Original Article

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In Silico Modeling and Production of New Vaccine Consisting of E7 Antigen Conjugated with Tetanus Toxin

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ABSTRACT

BACKGROUND AND OBJECTIVE: The cervical cancer is one of the main causes of death in women. Human Papilloma Virus (HPV) has been isolated in more than 98% of cervical cancer cases. The E7 has low capacity for induction immune response. Carrier proteins such as tetanus toxin are new approaches for increasing immune response against low immunogenic antigens. The aim of this study is production of new recombinant protein consisting of E7 antigen and tetanus toxin.

METHODS: This study is experimental study and recombinant fusion protein consisting of E7 and tetanus toxin produced in bacterial expression system. The coding sequence of fusion protein obtained from NCBI. Molecular weight and epitopes verified using ***Polyacrylamide gel*** and western blot analysis. For prediction of immunogenicity, epitope database and analysis resource (IEDB) was used.

FINDINGS: The presence of 42 kDa band in electrophoresis and epitopes in western blot analysis confirmed production of recombinant fusion protein. In silico modeling show that recombinant protein is stable (The parameter instability index (II) was near to 31.14) and has acceptable function in induction immune response (score of eliciting an immune response was close to 0.73).

CONCLUSION: This study was focused on modeling, optimization and production recombinant fusion protein consisting of E7 protein and tetanus toxin.

KEY WORDS: *Cervical cancer, Human papilloma virus E7, Recombinant protein, Tetanus toxin*.

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Introduction

Cervical cancer is a slowly progressive cancer that occurs in the genital tract (1). Human papilloma virus genome has been found almost in 98% of this cancer that the risk of the virus in cervical cancer incidence has raised (2). Among the viral proteins, E6 and E7 proteins play a crucial role in the formation of cancerous state of the infected cells (3). These proteins play pivotal roles in cellular signal transduction and cell cycle regulation (4). The most common treatment strategies are chemotherapy and radiotherapy against all types of cancer (5). Low efficiency, and deleterious side effects on surrounding healthy cells and reduction of effectiveness of bone marrow production of blood cells are some of the most negative effects of the treatment (6). The use of biological compounds as a secure and novel therapeutic strategy to control a wide range of diseases such as bacterial infections and cancer are considered (7,8). The use of vaccines is a new approach in the treatment of cervical cancer is that it is based on taking advantage of immune system cells can specifically targeted and therefore side effects of this therapy is minimum (9, 10).

Preliminary studies in animal models suggest the E7 protein has low immunogenicity properties provides a poor immune response (11,12). Theoretically using powerful as immunogenic carrier (Hapten-Carrier) will induce acceptable immune response against antigens with low immunogenicity property (13). C. tetani toxin is an example of this type of antigen carriers. The second amine area of domain C of the Clostridium toxin (Dom1) serves in the activation of killer T cells (Cytotoxic) (14).

The aim of this study was to produce a recombinant protein with a molecular structure of E7 protein and tetanus toxin (DOM1) as a potentially useful vaccine to enhance the immune response against the E7 antigen and cancer of the uterus area. The production of recombinant DNA technology in a bacterial expression system was used.

Methods

Design and optimization of gene construct encoding the fusion protein: the nucleotide structure of the fused protein of the N-terminal containing histidine label (His tag), restriction sites of entrokinase enzymes, the sequence of the first domain of the tetanus toxin, rigid linker and the sequence of the E7 protein, respectively. The sequence of restriction enzymes NcoI and Xho position were added at both ends of the coding sequences. Information on each of the sequences obtained from the NCBI database. To give maximum sustainable fusion protein, the fused protein sequence was optimized. DNA2 was used as optimization software, and most compatible were chosen to represent. The sequence GC content and codon adaptation index (CAI=Codon adaptation index) were also examined. PSIPRED and ITASSER servers were used to predict the spatial structure and the possible stability of the fusion protein (15-17). To predict the performance and potential of the protein epitope to induce immune response were analyzed by using database of analysis and immunological epitope (IEDB) (18). Finally, the optimized sequence for replicating in PUC 57 vector were ordered (Shin gene).

Expression of recombinant fused protein: PET-28a expression vector and BL21 bacteria were purchased from Pasteur Institute of Iran. The coding sequence was transferred to the PET-28a as cloning expression vector. In order to evaluate and confirm the gene transfer process, gene amplification test was used directly on the colonies (Direct Clony PCR) using T7 primers. Then the expression vector has been transferred into the susceptible bacteria (Competent) BL21 using the thermal shock. Recombinant protein expression was induced using IPTG 1 mM. After 5 to 6 hours recombinant protein expression pattern was evaluated using polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (19). Electrophoresis was done on 12% gel and stained the brumophenolblue color. In Western blotting the primary antibody anti-E7 (Santa Cruz) and secondary antibodies conjugated with HRP anti-mouse IgG (Sigma) were used.

Results

Optimization and simulation of sequence encoding the recombinant protein: After optimization, prediction results by software showed that codon adaptation index (CAI) increased of 62% to 83% and GC content from 33 percent to 49 percent. Secondary structure prediction also indicates that the rigid linker resulted in the formation of an alpha-helix structure between two different parts of the fusion protein (Fig 1). In addition, protein stability index (Instability Index) was calculated about 14.31. Predictions regarding to binding to molecules of histocompatibility class was shown in table 1. The results show that the fused protein has high performance to stimulate an immune response. Score of

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the immune response stimulation through histocompatibility class I molecules was estimated at around 0.73.

Figure 1. Three-dimensional structure of recombinant fusion protein. The formation of alpha helix structure between the E7 and tetani toxin

Comparison of different HLA alleles indicates that more alleles of HLA-A compared with HLA-B are able to provide antigenic determinants (epitopes) of the recombinant protein. Also evaluation of the different subtypes of HLA alleles indicates that the HLA-B * 08: 01 and HLA-A * 02: 06 have the highest potential to stimulate an immune response. Shear pattern of recombinant expression vector (PET-28a) suggests the presence of a band of approximately 1200 bp, which is fully consistent with the size of the designed nucleotide sequences (Fig 2A). The obtained results of gene amplification shows a DNA band of about 1360 bp that containing the 1181 bp of the designed sequence and 177 bp of the T7 primers (Fig 2B). These findings show that the sequence of designed vaccine has properly inserted in an expression vector.

Production of recombinant fused protein: Evaluation of recombinant protein production was performed using polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. A 42 kDa protein band which was entirely consistent with the pattern of recombinant protein was observed in electrophoresis (approximate weight of 110 Dalton was calculated for each amino acid) (Fig 3A). In addition, the results of Western blot showed that the protein antigen into account the characteristics of the E7 as well (Fig 3B). Finally, these findings indicate that successful production of recombinant proteins.

Table 1. Prediction of antigenic characteristics affinity of recombinant proteins for different alleles of HLA class I molecules. IC50 value which is lesser has a higher affinity for HLA molecules.

Allele	Seq_num	Start	End	Length	Peptide	ic50
HLA-A*02:01	1	305	313	9	YMLDLQPET	20.56
HLA-A*02:01	1	179	187	9	YLANKWVFI	22.65
HLA-A*02:01	1	371	379	9	LLMGTLGIV	26.13
HLA-A*02:06	1	103	111	9	FTVSFWLRV	1.034
HLA-A*02:06	1	179	187	9	YLANKWVFI	30.38
HLA-A*02:06	1	371	379	9	LLMGTLGIV	34.16
HLA-A*02:06	1	138	146	9	LSIGSGWSV	45.56
HLA-A*11:01	1	105	113	9	VSFWLRVPK	12.45
HLA-A*23:01	1	178	186	9	AYLANKWVF	15.12
HLA-A*24:02	1	178	186	9	AYLANKWVF	40.05
HLA-B*08:01	1	132	140	9	SMKKHSLSI	9.26

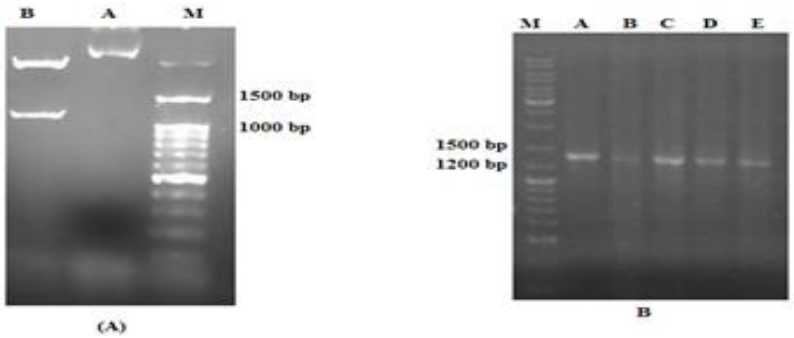

Figure 2. Electrophoresis pattern of recombinant vector pET 28 and direct gene amplification on a colony. A) Row A represents a non-cut vector. Presence of 1200 bp band in Row B indicates the presence of approximately designed fragment. B) Gene amplification pattern directly onto the colonies transformed with the recombinant vector pET 28. A band of approximately 1360 bp from the numbers of A to E represents a successful transform of recombinant designed fragment into the expression vector pET 28.

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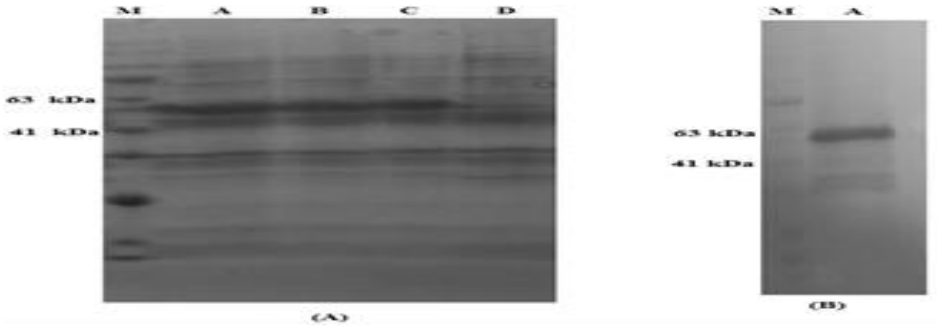

Figure 3. The expression pattern of produced proteins in transformed BL21 bacteria. A) Row D represents produced proteins in non- transformed bacteria. Row M is a molecular marker. Rows A to C are expression pattern of proteins produced in bacteria transformed with the recombinant vector. B) Protein production using the anti-E7 monoclonal antibody. A row represents proteins produced in bacteria transformed with the recombinant vector. Row M is a molecular marker

Discussion

In this study, after optimization of coding sequence, the CAI and GC were calculated 83% and 49%, respectively. The fused protein stability also was calculated 14/31 that according to the instructions of server is acceptable for protein structures to be stable. Expression pattern of fused protein on gel electrophoresis showed the presence of about 42 kDa band is the size of the designed protein.

Immunogenicity is one of the most important challenges in the way of designing vaccines against antigens, and among them, use of carriers with properties of high immunogenicity, such as fragment C toxin tetanus (Fragment C) is very effective approach to increase the immunogenicity of antigens such as E7 antigens with low immunogenicity properties (20). Studies on protein conjugated with the cholera toxin (OSP) and form recombinant heavy chain of tetanus toxin show that the vaccine not only capable of stimulating a humoral immune response against cholera toxin, but also provides memory cells (21). Another similar study on a vaccine by combining structural Her-2 antigen (breast cancer antigen), and C fragment of tetanus toxin had already been done, showed that the vaccine can create an immune response substantially against cancer cells (22).

Therefore, fusion protein containing E7 antigen protein structure and C fragment of tetanus toxin can be useful as a potential vaccine to protect against cervical cancer caused by human papillomavirus. Today, many expression systems for recombinant protein production are raised that the use of each depends on the purpose and structure of the target molecule. A bacterial expression system for the production of high volume, cost-effectiveness and the impact on immunological properties, especially against antigenic determinants of the line is the best expression systems (24, 23). In this study for better purification of recombinant protein, histidine tag (His Tag) was used in the amine section (25) as well as for removing the histidine sequence after purification, entrokinase enzyme cutting sites was placed in the sequence of the recombinant protein (26). To maintain the spatial structure and non-interference in the performance of antigenic characteristics both toxic part and E7 antigen joined together by a rigid linker (26). Optimization of sequence coding fused protein before cloning process ensures the successful production of recombinant proteins.

The most appropriate level of compatibility readings codon is 100 percent. Therefore, the closer number to 100 recombinant protein expression level is increased. GC percent of encoding sequence should be high leading to the stability of the structure and also should not be too low, which leads to non-specific binding. Evaluation of epitopes of expressed protein indicates that this protein has epitope of the E7 protein confirming the successful production of fusion proteins.

Theoretically antigenic characteristics of each non-overlapping detection by the immune system do not have any negative effect. Prediction of three-dimensional structure of fused protein reveals two E7 and toxic fragment separated from each other by an alpha helix structure without any overlap with each other. Another factor in the quality of the immune response against the vaccine are HLA molecules. Studies show that antigenic characteristics with high affinity for binding to HLA molecules induce greater immune response against these characteristics (27). Processing and presenting of antigens through histocompatibility class I molecules route is the main route for activation of toxic lymphocytes (TCD8) (28). Precise analysis shows the importance of affinity of antigenic factors for binding to HLA class I molecules is more than T helper cells (27). The results of this study suggests that designed protein has the greatest potential for antigen presentation. The immune response

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stimulation by this designed protein was nearly 73/0 in accordance with the instructions in the enterprise server which is an acceptable score to stimulate immune response. Due to the lack of comprehensive data bank on the type of high-frequency HLA molecules expression and affinity for Iranian population to bind to a different epitope,these alleles were not examined and can be cited as limitation of this study.

Treasures type of lymphocyte is an influential factor in quality of immune response against vaccines which also was examined in this study. According to the results obtained in this study and the mentioned constraints suggest that more studies on the quality of the immune response against the vaccine in laboratory models (In vitro) and animal models (In vivo) should be carried out. Based on available scientific resources, this is the first study on the design and production of recombinant forms of an efficient fusion protein containing structure of E7 proteins and tetanus toxin fragment for induction of immune response.

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