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Cloning and Expression of C2 and V Domains of ALCAM Protein in *E. coli* BL21 (DE3)

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Abstract

Introduction: ALCAM as a glycoprotein is a member of the immunoglobulin family and plays an important role in cell growth, survival and motility. This protein also contributes to the development of tumour invasion in colorectal and breast cancers and is typically considered as a cancer stem cell marker. ALCAM as a cell-surface antigen, with high expression in some cancers as colorectal and breast, has the potential to diagnose and treat these cancers.

Objectives: As with other membrane proteins, ALCAM represents a potential target for therapy of colorectal and breast cancers, therefore, this study is aimed to clone and express C2 and V domains to be used in practical, diagnostic and remedial plans.

Methods: In this study, the sequence of C2 and V domains were optimized for expression in prokaryotic host using online tools and cloned in pET-28a expression plasmids. *E. coli* BL21 (DE3) cells were transformed by pET-28a recombinant plasmids using heat shock method and the expression of recombinant V and C2 domains were examined by SDS-PAGE technique.

Results: The synthetic genes of C2 and V domains were located between *Nco*I/*Bam*HI and *Xho*I restriction sites and cloned into pBSK (+) vector. The presence of these genes in pET28a was determined by colony and confirmed by restriction digestion. Gens of C2 and V domains were expressed in *E. coli* BL21 DE3. The results of the SDS-PAGE technique confirmed the expression of recombinant 23 and 29 kDa C2 and V domains in a bacterial expression system.

Conclusions: The genes of C2 and V domains were expressed as the recombinant in *E. coli*. These recombinant fragments can be introduced as diagnostic and remedial candidates for screening and cancer-therapy in patients with colorectal and breast cancers.

Keywords: Cloning and expression; Recombinant protein; ALCAM; V and C2 domains

Introduction

Activated leukocyte cell adhesion molecule (ALCAM), also known as CD166, is a glycoprotein of the immunoglobulin superfamily comprised of a 500-amino acid extracellular domain (including 5 extracellular domains, two types of V and three types of C2), a 22 amino acid trans-membrane domain and a short 34-amino acid cytoplasmic domain [1-3].

The encoding gen of ALCAM is located in the long arm of chromosome 3(3q13.1-q13.2). It is comprised of 16 exons, with the size of 150 kb and weight of 110 kDa [4].

The V region is composed of two parts, V1 with the length of 93 amino acids and V2 with the length of 110 amino acids, which are linked to each other with a 4-amino acid linker peptide. C2 region comprises three domains with the lengths of 84, 77 and 86 amino acids, in order, which are linked with two 4 and 5-amino acid linker peptides.

ALCAM plays an important role in the survival, growth [5] and migration of cells [6,7] and cellular haemophilic (ALCAM-ALCAM) and heterophilic (ALCAM-CD6) reactions [8-13]. In addition, this protein performs a vital role in invasion and development of tumour in colorectal [14-16] and breast cancers [6].

ALCAM is assumed to be a surface-exposed protein in cancer stem cells such as colorectal [17,18], prostate [19,20], oesophageal squamous cells [4] and the breast [21-23]. Antigens are cell-surface markers,

which can be used in the diagnosis of cell groups forming an organ [24]. Considering the fact that ALCAM is a marker of cancer stem cells, it can be used to prognosticate the response to therapy, the regulation of cancer stem cells and the treatment.

During the formation of mass lesion, as the cancer cells should bind to each other, they use adhesion molecules to stay together. As ALCAM is a member of immunoglobulin family with a high expression in colorectal, prostate, oesophageal squamous cells, breasts, bladder and melanoma, this protein is assumed to play a crucial role in the metastasis of these cancers [4].

Today cancer is assumed as one of the serious problems and challenges of health and treatment all around the world. It is becoming increasingly more important in our country as it is the third reason for mortality and the second big non-transmissible chronic disease in Iran [25].

ALCAM plays a crucial role in the invasion and the development of tumour in some cancers and is assumed as a cancer stem cells marker. Besides, V and C2 domains of this protein are located in extracellular region with appropriate size. They perform an important role in the reactions and interactions of the protein, for either haemophilic or heterophilic reactions. They are 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 V and C2 domains to be used in practical, diagnostic and remedial plans.

Methods

Hosts and plasmids

E. coli TOP10 and BL21 (DE3) strains were used as cloning and expression hosts, respectively. Plasmid of pET-28a (+) was used as expression vector.

Codon optimization and gene synthesis

The sequences of V and C2 domains were obtained from Swiss-port, UniProtKB and NCBI (National Center of Biotechnology Information) online databases.

To have access to the utmost level of appropriate expression in the prokaryotic host (*E. coli*), the 662 and 812-bp nucleotide sequences of V and C2 domains were optimized by gene-script Company (NJ, USA).

Optimized structures containing Open Reading Frame (ORF) of V and C2 domains had a 6xHis-tag in their c-terminals to facilitate their purification process. *Nco*I/*Bam*HI and *Xho*I restriction sites were added to the 5' and 3' of the sequence, respectively. The optimized structures were chemically synthesized and inserted into pBSK cloning vector (Biomatik, Canada).

Sub-cloning of V and C2 domains in pET-28a expression plasmid

E. coli TOP10 was transformed by pBSK vector containing V and C2 to replicate the gens. Followed by plasmid extraction (using Plasmid Miniprep Kit, Fermentas, Lithuania), the plasmids were double digested by *Xho*I and *Nco*I enzymes (Fermentas, Lithuania). pET-28a vector was digested with the same enzymes. Digested fragments were examined by electrophoresis on agarose gel and then pET-28a vector and V and C2 fragments were purified (Fermentas, Lithuania). Finally, V and C2 fragments were ligated to pET-28a using T4 DNA ligase enzyme (Fermentas, Lithuania).

Transformation of recombinant vectors (pET-28a-V domain and pET-28a-C2)

To express the genes of V and C2 domains of ALCAM, *E. coli* BL21 (DE3) was applied. In this study, Calcium Chloride method was used to prepare the *E. coli* BL21 (DE3) host competent cells [26].

Considering the recombinant structures, plasmid double digestion with *Xho*I and *Nco*I enzymes were used to confirm the accuracy of the transformation.

Expression of recombinant vectors (pET-28a-V domain and pET-28a-C2)

Considering the presence of lac promoter in pET-28a plasmid, IPTG inductor was used to induct protein expression. 20 µL of transformed BL21 (DE3) was added to 2 ml of LB medium containing kanamycin antibiotic (100 µg/mL) and incubated in a shaker incubator for an overnight at 37°C and 150 rpm. Then, 20 µL of an overnight pre-cultured transformed bacterial cells was inoculated to 2 ml fresh LB medium containing kanamycin (100 µg/mL) and incubated in a shaker incubator at 37°C for 2 h and 150 rpm until the bacterium concentration reaches 0.8 OD.

20 µL of IPTG inductor (100 µg/mL) was added to the medium to induce the protein expression. It was then incubated in a shaker incubator for 6 h at 37°C and 150 rpm. Followed by centrifugation for 5 min at 4°C and 5000 rpm, supernatant was discarded and 60 µL of 8 M Urea was added to the remaining sediment.

Analysis of V and C2 domains expression

To separate cellular protein bands and to track the recombinant proteins in transformed cells and to measure the amount of the expression of selected protein, the expression of V and C2 domains were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Considering that the molecular weight of the recombinant proteins was 23 and 29 kDa, 15% gel was used. To run the specimens, the mixture (Urea and sediment) was dissolved in sample buffer (1x SDS-PAGE). Specimens containing V and C2 domains and molecular weight marker were heated at 95°C for 7 min. Then, the specimens and the marker were loaded in 15% SDS-PAGE gel and run with stable voltage of 110. The gel was dyed with Coomassie brilliant blue R-250 in slow rotation for 2 h. Finally, gel was washed with water for 2 h.

Results

The synthesis of V and C2 domains was performed on the basis of *E. coli* codon usage. Amino acid sequences of wild type and optimized sequences were compared for Peer to Peer (Figure 1). The synthetic genes of V (662 bp) and C2 (812 bp) domains were located between *Nco*I/*Bam*HI and *Xho*I restriction sites and cloned into pBSK (+) vector (Figure 2).

The genes were replicated by *E. coli* top 10 transformants harboring pBSK (+) vectors. Genes of V and C2 domains were digested and successfully sub-cloned into similarly digested pET-28a expression vector (Figure 3). In the following, *E. coli* BL21 (DE3) cells were transformed by recombinant constructs. The LB agar medium

contained kanamycin (100 µg/mL), used as an antibiotic to ensure the screening process. Transformant colonies were observable after 18 to 20 h of incubation in 37°C.

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Optimized     1     MAWYTVNSAYGDTIIIPCRLDVPQNLMFGKWKYEKPDGSPVFIAFRSSTKKSVQYDDVPE
Original      1     MAWYTVNSAYGDTIIIPCRLDVPQNLMFGKWKYEKPDGSPVFIAFRSSTKKSVQYDDVPE

Optimized    61     YKDRLNLSENYTLSISNARISDEKRFVCMLVTEDNVFEAPTIVKVFKQPSKPEIVSKALF
Original     61     YKDRLNLSENYTLSISNARISDEKRFVCMLVTEDNVFEAPTIVKVFKQPSKPEIVSKALF

Optimized   121     LETEQLKKLGDCISEDSYPDGNITWYRNGKVLHPLEGAVVIIFKKEMDPVTQLYTMTSTL
Original    121     LETEQLKKLGDCISEDSYPDGNITWYRNGKVLHPLEGAVVIIFKKEMDPVTQLYTMTSTL

Optimized   181     EYKTTKADIQMPFTCSVTYYGPSGQKTIHHHHHHH*
Original    181     EYKTTKADIQMPFTCSVTYYGPSGQKTIHHHHHHH*
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Figure 1A: Protein Alignment: Amino acid sequences of wild type and optimized sequences were compared for Peer to Peer: V domain.

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Optimized     1     MAPTEQVTIQVLPPKNAIKEGDNITLKCLGNGNPPPEEFLFYLPGQPEGIRSSNTYTLTD
Original      1     MAPTEQVTIQVLPPKNAIKEGDNITLKCLGNGNPPPEEFLFYLPGQPEGIRSSNTYTLTD

Optimized    61     VRRNATGDYKCSLIDKKSMIASTAITVHYLDLSLNPSGEVTRQIGDALPVSCTISASRNA
Original     61     VRRNATGDYKCSLIDKKSMIASTAITVHYLDLSLNPSGEVTRQIGDALPVSCTISASRNA

Optimized   121     TVVWMKDNIRLRSSPSFSSLHYQDAGNYVCETALQEVEGLKKRESLTLIVEGKPQIKMTK
Original    121     TVVWMKDNIRLRSSPSFSSLHYQDAGNYVCETALQEVEGLKKRESLTLIVEGKPQIKMTK

Optimized   181     KTDPSGLSKTIICHVEGFPKPAIQWTITGSGSVINQTEESPYINGRYYSKIIISPEENVT
Original    181     KTDPSGLSKTIICHVEGFPKPAIQWTITGSGSVINQTEESPYINGRYYSKIIISPEENVT

Optimized   241     LTCTAENQLERTVNSLNVSHHHHHH*
Original    241     LTCTAENQLERTVNSLNVSHHHHHH*
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Figure 1B: Protein Alignment: Amino acid sequences of wild type and optimized sequences were compared for Peer to Peer: C2 domain.

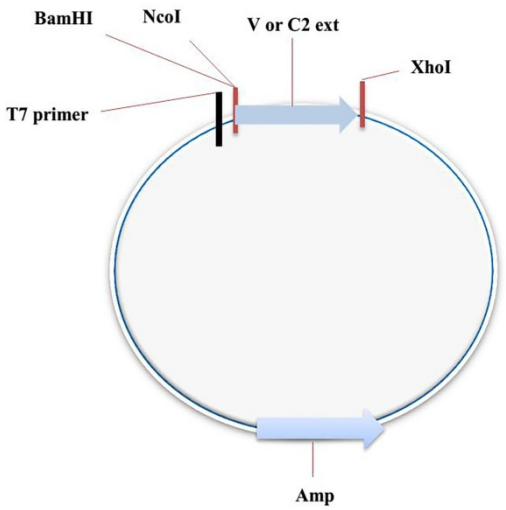

Figure 2: The schematic figure of pBSK (+) simple-Amp-V or C2 domains construct.

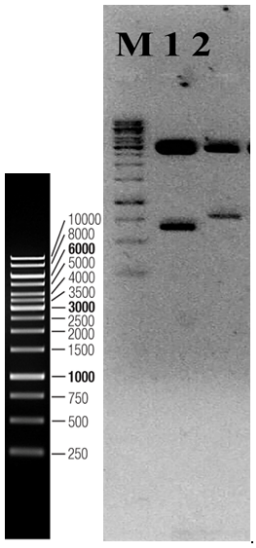

Figure 3: Double digestion of pBSK (+) vector (with *Nco*I and *Xho*I) (M: GeneRuler™ 1 kb ladder (Fermentas, Lithuania); Lane 1: Double digested pBSK (+) simple-Amp-V domain; Lane 2: Double digested pBSK (+) simple-Amp-C2 domain).

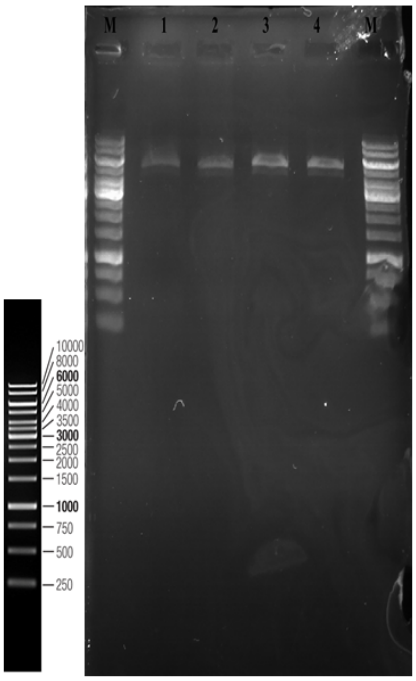

Figure 4: The extraction of transformed plasmid (pET-28a Recombinant Vector) (M: GeneRuler™ 1 kb ladder; Lane 1 and 2: Transformed plasmid of pET-28a-V domain; Lane 3 and 4: Transformed plasmid of pET-28a-C2 domain).

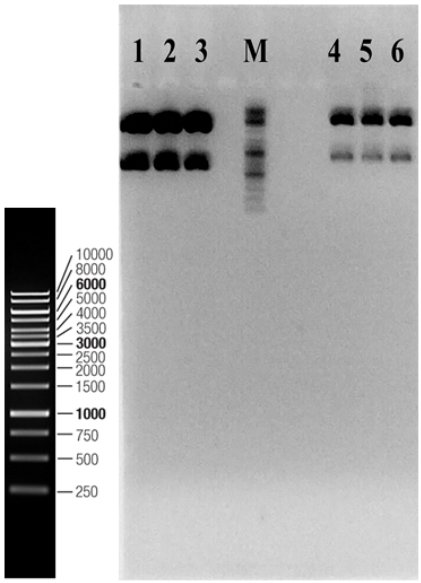

Figure 5: The double digestion of pET-28a recombinant vector (with NcoI and XhoI) (M: GeneRuler™ 1 kb ladder; Lane 1, 2 and 3: Double digestion of pET-28a-V domain; Lane 4, 5 and 6: Double digestion of pET-28a-C2 domain).

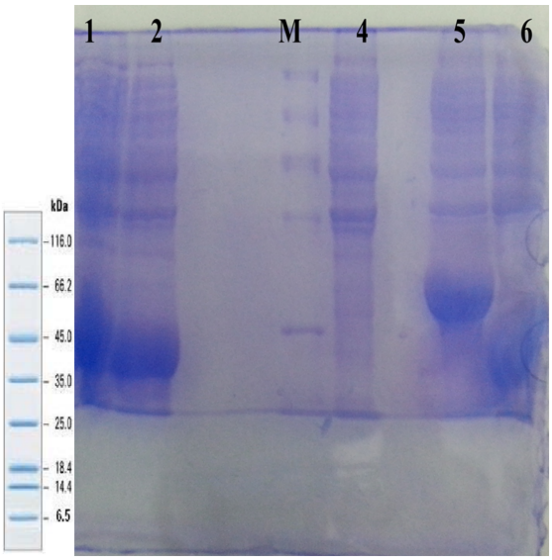

Figure 6: Expression analysis of recombinant V and C2 domains produced in *E. coli* BL21 (DE3) by SDS-PAGE (Lane M: protein marker (CMG, Iran); Lane 1 and 2: Induced *E. coli* BL21(DE3) harboring recombinant V domain with IPTG; Lane 4: Non-induced *E. coli* BL21(DE3); Lane 5 and 6: Induced *E. coli* BL21 (DE3) harboring recombinant C2 domain with IPTG).

To confirm the presence of recombinant pET-28a+V and pET-28a +C2 plasmids in the bacteria and absence of any contamination in the medium, it is necessary to confirm the presence of the intended plasmid. For this purpose, the plasmid was extracted from a medium containing transformed *E. coli* BL21 (DE3) (Figure 4). Then for confirming the presence of the gens of V and C2 domains in the transformed pET-28a expression vector, enzyme double digestion was conducted (Figure 5).

The recombinant colonies were selected, inoculated in the LB medium and then inducted by IPTG. SDS-PAGE technique was used to examine the expression of V and C2 domains. As the molecular weight of the recombinant proteins of V and C2 is about 23 and 29 kDa respectively, 15% gel was used (Figure 6).

Discussion

ALCAM is one of the members of a small subgroup of trans-membrane glycoproteins in the IgSF (immunoglobulin superfamily), which plays an important role in some cellular activities such as cell development, survival and motility [27]. The high expression of this protein has been seen in the colorectal, prostate, oesophageal squamous cells and breast cancer. This fact can be related to the generation of tumour and their development in these cancers.

ALCAM is considered as a stem cell marker in colorectal [28-32] prostate [33,34] and glioblastoma cancer [35]. Cancer stem cells are a small subset of cancer cells that have considerable ability in self-renewal and differentiation [36-48]. Today the methods commonly used in chemical therapy for the treatment of cancer cam only target the differentiated or differentiating cells forming a major part of the tumour mass. In these techniques, efficiency and success primarily depend on the cut off tumour size. In these techniques, the fact that these cells only form the tumour volume but they cannot generate new cell and do not play a role in the disease progression and tumour development, is not noticed. Cancer cell population, which causes tumour development and relapse of disease, stay intact and are negligible. Given that, it can explain why tumours are renewed even after they have been destroyed by anti-cancer drugs [17]. What should be considered for the production of new anti-cancer drugs is that they should be selected for the elimination of the stem cells not for their ability to eliminate all cells and not to reduce the size of tumours. Considering these facts, and as ALCAM is assumed as stem cell marker and is expressed highly in colorectal cancer, it can be an appropriate remedial potential to treat them.

Adhesion molecules are classified into 5 main categories: mucin, selectin, integrin, cadherin and immunoglobulin. They play role in mechanisms such as 'tumour cell-endothelial cell adhesion', 'tumour cell-matrix adhesion' and 'tumour cell-tumour cell adhesion'. The presence of these adhesion molecules is necessary at different times to form the primary tumour or metastases [4,49].

ALCAM is a cell-surface antigen that is proposed as the antigen of cancer stem cell in colorectal and breast cancers [50,51]. Recently, the recognition of the tumour 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 [52].

In this study, with the induction of the V and C2 domains of ALCAM 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 (diagnostic kit) and cancer remedial techniques (production of vaccine).

Considering that differential posttranslational glycosylation of CD166/ALCAM did not affect the homophilic binding properties [27], *E. coli* BL21 (DE3) may be a proper host with lower costs and increased expression.

Conclusion

ALCAM as the stem cell marker plays an important role in the development of tumour invasion in some cancers. As V and C2 are extracellular domains in this protein and as they perform crucial role in forming its reactions. Besides they are of the appropriate size, they can be proper candidates for potential remedial and diagnostic targets for colorectal and breast cancers.

The results of this study revealed the high expression of V and C2 domains of ALCAM gen by pET28a expression vector in the *E. coli* expression system.

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