In Silico Analysis, Cloning and Expression of Recombinant CD166 in E. coli BL21 (DE3) as a Marker for Detection and Treatment of Colorectal Cancer

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Abstract

Introduction: Colorectal cancer is the third most common type of tumors, with more than 1.2 million new cases resulting in 600 thousand deaths annually and ranks fourth in terms of mortality worldwide. The activated leukocyte cell adhesion molecule (ALCAM) also called CD166 is a marker of colorectal cancer (CRC) stem cells. The expression of CD166 increases in colorectal cancer. Also with advancement of illness in different stages of cancer, this expression increased. So, it could be a reasonable marker for Detection and Treatment of Colorectal cancer. The purpose of this study is to produce recombinant protein CD166 for cancer therapy or early detection of colorectal cancer cells.

Methods: In this study, the sequence of CD166 was optimized for expression in E. coli using online tools and cloned into pET28a as an expression vector. The recombinant pET28a was transformed into the E. coli BL21DE3 using heat shock method and expression of recombinant CD166 was examined using SDS-PAGE.

Results: The synthetic gene of CD166 was located between NcoI/BamHI and XhoI restriction sites and cloned into pBSK (+) vector. The presence of this gene in pET28a was determined by colony and confirmed by restriction digestion. Gene of CD166 were expressed in E. coli BL21 DE3. The results of the SDS-PAGE technique confirmed the expression of recombinant 53 kDa CD166 in a bacterial expression system.

Conclusion: A portion of the CD166 gene was expressed as a recombinant in E. coli. This could be a good candidate to produce a vaccine for cancer therapy or colorectal cancer diagnostic test.

Keywords: Colorectal cancer; Cloning and Expression; Recombinant Protein; CD166/ALCAM

Introduction

Cancer is one of the oldest diseases occurring among humans and animals with a history longer than the prehistoric era. Developing countries account for at least two-thirds of cancers in the world [1]. Colorectal cancer is fourth common type of cancer with an estimated of 1/2 million new diagnosed cases annually. This disease is the third cause of death among the cancers in the world [2-4].

Factors which increase the risk of colorectal cancer may be considered as age over 50, colorectal polyps, family history of colorectal cancer, genetic disorders, history of being diagnosed with cancer, Krone or inflammatory bowel disease, diet, smoking, etc. [5].

Molecular or genetic changes can also be involved in colorectal cancer but some genetic changes can increase the risk of colorectal cancer such as changes in HNPCC or APC2 genes [6]. Symptoms of colorectal cancer are diarrhea, constipation, blood in the stool, a completely empty bowel feeling, narrowing of the stool and losing weight without reason.

Colorectal cancer may be treated using various methods depending on the location of the tumor, in the rectum and colon, and the stage of the disease, such as surgery, radiotherapy, chemotherapy or a combination of all these methods, in which surgery is the most common treatment method [6].

CD166, also known as ALCAM (Activated Leukocyte Cell Adhesion Molecule), is a marker of colorectal cancer stem cells, which emerges by aggressive tumors. Adhesion molecules like the CD166 are important for cell survival, cell growth and motility, and for invasion during tumor progression and metastases [7-12].

The presence of CD166 at the tumor surface indicates the shortening of survival [13]. When the cells become cancerous, CD166, which is located on the outer surface of colorectal cancer cells, is noticeably overexpressed at the surface [14].

CD166 is a member of the immunoglobulin superfamily and was identified by expression cloning based on its ability to bind to CD6 making use of COS cells transfected with cDNA libraries. CD166 consists of five extracellular immunoglobulin domains (two NH2-terminal, membrane-distal variable-Vf-type folds and three membrane-proximal constant-[C2]-type immunoglobulin folds), a transmembrane region, and a short cytoplasmic tail. ALCAM is able to mediate homophilic as well as heterophilic (CD6) interactions. The gene...
CD166 is located on the long arm of human chromosome 3 (3q13.1-q13.2). It is organized into 16 exons that span nearly 150 kb of DNA [15-17].

In addition, this protein is considered to be a recognizable anti-gene that may lead to the production of antibody in an immunized mouse with human intestine cancerous cells [18]. The CD166 protein is also considered as a well-known molecular marker for targeted treatment used for patients suffering colorectal cancer [19,20]. In this research bioinformatics analysis, cloning and surface expression of CD166 were applied to study and to analyze the potential of optimization of diagnosis methods such as generating diagnostic kits to diagnose the colorectal cancer cells before becoming malignant and the possibility producing a vaccine for colorectal cancer.

CD166 plays a crucial role in the invasion and the development of tumor in colorectal cancer and is assumed as a cancer stem cells marker. Besides, the extracellular of this protein has a region with appropriate size. it performs an important role in the reactions and interactions of the protein, for either hemophilic or heterophilic reactions. CD166 is composed of two components of 93 and 110-amino acids and three components of 84, 77 and 86 amino acids, given that, this experiment is aimed to clone and express CD166 to be used in practical, diagnostic and remedial plans.

Materials and Methods

Materials

Luria Bertani (LB) media was prepared according to Sambrook and Russel. Antibiotic screening was performed on LB agar plates using ampicillin at 100 μg/mL and Kanamycin at 20 μg/mL which was obtained from Sigma, USA. Concentrations of IPTG (100 μg/mL) were used for the induction of CD166 gene. This reagent was purchased from Merck, Germany. The restriction enzymes were purchased from Fermentas (Lithuania).

Bioinformatics analysis and optimization and chemical synthesis of gene CD166

The sequence of the specified gene was estimated via uniport KB/Swiss-Port website and the national center of biotechnology information (NCBI). Afterwards, after 3’ sequences, 6 histidine amine acids (His-Tag) were inserted followed by TAA stop codon. In the end, Ncol and BamHI enzymes restriction sites were embedded at 5’ terminal and Xhol enzyme restriction site at 3’ terminal. In the following, codon optimization was performed using American GenScript Company to gain access to the most correct expression in prokaryotic host. In the following, the gene was chemically synthesized and delivered as a clone in pBSK (+) vector (Biomatik Corporation, Canada).

Amplify the CD166 gene

In order to amplify the CD166 gene, the E. coli TOP10 was transformed with plasmid containing CD166 gene using heat shock method. Then, plasmid extraction, a double digestion was done with Ncol and Xhol.

Gene CD166 sub cloning

E. coli BL21 (DE3) was used as an expression host cell. Digested fragments were analyzed by agarose gel electrophoresis and PET28a expression vector and CD166 fragments were purified. Finally, CD166 sequence was ligated to the PET28a using T4 DNA ligase. After an overnight incubation at 37°C, the plasmid extraction from appeared colonies were performed.

Transformation of recombinant vector (pet-28a-CD166)

In this research, the competent E. coli BL21 (DE3) host cells were prepared with calcium chloride. The accuracy of transformation was verified by double digestion of plasmid with Ncol and Xhol enzymes.

Expression of CD166 gene

Protein expression was analyzed using SDS-PAGE analysis technique. E. coli BL21 (DE3) harboring the CD166 pet-28a (+) positive colony was grown in 100 ml LB broth at 37°C including 20 μg/ml Kanamycin to achieve an optical density (OD) of 0.8 and at this time, 20 μL IPTG (100 μg/mL) was added to 50 ml LB broth. At 37°C, incubation was continued. The samples from induced culture for 6 h at 150 rpm and 37°C after induction. Samples were accumulated, centrifuged at 5000 rpm for 5 min and 4°C. Supernatant was discarded and 80 μL Urea (8 M) was added to the precipitate. To run the samples, the mixture (precipitated sample and Urea) was dissolved in 1 × SDS-PAGE sample buffer. The prepared samples and protein molecular weight marker was heated for 7 min at 95°C. Then each sample and marker was loaded on the 12% SDS-PAGE gel and run on the constant voltage of 100 V.

Results

Sequence optimization and gene synthesis

In the native nucleotide sequence, there were parameters that could decrease the efficiency of expression system in the E. coli host cell. Thus, these parameters were substituted to optimize clones to a higher efficiency system. The parameters changed through optimization were Codon Adaptation Index (CAI) and frequency of optimal codons (FOP) and the GC content adjustment.

CAI is variable between 0 and 1, in which the closer index to 1 shows better expressional conditions. In the native sequence the CAI was 0.61 which was changed to 0.88 By changing the nucleotides (Figure 1).
The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. In the native sequence the FOP was 43 which was changed to 70 (Figure 2).

A suitable sequence has a G-C Content Adjustment of 30% to 70% (50% on average). Through codon optimization, the G-C content adjustment was increased from 41.59 to 48.56, which results in an increase in the half-life of mRNA of the gene (Figure 3). Considering the modified parameters, nucleotide sequence of original and optimized sequences was compared for Peer to Peer (Figures 4 and 5).

**Amplify the CD166**

To amplify the recombinant pBSK plasmid containing CD166 DNA, transformation process was carried out for *E. coli* top 10 competent cell and the colonies grown on medium containing ampicillin antibiotic were selected (Figure 6).

Followed by confirming pBSK plasmid containing the CD166 gene using the electrophoresis method, double digestion technique was applied to confirm the presence of CD166 gene in the plasmid, considering NcoI and XhoI enzyme restriction sites embedded at the two ends of synthesized gene (Figure 7).

**Sub-cloning of CD166 in pET28a**

CD166 DNA was digested and it was ligated into similarly digested pET-28a expression plasmid. In the following, *E. coli* BL21 (DE3) competent cells were transformed by recombinant construct. Afterwards, the transformed bacteria were inoculated in a medium containing kanamycin and incubated for an overnight. Plasmid extraction was performed and was confirmed on gel electrophoresis. After confirming the presence of recombinant pET-28a plasmid, enzyme double digestion technique was performed. Observing a 1463 bp band related to the target gene and a 5369 bp band to the pET-28a expression plasmid indicated the cloning of target gene into the pET-28a plasmid (Figures 8 and 9).

**Discussion**

With nearly 135,000 new cases diagnosed annually, CRC is the third most prevalent cancer in the United States [21]. Concentrate on cancer initiation and development has dominated the effort to better understand disease pathology and guide therapeutic approaches. Hence, the cancer stem cell (CSC) hypothesis, which suggests that cancer is driven by cells harboring stem cell-like qualities, offers one explanation for why many current therapeutic approaches ultimately result in relapse of disease. In this hypothesis, some CSCs may be quiescent and, thus, evade eradication by standard cytotoxic therapies designed to target proliferating cells. These surviving cells can then proceed to support tumor growth and have the potential to initiate metastatic or recurrent disease [22-32].

ALCAM has been reported to be involved in tumorigenesis of CRC and to function as a CSC marker. Correlation of the CD166 expression pattern with aggressive disease has led to efforts for targeting this molecule as a cancer therapeutic.

Bioinformatics is gathering, implementation and analysis of a large quantity of biological data, which its most essential application is sequence analysis. Another practical application of bioinformatics is the optimization of newly composed gene considering the host cell, which was done in this research. Codon optimization leads to a better expression, precision, and performance of the target gene in the host cell.

Monoclonal antibodies (MAbs) are a relatively new innovation in cancer treatment. At present, some monoclonal antibodies have increased the efficacy of the treatment of certain tumors with acceptable safety profiles. CD166 is a cell-surface antigen that is proposed as the antigen of cancer stem cell in CRC [33]. Recently, the recognition
Figure 4: DNA alignment; Comparing nucleotide for peer to peer in the primary and optimized sequences.

Figure 5: The map of pBSK plasmid containing CD166 gene.
of the tumor related antigen has introduced a new foundation in the immunotherapy of special antigens. The major aim of vaccine experiments initiated from the last decade was to induce a specific immune response against the cancer antigens [34-37].

In this study, with the induction of CD166 protein in E. coli BL21 (DE3), to take initial steps to purify and separate, we made an effort to take initial steps to purify and separate them in a way to be used for in vitro induction of immune cells. The results of this study can be used to improve the diagnostic approaches and cancer remedial techniques.

Conclusion

By analyzing the expression of CD166 protein in normal and tumor tissues, the excess expression of CD166 protein was demonstrated in the colorectal cancer, as result this protein is considered to be the exclusive marker of colorectal cancer. Considering the correlation between CD166 protein and tumor growth, this protein is suggested to be a marker for fighting methods against colorectal cancer. In this research, underlying studies have been provided using bioinformatics analysis, cloning and expression of the CD166 protein, in order to employ this protein as a vaccine and to produce colorectal cancer detection kits.

References

1. Cancer Facts and Figures. American Cancer Society. Accessed on Feb 23, 2017.
2. Haggar FA, Boushey RP (2009) Colorectal cancer epidemiology: Incidence, mortality, survival, and risk factors. Clin Colon Rectal Surg 22: 191-197.
3. Forghanifard MM, Moghbeli M, Raeisossadati R, Tavassoli AL, Javdani Mallak A, et al. (2013) Role of SALL4 in the progression and metastasis of colorectal cancer. J Biomed Sci 20: 6.
4. Peravali R, Hall N (2015) Colorectal cancer: Features and investigation. Medicine 43: 299-302.
5. Kim E, Coelho D, Blachier F (2013) Review of the association between meat consumption and risk of colorectal cancer. Nutr Res 33: 98-994.
6. Pitule P, Čedíková M, Třeška V, Králíčková M, Liška V (2013) Assessing colorectal cancer heterogeneity: One step closer to tailored medicine. J Appl Biomed 11: 115-129.
7. Weidle UH, Eggle D, Klostermann S, Swart GW (2010) ALCAM/CD166: cancer-related issues. Cancer Genomics Proteomics 7: 231-244.
8. Ofori Acquah SF, King JA (2008) Activated leukocyte cell adhesion molecule: A new paradox in cancer. Transl Res 151: 122-128.
9. King JA, Ofori Acquah SF, Stevens T, Al Mehdi AB, Fodstad O, et al. (2004) Activated leukocyte cell adhesion molecule in breast cancer: prognostic indicator. Breast Cancer Res 6: 478-487.
10. Brunner TB, Kunz-Schughart LA, Grosse-Gehling P, Baumann M (2010) Cancer stem cells as a predictive factor in radiotherapy. Semin Radiat Oncol 22: 151-174.
11. Jia G, Wang X, Yan M, Chen W, Zhang P (2016) CD166-mediated epidermal growth factor receptor phosphorylation promotes the growth of oral squamous cell carcinoma. Oral Oncol 1-11.
12. Ihnena M, Kohler N, Kerstenc JF, Milde Langoscha K, Becka K, et al. (2010) Expression levels of activated leukocyte cell adhesion molecule (ALCAM/
CD166 in primary breast carcinoma and distant breast cancer metastases. Dis Markers 28: 71-78.

13. Hansen AG, Freeman TJ, Arnold SA, Starchenko A, Jones Paris CR, et al. (2013) Elevated ALCAM shedding in colorectal cancer correlates with poor patient outcome. Cancer Res 73: 1-10.

14. Fujiiwa K, Ohuchida K, Sada M, Horioka K, Ulrich CD, et al. (2014) CD166/ALCAM expression is characteristic of tumorigenicity and invasive and migratory activities of pancreatic cancer cells. PLoS One 9: e107247.

15. Miyata T, Yoshimatsu T, So T, Oyama T, Uramoto H, et al. (2015) Cancer stem cell markers in lung cancer. Personalized Medicine Universe 4: 40-45.

16. Ni C, Zhang Z, Zhu X, Liu Y, Qu D, et al. (2013) Prognostic value of CD166 expression in cancers of the digestive system: A systematic review and meta-analysis. PLoS One 8: e70958.

17. Sun Y, Wang Y, Cao Q, Yu H, Zheng D, et al. (2015) Expression and role of CD166 in the chronic kidney disease. Iran J Pediatr 25: e543.

18. Levin TG, Powell AE, Davies PS, Silk AD, Dismuke AD, et al. (2010) Characterization of the intestinal cancer stem cell marker CD166 in the human and mouse gastrointestinal tract. Gastroenterology 139: 2072-2082.

19. Dana H, Mahmoodi G, Marmari V, Mazraeh A, Ebrahimie M (2016) An overview of cancer stem cell. J Stem Cell Res Ther 1: 00029.

20. Tachezy M, Effenberger k, Zander H, Minner S, Gebauer F, et al. (2012) ALCAM (CD166) expression and serum levels are markers for poor survival of esophageal cancer patients. Int J Cancer 131: 396-405.

21. Dana H, Marmari V, Mahmoodi G, Mahmoudzadeh H, Ebrahimie M, et al. (2016) CD166 as a Stem Cell Marker? A Potential Target for Therapy Colorectal Cancer?. J Stem Cell Res Ther 1:00041.

22. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ (2005) Opinion: The origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 5: 899-904.

23. Nguyen LV, Vanner R, Dirks P, Eaves CJ (2012) Cancer stem cells: An evolving concept. Nat Rev Cancer 12: 133-143.

24. Kreso A, Dick JE (2014) Evolution of the cancer stem cell model. Cell Stem Cell 14: 275-291.

25. Chen LS, Wang AX, Dong B, Pu KF, Yuan LH, et al. (2012) A new prospect in cancer therapy: Targeting cancer stem cell to eradicate cancer. Chin J Cancer 31: 564-572.

26. Bu Y, Cao D (2012) The origin of cancer stem cells. Front Biosci (Schol Ed) 4: 819-830.

27. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414: 105-111.

28. Medema JP (2013) Cancer stem cells: The challenges ahead. Nat Cell Biol 15: 338-344.

29. Sanchez Garcia I, Vicente Duenas C, Cobaleda C (2007) The theoretical basis of cancer-stem-cell-based therapeutics of cancer: Can it be put into practice. Bioessays 29: 1269-1280.

30. Li Q, Rycal K, Chen X, Tang DG (2015) Cancer stem cells and cell size: A causal link. Semin Cancer Biol 35: 191-199.

31. Tang DG (2012) Understanding cancer stem cell heterogeneity and plasticity. Cell Res 22: 457-472.

32. O'Brien C A, Kreso A, Jamieson CH (2010) Cancer stem cells and self-renewal. Clin Cancer Res 16: 3113-3120.

33. Scott AM, Allison JP, Wolchok JD (2012) Monoclonal antibodies in cancer therapy. Cancer Immun 12: 14.

34. Chan AC, Carter PJ (2010) Therapeutic antibodies for autoimmunity and inflammation. Nat Rev Immunol 10: 301-316.

35. Van den Eynde BJ, Scott AM (1998) Tumor Antigens. Encyclopedia of Immunology 2424-2431.

36. Weiner LM, Surana R, Wang S (2010) Monoclonal antibodies: Versatile platforms for cancer immunotherapy. Nat Rev Immunol 10: 317-327.

37. Schliemann C, Neri D (2010) Antibody-based vascular tumor targeting. Recent Results Cancer Res 180: 201-216.