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Cloning and expression of the V-domain of the CD166 in prokaryotic host cell

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Original Article

Abstract

Purpose: CD166/ALCAM (Activated leukocyte cell adhesion molecule) as an immunoglobulin is implicated in cell migration. It is also involved in tumorigenesis of CRC (colorectal cancer) and known as a cancer stem cell marker. CD166, as a membrane protein, potentially represents either diagnostic or therapeutic capacities for CRC. **Methods:** In this study, the sequence of V domain was optimized for expression in prokaryotic host using online tools and cloned into pET-28a plasmid. The recombinant pET28a was transformed into the *E. coli* BL21DE3 using heat shock method and expression of recombinant V domain was examined using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). **Results:** The results confirmed protein expression of recombinant 22.77 kDa V domains in bacterial expression system. **Conclusion:** V domain of the CD166 was expressed successfully in *E. coli* bacteria. This recombinant fragment can be introduced as a suitable diagnostic and therapeutic candidate for screening and cancer-therapy of CRC patients, respectively.

Keywords: Cloning and Expression, ALCAM / CD166, V domain, Recombinant Protein, Colorectal Cancer

1. Introduction

CD166/ALCAM (Activated leukocyte cell adhesion molecule) is an immunoglobulin molecule implicated in cell migration.[1, 2] It is also involved in tumorigenesis of colorectal cancer (CRC) where functions as a cancer stem cell marker.[3-5]

CD166, as a member of immunoglobulin superfamily, was identified by expression cloning based on its ability to bind to the CD6, making use of COS (an acronym for CV-1 in Origin with SV40 genes) cells transfected with cDNA libraries.[6, 7] CD166 is a single-pass membrane protein with an extracellular domain consisting of 500-amino acid polypeptide, and a molecular weight of 105 kDa. The gene encoding CD166 is located on the long arm of chromosome 3 (3q13.1-q13.2) and is composed of 16 exons, with a size over 200 kb.[8] The protein mediates heterophilic (ALCAM-CD6) and homophilic (ALCAM-ALCAM) cell-cell interactions.[9-12]

ALCM consists of five extracellular domains including two V-types and three C-types domains.[13, 14] V domain consists of two parts including V_1 and V_2 which are consisting of 93 and 110-amino acids length, respectively. Functional domain mapping studies determined that existence of the NH2-terminal V-type domain is required for both cell-cell (hemophilic and hetrophilic) interactions. This observation is in direction with previous mapping studies on immunoglobulin superfamily cell adhesion molecules, which most frequently identified the N-terminal immunoglobulin superfamily domain as the major ligand-binding domain.[15]

Several methods based on genomics and proteomics introduced CD166 as an oncology-related target, and identified it as a surface antigen in CRC.[8] This protein play a main role in CRC progression and migration.[3, 16] During mass lesion, cancerous cells must be attached to another cell. Adhesion molecules can be involved in different types of cell adhesions including tumor cell–endothelial cell adhesion, tumor cell–matrix adhesion, or tumor cell–tumor cell adhesion playing essential roles in different stages of primary tumor formation or metastasis.[11]
CRC is the third most common type of tumors, with more than 1.2 million new cases resulted in 600

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thousand deaths annually and ranks fourth in terms of mortality worldwide.[17] In North America and Western Europe, CRC is the second most common cause of cancer-related death.[18, 19] According to the report of national Comprehensive of cancer control Ministry of Health and Medical Education, the range of CRC mortality in Iran is the third and fifth common cancer in Iranian woman and man, respectively.[20] The greatest problem is the high capacity of CRC to form secondary tumors, particularly in the lung and liver.[17, 21-23]

Based on functional mapping analyses, V domain in CD166 extracellular region is of great importance for both heterophilic and hemophilic types of cell-cell adhesion. It is identified as the major ligand-binding domain at the N-terminal immunoglobulin domain, comprising two parts, V_1 and V_2, consisting of 93 and 110-amino acids length, respectively. Therefore, our aim in this study was to cloning and expression of recombinant V domain to use in diagnostic or therapeutic applications.

2. Methods and Materials

2.1. Hosts and plasmids

E. coli BL21DE3 and TOP10 strains were used as host for expression and cloning, respectively. The pET - 28a (+) plasmid was employed for gene expression.

2.2. Codon optimization and gene synthesis

Sequence encoding V domain was obtained from online databases including Swiss-port, Uniprot KB and National Center for Biotechnology Information (NCBI, Gene ID: 214).

Codon optimization was performed based on *E. coli* codon usage and confirmed by genescript Company (NJ, USA). The codon-optimized construct containing the ORF encoding V domain was tagged at C-terminal with 6xHis-tag to facilitate its protein purification. *NcoI/BamHI* and *XhoI* restriction sites were added to the 5' and 3' of the sequence, respectively. The optimized sequence was synthesized and inserted into the pBSK (+) vector (Biomatik, Canada).

2.3. Sub-cloning of V domain in pET28a expression plasmid

To amplify V-domain, *E. coli* TOP10 was transformed by pBSK (+) vector. After plasmid extraction (Fermentas, Lithuania), a double digestion was done with *NcoI* and *XhoI* (Fermentas, Lithuania). The expression vector pET28a was also digested with same enzymes. Double digestion was carried out at 37 ºC. Digested fragments were analyzed by agarose gel electrophoresis and pET28a expression vector and V-Domain fragments were purified (Fermentas, Lithuania). Finally, V domain sequence was ligated to the pET28a using T4 DNA ligase (Fermentas, Lithuania).

2.4. Transformation of Recombinant Vector (pet-28a- V domain)

In this study, competent *E. coli* BL21 (DE3) host cells were prepared according to Calcium Chloride method.[24] The accuracy of transformation was verified by double digestion of plasmid with *NcoI* and *XhoI* enzymes.

2.5. Expression

20 µL of an overnight pre-cultured transformed bacterial cells was inoculated to 2 ml fresh LB medium containing kanamycin (100 $\mu g.mL^{-1}$) and incubated in a shaker incubator for 2 h at 150 rpm (37 ºC) until reaching the optimized optical density (0.8). 20 µL IPTG (100 $\mu g.mL^{-1}$) was added to the medium to induce protein expression and incubation was performed in the shakers incubator for 6 h at 150 rpm and 37 ºC. The mixture was centrifuged for 5 min at 5000 rpm and 4 °C. Supernatant was discarded and 60 µL Urea (8 M) was added to the precipitate.

2.6. Analysis of V domain expression

Expression of the V-domain was evaluated by SDS-PAGE. To run the samples, the mixture (precipitated sample and Urea) was dissolved in 1x SDS-PAGE sample buffer. Protein molecular weight marker and the prepared sample were heated at 95ºC for 5 min. Afterward, marker and sample were loaded on the 12% SDS-PAGE gel and run with the constant voltage of 100 V. The gel was stained with Coomasie brilliant blue R-250 for 2 h and washed with water followed by destaining for 2 h

3. Results

The synthetic V-domain gene was assembled based on the *E. coli* codon usage. The reference sequence of the gene employs tandem rare codons which can decrease the productivity of translation or even disengage the translational machinery. The codon usage bias of the gene was changed in *E.coli* by upgrading the Codon Adaptation Index (CAI) and Frequency of Optimal Codons (FOP) from 0.62 to 0.88 and 44 to 68, respectively. CAI of > 0.8 is considered as good, in terms of high gene expression level. Moreover, 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism (Figure 1).

The GC content was changed from 42.17% to 47.69% in optimized sequence. Between 30-70% is ideal percent range of GC content (Figure 2). Nucleotide sequence of original and optimized sequences were compared for Peer to Peer (Figure 3).

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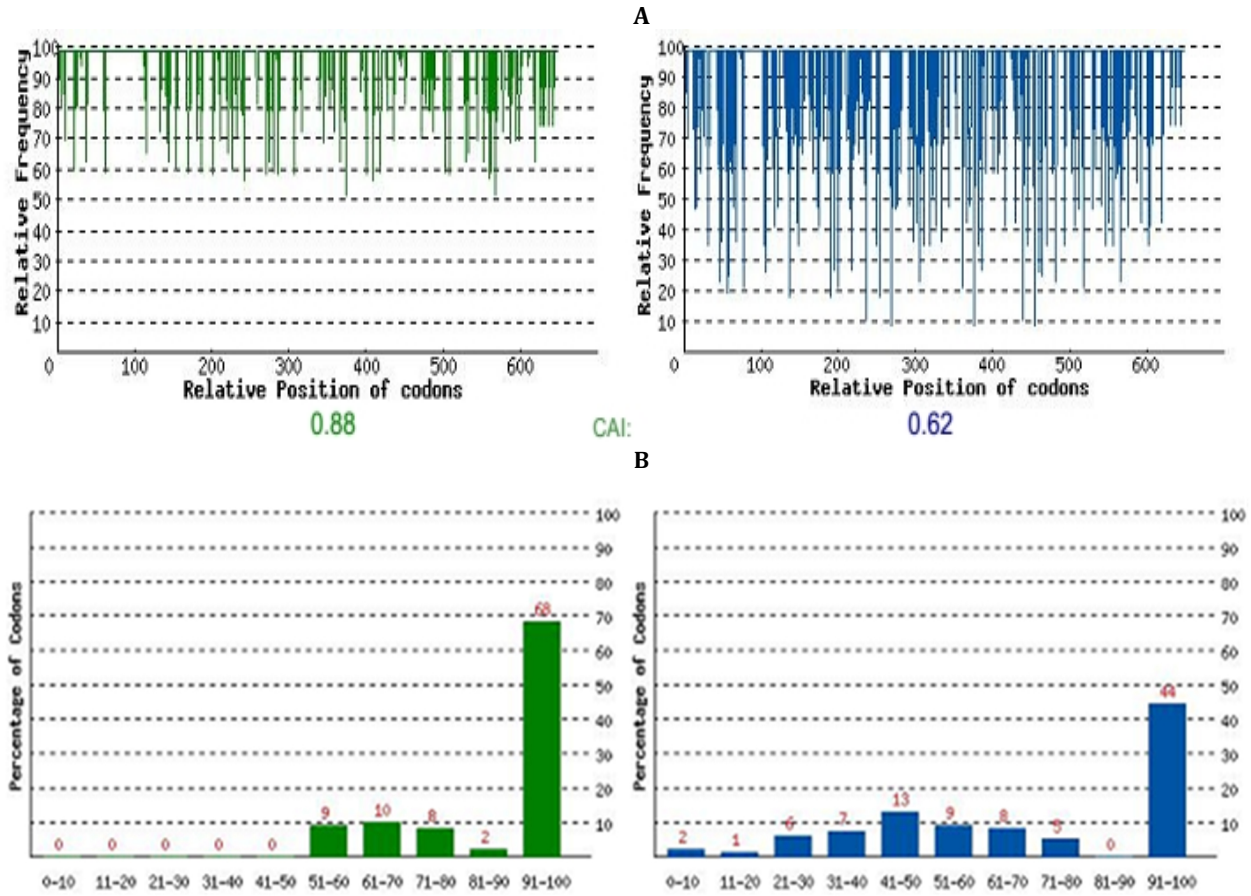

Figure: 1. Codon usage bias in E. coli: (A) Codon Adaptation Index (CAI); Right: before optimization, Left: after optimization; (B) Frequency of Optimal Codons (FOC); Right: before optimization, Left: after optimization.

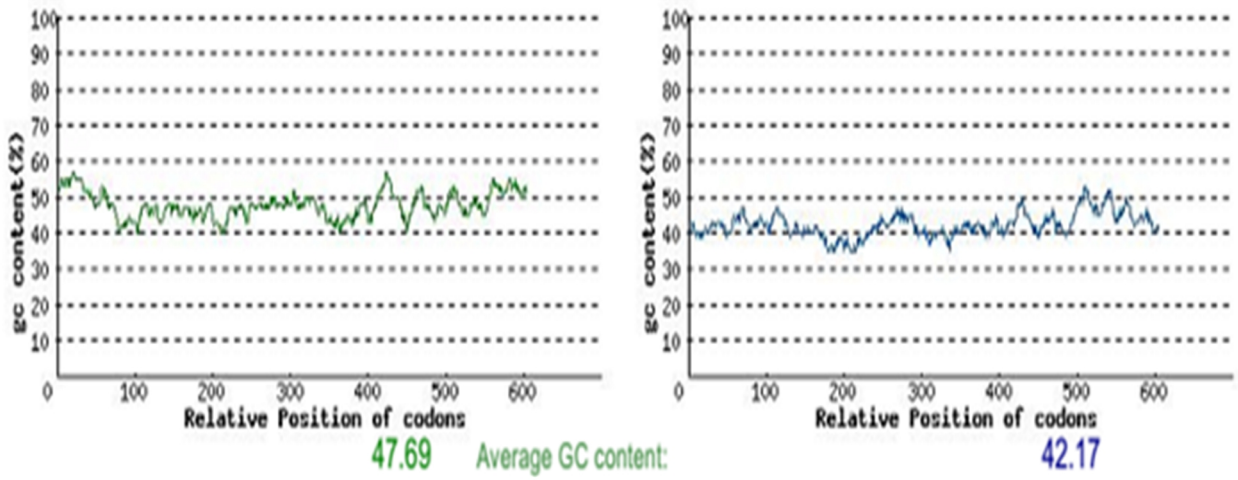

Figure: 2. GC Content adjustment; Right: before optimization, Left: After optimization.

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Optimized      9   ATGGCCTGGTACACCGTGAACTCCGCCTATGGTGATACGATTATTATTCCGTGCCGCCTG
Original       9   ATGGCATGGTATACTGTAAATTCAGCATATGGAGATACCATTATCATACCTTGCCGACTT

Optimized     69   GACGTGCCGCAGAACCTGATGTTTGGCAAATGGAAATATGAAAAACCGGATGGTAGTCCG
Original      69   GACGTACCTCAGAATCTCATGTTTGGCAAATGGAAATATGAAAAGCCCGATGGCTCCCCA

Optimized    129   GTGTTTATTGCGTTCCGTAGCTCTACGAAAAAATCCGTGCAGTATGATGACGTTCCGGAA
Original     129   GTATTTATTGCCTTCAGATCCTCTACAAAGAAAAGTGTGCAGTACGACGATGTACCAGAA

Optimized    189   TACAAAGACCGCCTGAACCTGTCAGAAAATTACACCCTGAGTATTTCCAACGCACGTATC
Original     189   TACAAAGACAGATTGAACCTCTCAGAAAACTACACTTTGTCTATCAGTAATGCAAGGATC

Optimized    249   TCGGATGAAAAACGCTTTGTTTGCATGCTGGTCACGGAAGACAATGTCTTTGAAGCGCCG
Original     249   AGTGATGAAAAGAGATTTGTGTGCATGCTAGTAACTGAGGACAACGTGTTTGAGGCACCT

Optimized    309   ACCATTGTCAAAGTGTTCAAACAGCCGAGCAAACCGGAAATCGTGTCTAAAGCCCTGTTC
Original     309   ACAATAGTCAAGGTGTTCAAGCAACCATCTAAACCTGAAATTGTAAGCAAAGCACTGTTT

Optimized    369   CTGGAAACGGAACAACTGAAAAAACTGGGCGATTGTATTTCAGAAGATTCGTATCCGGAC
Original     369   CTCGAAACAGAGCAGCTAAAAAAGTTGGGTGACTGCATTTCAGAAGACAGTTATCCAGAT

Optimized    429   GGCAACATCACCTGGTACCGTAATGGTAAAGTTCTGCATCCGCTGGAAGGCGCAGTGGTT
Original     429   GGCAATATCACATGGTACAGGAATGGAAAAGTGCTACATCCCCTTGAAGGAGCGGTGGTC

Optimized    489   ATCATCTTCAAAAAAGAAATGGACCCGGTCACCCAGCTGTATACCATGACGAGTACCCTG
Original     489   ATAATTTTTAAAAAGGAAATGGACCCAGTGACTCAGCTCTATACCATGACTTCCACCCTG

Optimized    549   GAATACAAAACCACGAAAGCTGACATCCAAATGCCGTTCACCTGCTCTGTTACCTACTAC
Original     549   GAGTACAAGACAACCAAGGCTGACATACAAATGCCATTCACCTGCTCGGTGACATATTAT

Optimized    609   GGTCCGAGCGGTCAGAAAACGATTCACCACCACCATCACCATCACTAA
Original     609   GGACCATCTGGCCAGAAAACAATTCATCATCACCATCACCATCACTAA
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Figure: 3. DNA Alignment**;** Comparing nucleotide for peer to peer in the primary and optimized sequences.

The synthetic gene was constructed from 662 bp located between *NcoI* and *XhoI* restriction sites and coloned into pBSK (+) vector (Figure 4).

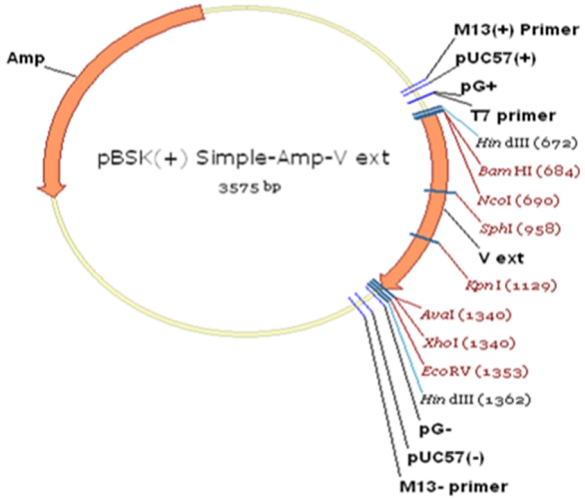

Figure: 4. Schematic figure of construct map pBSK(+) simple-Amp-V dmain

The V-domain gene was amplified by transformed pBSK (+) vector into *E. coli* top10. It was digested and subcloned into pET-28a expression vector successfully (Figure 5).

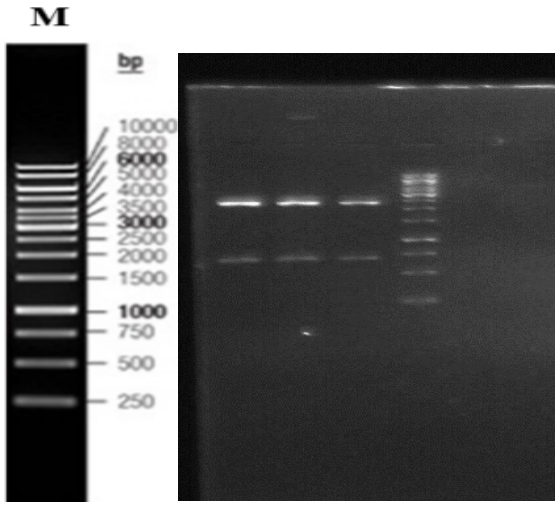

Figure: 5. Double digestion of pBSK (+) vector (with the NcoI and the XhoI);M, GeneRuler™ 1 kb ladder (Fermentas, Lithuania) and Lane 1, 2 and 3 Double digested plasmid.

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E. coli BL21 (DE3) was transformed by recombinant construct. The LB agar was supplemented with kanamycin (100 µg.mL-1) as antibiotic to assurance the screening process. Transformed colonies were observed after 18 to 20 h incubation at 37 ºC. After extraction of the transformed plasmid, double digestion verified the accuracy of transformation (Figure 6).

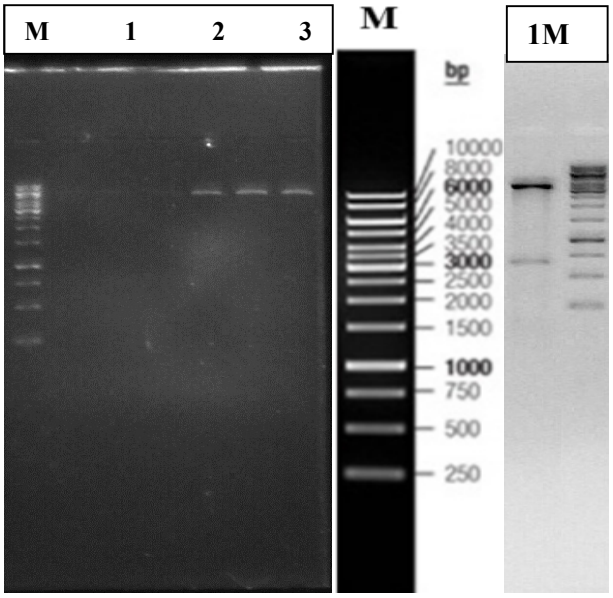

Figure: 6. (A) Extraction of transformed plasmid (pET-28a Recombinant Vector); M, GeneRuler™ 1 kb ladder; Lane 1, 2 and 3: Extraction of transformed plasmid; (B) The double digestion of pET-28a recombinant vector (with the NcoI and the XhoI); M, GeneRuler™ 1 kb ladder; Lane 1, double digestion.

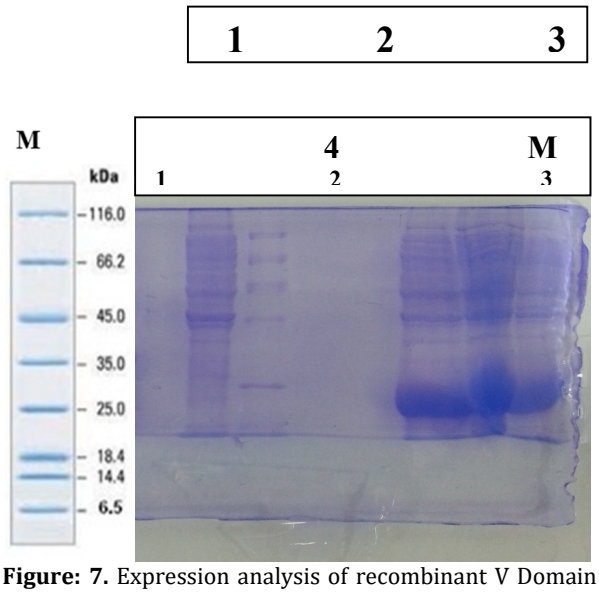

Figure: 7. Expression analysis of recombinant V Domain produced in E. coli BL21 (DE3) by SDS-PAGE; line M, protein marker (CMG, Iran); line1, 2 and 3: Induction of E. coli BL21 (DE3) with IPTG; lane 4: Non- induced E. coli BL21 (DE3).

Recombinant clones were selected, grown in LB Media and induced with IPTG. The expression of recombinant V domain was examined by SDS-PAGE. Since the weight of the recombinant protein together with 6x His-tag is approximately 23 kDa, 12% SDS-PAGE gel was used (Figure 7).

4. Discussion

CD166, as an immunoglobulin molecule, is involved in tumorigenesis of CRC.[2, 3, 25-28] The protein mediates heterophilic and homophilic cell-cell interactions and implicated in cell migration.[1, 9] As well as other specific membrane proteins, CD166 can be applied potentially as a suitable target for cancer therapy.[16] CD166 as a cell surface antigen, can be used for several purposes such as predicting the response to treatment and sorting cancer stem cells.[29, 30]

The specific expression pattern of CD166 suggests this marker as an effective therapeutic target for CRC. CD166 is significantly overexpressed in CRC while normally expressed in intestinal cells surface.[31] It is also known as a cancer stem cell marker protein and believed to have unique ability to develop self-renewal of cancer cells leading to form the source of many human tumors.[32]

Common methods of chemotherapy target differentiated or differentiating cells that form the bulk of the tumor. But it should be noted that these cells are only the size of the tumor and are not able to produce new cells and have no role in disease progression and tumor growth, while the population of cancer cells that cause cancer and tumor growth remains intact and out of sight, thereby causing recurrence of the disease. So given that CD166 is introduced as a surface cell stem marker for CRC, it can be an appropriate candidate to be focused for diagnostic and therapeutic applications.[33, 34]

It is also one of the main components for CRC metastasis to the liver and lung tissues.[15] Cancer cells use sticky molecules to be connected to each other during formation of mass lesions. Adhesion molecules are involved in interaction with the extracellular matrix, cell-cell adhesion, local invasion, dissemination by intra-vascularization, as well as formation of metastases via the blood or lymphatic system in distant organs. Therefore, a set of the functions of a adhesion molecules is a strategy with impact for intervention in the progression of cancer.[7, 35]

Based on functional mapping studies, the presence of V zone is essential for both CD166 heterophilic and hemophilic reactions. This observation was in line with mapping studies of immunoglobulin superfamily cell adhesion molecules, which most frequently identified the V domain as the major ligand-binding domain. Given the importance of V domain in promoting CD166 reactions and its presence in extracellular region,

cloning and expression of V domain seems necessary to present it as a platform for diagnostic and therapeutic reasons.

Cancer stem cell markers are used for different purposes such as early diagnosis screening, determining the origin of cancer, either prognosis or diagnosis of tumor invasion, analyzing the effectiveness of the treatment, and finding a suitable treatment and diagnosis of cancer recurrence after surgery and chemotherapy. In this study, we aimed to produce the V domain in suitable prokaryotic host to provide necessary conditions for its purification and isolation. This recombinant protein can be used in laboratory for cellular immunity induction and confirmation of its immunogenicity under natural conditions. Furthermore, it can be used to improve diagnostic procedures (diagnostic kits) or therapies (vaccines) for colorectal cancer.

According to precious specificity and ability of the immune system, vaccination is in theory the most accurate and strong approach for controlling cancer.[36] The long-standing target in cancer immunology and medical oncology has been special activation of the immune system to control cancer growth in vivo. The use of antigen-specific vaccination is the eventuality of monitoring special vaccine-induced immune responses.[37]

In addition, given that post-translational modifications of glycosylation does not affect the binding properties of hemophilic CD166[15] (Figure 8); therefore, the choice of *E. coli* as host for expression can be an appropriate option for reducing costs and increasing the expression.

5. Conclusion

CD 166, as a stem cell marker, plays important roles in tumor progression and invasion of colorectal cancer. Since the V domain of CD166 is located on the extracellular region playing very important role in interactions, and enjoy a proper size, it can be a good choice as a potential target for the treatment and diagnosis of colorectal cancer. The result of this study demonstrated high level of V domain expression in *E. coli* system using pET28a expression vector.

Conflict of Interest

All of authors declare no potential conflict of interest.

References

1. King JA, Ofori-Acquah SF, Stevens T, *et al*. Activated leukocyte cell adhesion molecule in breast cancer: prognostic indicator. Breast Cancer Res. 2004;6:478-87.
2. Masedunskas A, King JA, Tan F, *et al*. Activated leukocyte cell adhesion molecule is a component of the endothelial junction involved in transendothelial monocyte migration. FEBS Lett. 2006;580(11):2637-45
3. Wang J, Gu Z, Ni P, *et al*. NF-kappaB P50/P65 hetero-dimer mediates differential regulation of CD166/ALCAM expression via interaction with micoRNA-9 after serum deprivation, providing evidence for a novel negative auto-regulatory loop. Nucleic Acids Res. 2011;39(15):6440-55.
4. Klonisch T, Wiechec E, Hombach-Klonisch S, *et al.* Cancer stem cell markers in common cancers – therapeutic implications. Trends Mol Med. 2008;14(10):450-60.
5. Dalerba P, Dylla S, Park I, *et al.* Phenotypic characterization of human colorectal cancer stem. Cell pans. 2007;24:10158-63.
6. Bowen MA, Pate DD, Li X, *et al.* Cloning, mapping, and characterization of Activated Leukocyte-Cell Adhesion Molecule (ALCAM), a CD6 Ligand. J Exp Med. 1995;181(6):2213-20.
7. Bowen MA, Bajorath J, Siadak AW, *et al*. The Amino-terminal Immunoglobulin-like Domain of Activated Leukocyte Cell Adhesion Molecule binds specifically to the membrane-proximal scavenger receptor cysteine-rich domain of CD6 with a 1:1 stoichiometry. J Biol Chem. 1996;271(29):17390-6.
8. Weidle UH, Eggle D, Klostermann S, *et al*. ALCAM/CD166: Cancer-related Issues. Cancer Genomics Proteomics. 2010;7:231-44.
9. Wiiger MT, Gehrken HB, Fodstad Q, *et al*. A novel human recombinant single-chain antibody targeting CD166/ALCAM inhibits cancer cell invasion in vitro and in vivo tumour growth. Cancer Immunol Immunother. 2010;59:1665–74.
10. Bowen M.A, Aruffo A. Adhesion molecules, their receptors, and their regulation: Analysis of CD6-Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166) Interactions. Transplant Proc. 1999;31(1-2):795-6.
11. Ofori-Acquah SF, King JA. Activated leukocyte cell adhesion molecule: a new paradox in cancer. Translational Research. 2008;151:122–28.
12. Fanali C, Lucchetti D, Farina M, *et al*. Cancer stem cells in colorectal cancer from pathogenesis to therapy: Controversies and perspectives. World J Gastroentero. 2014;20(4):923-42.
13. Bowen MA, Aruffo AA, Bajorath J. Cell surface receptors and their ligands: In Vitro analysis of CD6-CD166 interactions. PROTEINS. 2000;40: 420–8
14. Skonier JE, Bowen MA, Aruffo A, *et al*. CD6 recognizes the neural adhesion molecule BEN. Protein Science. 1997;6:1768-70.

 ISSN 2330-4049

15. Swart GW. Activated leukocyte cell adhesion molecule (CD166/ALCAM): Developmental and mechanistic aspects of cell clustering and cell migration. Eur J Cell Biol. 2002;81:313–21.
16. Tachezy M, Zander H, Gebauer F, *et al*. Activated leukocyte cell adhesion molecule (CD166)-Its prognostic power for colorectal cancer patients. J Surg Res. 2012;177:15-20.
17. Pitule P, Čedíková M, Třeška V, *et al*. Assessing colorectal cancer heterogeneity: one step closer to tailored medicine. J Appl Biomed. 2013;11:115–29.
18. Park JH, McMillan DC, Horgan PG, *et al*. The impact of anti-inflammatory agents on the outcome of patients with colorectal cancer. Cancer Treat Rev. 2014;40:68–77.
19. Mejia A, Schulz S, Hyslop T, *et al*. Molecular staging individualizing cancer management. J Surg Oncol. 2012;105(5):468–74.
20. Rasaf MR, Ramezani R, Mehrazma M, *et al*. Inequalities in cancer distribution in Tehran; A disaggregated estimation of 2007 incidence by 22 districts. Int J Prev Med. 2012;3:483-92.
21. Ouakrim DA, Lockett T, Boussioutas A, *et al*. Screening participation predictors for people at familial risk of colorectal cancer a systematic review. Am J Prev Med. 2013;44(5):496–506.
22. Kim E, Coelho D, Blachier F. Review of the association between meat consumption and risk of colorectal cancer. Nutrition Research. 2013;33:983–94.
23. Moshfeghi K, Mohammad-Beigi A, Hamedi-Sanani D, *et al*. Evaluation the role of nutritional and individual factors in colorectal cancer. Zahedan J Res Med Sci. 2011;13(4):12-7.
24. Darabi P, Galehdari H, Khatami SR , *et al*. Codon optimization, cloning and expression of the human Leukemia Inhibitory Factor (hLIF) in E. Coli. Iran J Biotech. 2013;11(1):47-53.
25. Ni C, Zhang Z, Zhu X, *et al*. Prognostic value of CD166 expression in cancers of the digestive system: a systematic review and meta-analysis. PLoS One. 2013;5:8(8):e70958.
26. Wang F, Scoville D, He XC, *et al*. Isolation and Characterization of intestinal stem cells based on surface marker combinations and colony-formation assay. Gastroenterology. 2013;145(2):383–95.
27. Vaiopoulos A G, Kostakis I D, Koutsilleris M, *et al*. Concise review: colorectal cancer Stem Cells. Stem Cells. 2012;30:363–71.
28. Sanders MA, Majumdar APN. Colon cancer stem cells: implications in carcinogenesis. Front Biosci (Landmark Ed). 2014;16:1651–62.
29. Liu AY, Roudier MP, True LD. Heterogeneity in primary and metastatic prostate cancer as defined by cell surface CD profile. Am J Pathol. 2004;165:1543-56.
30. Dana H, Marmari V, Mahmoodi G, *et al*. CD166 as a Stem Cell Marker? A potential target for therapy colorectal cancer? J Stem Cell Res Ther. 2016;1(6):00041.
31. Levin TG, Powell AE, Davies PS, *et al*. Characterization of the intestinal cancer Stem Cell Marker CD166 in the human and mouse gastrointestinal tract. Gastroenterology. 2010;139:2072–82.
32. Shiozawa Y, Nie B, Pienta KJ, *et al*. Cancer stem cells and their role in metastasis. Pharmacol Ther. 2013;138:285–93.
33. Botchkina C. Colon cancer stem cells – From basic to clinical application. Cancer Letters. 2013;338:127–40.
34. Dana H, Mahmoodi G, Marmari V, *et al*. An overview of cancer Stem Cell. J Stem Cell Res Ther. 2016;1(4):00029.
35. Noori Daloii MR, Fazilaty H, Tabrizi M. Cancer metastasis, genetic and micro environmental factors of distant tissue: a review article. Tehran Univ Med J(TUMJ). 2013; 70(11): 671-83.
36. Noh KH, Lee Y-H, Jeon J-H, *et al*. Cancer vaccination drives Nanog-dependent evolution of tumor cells towards an immune-resistant and stem-like phenotype. Cancer Research. 2012;72(7):1717-27.
37. Jager E, Jager D, Knuth A. Antigen-specific immunotherapy and cancer vaccines. Int J Cancer. 2003;106:817–20.

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