سنتز و برای دست‌یابی به وکتور بیانی نوترکیب درون وکتور بیانی pcDNA3.1+ الحاق گردید. جهت تأیید واکنش لیگاسیون، Colony PCR، توالی یابی و هضم انجام شد.*

نتایج: *نتایج کلونی PCR، یک قطعه به طول ۱۱۳۲ kb را نشان داد. همچنین تعیین توالی و هضم صحت واکنش الحاق را تأیید کرد.*

نتیجه‌گیری: *ما به یک pcDNA3.1+ نوترکیب حاوی ژن hCD20 به عنوان یک ناقل بیانی کارآمد در بیان پروتئین نوترکیب در سلول پستاندار دست یافتیم که می‌تواند جهت تولید آنتی بادی مونوکلونال علیه CD20 انسانی استفاده شود.*

کلیدواژه‌ها: *آنتی ژن لمفوسیت B، آنتی بادی مونوکلونال، لنفومای غیر – هوجکینی*

نویسنده مسئول:
دکتر مجتبی طهمورث‌پور
دانشکده کشاورزی دانشگاه فردوسی مشهد
مشهد – ایران
تلفن: ۹۱۵۱۱۵۹۹۱۱ ۹۸+
پست الکترونیکی:
m_tahmooresepur@yahoo.com

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