Research in Pharmaceutical Sciences, August 2017; 12(4): 265-273
Received: April 2017
Accepted: May 2017

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Immunization of mice by a multimeric L2-based linear epitope (17-36) from HPV type 16/18 induced cross reactive neutralizing antibodies

Farhad Motavalli Khiavi[1], Arash Arashkia[1], Maryam Nasimi[2], Mehdi Mahdavi[3], Majid Golkar[4], Farzin Roohvand[1,*], and Kayhan Azadmanesh[1,*]

[1]*Department of Virology, Pasteur Institute of Iran, Tehran, I.R. Iran.*
[2]*Department of Dermatology, Tehran University of Medical Sciences, Tehran, I.R. Iran.*
[3]*Department of Immunology, Pasteur Institute of Iran, Tehran, I.R. Iran.*
[4]*Department of Parasitology, Pasteur Institute of Iran, Tehran, I.R. Iran.*

Abstract

Current licensed and commercially available prophylactic human papillomavirus (HPV) vaccines (Cervarix and quadrivalent/nine valents Gardasil) are based on major capsid protein L1 virus-like particles (VLPs) production which are expensive and type specific. Minor capsid L2-RG1 linear epitope (17-36) is a known candidate for induction of cross-neutralizing antibodies to develop low-cost pan-HPV vaccines. Herein, we report construction and expression of a three tandem repeats of L2-RG1 derived from HPV16 and 18 fused with the same head to tail pattern (HPV16:17-36×3+ HPV18:17-36×3; hereafter termed dual-type fusion L2 peptide) in *E. coli* and provide the results of its immunogenicity in mice. SDS-PAGE and western blot analyses indicated proper expression of the peptide that could be further purified by Ni-NTA affinity chromatography via the located C-terminal 6xHis-tag. Mice immunized by formulation of the purified peptide and Freund adjuvant raised neutralizing antibodies which showed proper cross reactivity to HPV L2 (11-200) of types: 18, 16, 31 and 45 (which totally are responsible for 90% of cervical cancers) and efficiently neutralized HPV18/16 pseudoviruses *in vitro*. Our results imply the possibility of development of a simple, low-cost preventive HPV vaccine based on this dual-type fusion L2 peptide in bacterial expression system with broad spectrum.

Keywords: Human papilloma virus; Cross neutralization; Pseudovirus and L2 peptide

INTRODUCTION

Human papilloma virus (HPV), a non-enveloped virus with an icosahedral capsid and covalently closed double stranded DNA genome, encodes for major (L1) and minor (L2) capsid proteins. Currently, close to 200 HPV genotypes have been identified that generally cause benign epithelial warts on skin or mucosa (1). However, persistent infection with about fifteen types, specially HPV types: 16, 18, 31, and 45 eventually progress to carcinoma (1). In fact, these four types are among the most recognized high-risk HPV types, accounting for incidence of more than 90% of global cervical carcinomas (2). Additionally, recent meta-analysis indicated that HPV 16 and 18 are the most prevalent high risk types in Iranian women (3).

Currently, two licensed recombinant prophylactic HPV vaccines are available in the market; "Gardasil" and "Cervarix". Both of these vaccines are derived from L1 major capsid protein of HPV using the yeast (*Saccharomycec*) and insect (*baculovirus*) expression systems, respectively.

Both vaccines contain L1-based virus like particles (VLPs) of types 16/18, while "Gardasil" further encodes those of 6 and 11 types that cause 90% of benign mucosal warts (4).

Recently, A nine-valent HPV vaccine containing L1-based VLPS of types 6/11/16/18/31/33/45/52/58 was also approved for human applications (5).

*Corresponding authors:
K. Azadmanesh, Tel/Fax: +98-2166496682
Email: azadmanesh@pasteur.ac.ir
F. Roohvand, Tel/Fax: +98-2166496682
Email: rfarzin@pasteur.ac.ir

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.212043

Although, these HPV L1-based vaccines elicit type-specific, high titer and protective neutralizing antibody (NAb) responses against the HPV types included in the vaccine but limited cross-protections are provided against other high-risk or clinically important HPV types (6).

In addition, the high price of the L1-based VLP vaccines (due to the complexity of multi-valent VLP-production process) is not affordable for many developing and low-income countries. Therefore, these vaccines are not included in national immunization program of many countries (e.g., Iran) where much higher rates of HPV infection compared with developed countries occur (7).

Search for an alternative vaccine with broaden spectrum to cover most HPV types, attracted the attentions to the L2 minor capsid protein. Remarkable role of L2 as a prophylactic antigen has been introduced in the early 1990s through vaccine studies in animal models (2). Results of these studies indicated that N-terminal region of HPV L2 (amino acid 11-200) contained conserved pan-type linear B-cell epitopes capable of eliciting neutralizing antibodies (Nabs) even in the isolated forms, to prevent progression of papillomavirus infection in experimental animal challenge studies (8). Accordingly, several protective HPV L2 epitopes comprising residues 1-88, 17-36, 65-81, 108-120, 94-122 were introduced of which the 17-36 epitope (initially identified by the mouse monoclonal antibody "RG1"), was shown to be capable of providing broad cross-neutralizing activity against heterologous papillomavirus types (9). Although antibodies (Abs) elicited by immunization via RG1-epitope showed pan-type neutralization properties but the titers of Abs, especially for heterologous types were extremely low.

In this context, several attempts to enhance the immunogenicity of this epitope were undertaken, such as; use of different bacterial scaffolds or bacteriophages and viruses (to display the RG1-epitope) or fusion to modified IgG1 Fc fragments or lipopeptides (2,10). Although application of these formulation and modalities enhanced the immunogenicity of RG1 epitope, but the employed approaches were still rather complex with potentially high cost of production. Still another approach, to develop low-cost vaccines with improved immunogenicity, multimeric, concatenated RG1-encoding peptides of 22 clinically relevant HPV genotypes [1, 2, 63, 5, 8 (cutaneous types), 6, 11 (mucosal low risk types) and 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82 (high risk oncogenic types)] were produced. Immunization studies by this 49 kDa peptide produced in bacterial expression system and formulated with strong adjuvants, indicated the induction of pan-HPV Nabs in mice and rabbits, albeit raised Abs showed better neutralization activity against the endogenous vaccine derived epitopes rather than heterologous types (11). In addition, the stability and proper expression of such complex antigen formulations in bacterial systems was a limiting factor, since increased copies of L2-RG1 might result in protein degradation (10).

Prior studies indicated that application of three or more tandem repeats of the same L2 (amino acid 20-38) epitope in the context of linear protein induced more potent immune response in comparison with the same residues in mono-peptide form (12). Considering that HPV16/HPV-31 and HPV-18/HPV-45 pairs are closely related to each other (11, 13), it might be expected that Nabs raised against HPV 16/18 L2-RG1 should also be at least very effective against HPV 31/45. If this proposition could be true, such an immunogen might have the potential to be used for vaccination against more than 90% of cervical carcinomas due to the frequency of these four types in HPV-induced cancers as mentioned above (14).

The aim of the present study was the development of a simple and low-cost HPV vaccine (at least effective against types 16, 18, 31, 45) in bacterial expression system especially for developing countries with lower financial resources.

Then, we constructed a three tandem repeats of L2-RG1 epitope (amino acids 17-36) derived from HPV16 and 18 fused with the same head to tail pattern (hereafter termed dual-type fusion L2 peptide) and evaluated its immunogenicityin mice.

MATERIALS AND METHODS

Generation of dual-type fusion L2 construct

The L2 multimeric construct encompassing the DNA sequences of the three head to tail repeats of HPV L2 RG-1 epitope "17-36 residues (17-36×3)" derived from HPV16 (accession number; NC_001526) fused to the same (17-36×3) region of HPV18 (accession number; NC_001357) was codon optimized for *E. coli* expression and synthesized by Biomatik gene synthesis company (Canada) with 5' *EcoR*I and 3'*Xho*I sites. The aforementioned L2 RG-1 sequence (dual-type fusion L2 peptide) was subcloned into the pET28a (+) expression vector (Novagen, San diego, USA), upstream of the C-terminal His-tag sequence (Fig. 1A). The final recombinant construct was confirmed by restriction analyses followed by agarose gel electrophoresis and DNA sequencing reactions. All molecular and cloning procedures were performed according to standard protocols (15).

Expression, purification and analyses of dual-type fusion L2 peptide

The recombinant dual-type fusion L2 peptide was expressed in *E. coli* BL21 (Rosetta DE3) by IPTG induction (1 mM) and purified by Ni-NTA (Qiagen, USA) affinity chromatography according to the denaturing protocol of the manufacturer from the sonicated bacterial lysate (Qiagen, Germany). Subsequently, the recombinant polypeptide was renatured by dialysis against phosphate buffer saline (PBS) and quantified using Bradford protein assay (Thermo Fisher Scientific, USA). The endotoxin level of the purified protein was quantified by QCL-1000 Chromogenic Limulus amoebocyte lysate test (Lonza, USA) according to the manufacturer protocols. The recombinant protein was stored at −70 °C until use. Expression of the dual-type fusion L2 peptide was analyzed by 1% SDS-PAGE according to standard protocols (15). Western blot analysis was carried out by transferring the protein bands from polyacrylamide gel to nitrocellulose membrane via semi-dry blotting apparatus (Biorad, USA). Membranes were blocked with 5% blocking buffer (over-night at 4 °C) and washing steps were performed and finally the membrane was incubated with anti-HPV-16 L2 (1-40 residues) anti-mouse monoclonal antibody (Santa Cruz, USA) for 60 min at room temperature. At the final step, color visualization of antigen-antibody reaction was accomplished using 3, 3'-diaminobenzidine tetra-hydrochloride solution (DAB) (Abcam, USA).

Immunization of mice

All animal experiments were performed in accordance with the international animal care ethics. Groups of five female Balb/c (H-2^d) mice, 4-6 weeks age were vaccinated three times at two-weak intervals. Mice were immunized subcutaneously (s.c.) with 25 µg of the dual-type fusion L2 peptide, formulated in "complete Freund" adjuvant (CFA) for the priming dose and boosted in "incomplete Freund" adjuvant (IFA) (Sigma, USA) in 100 µL total immunogen volume (Table 1). Control groups (G2 and G3) were administered with 100 µL of sterile PBS and CFA/IFA with the same procedure, respectively. Blood samples were collected from all experimental groups through retro-orbital bleeding two weeks after the final injection and after separation of serum they were stored at -70 °C.

Enzyme-linked immunosorbent assays

A homemade ELISA was designed to assess the titer of the cross-reactive antibody (IgG) of immunized mice. To this end, ELISA 96-well plates (Nunc, Rochester, NY, USA) were coated with 500 ng/well of recombinant L2 amino acids 11-200 from HPV types 16, 18, 31, and 45 overnight. Information on these HPV type-specific proteins are provided elsewhere (11).

Table 1. Group of immunized mice.

Groups	Immunogen
G1	(HPV16)17-36×3+ (HPV18)17-36×3 in CFA/IFA
G2 (control group)	PBS
G3 (control group)	CFA/IFA

CFA and IFA denote the complete and incomplete Freund adjuvants respectively

The coated plates were blocked with 1% bovine serum albumin (BSA) at 4 °C and incubated with ten-fold serially diluted serum from vaccinated mice for 1 h at room temperature. After washing with PBST (PBS 0.05 % tween), 100 µL of HRP-conjugated goat anti-mouse immunoglobulin G (Abcam, USA) (1:20000) was added to each well and incubated for 1 h at room temperature. After extensive washing steps, reactions were developed by adding 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Abcam, USA), and color development were stopped by 2 N sulfuric acid. Finally, the optical densities of wells were measured at 450 nm by ELISA reader (BioTek, USA).

In vitro neutralization assay

HPV18/16 pseudoviruses were used for neutralization assay. The generated HPV18/16 pseudoviruses for this assay were based on co-transfection of a bi-cistronic L1 and L2 expression construct (HPV16 L1/L2 and HPV18 L1/L2, respectively) and a GFP-expression packaging plasmid (pfwB) (kindly gifted from Prof. Muller) into the 293 FT cells as described by Seitz, *et al* (16). The papillomavirus pseudovirion neutralization assay was done as described before with few modifications (17).

Briefly, 20,000 293FT cells were seeded in each well of a 96 well plate (SPL, South Korea). The day after, pooled sera from each immunized group (5 mice per group) were serially diluted (at starting dilution 1:10) in culture media.

Subsequently, 20 µL of diluted sera were mixed with 80 µL of diluted pseudovirions (1:100), incubated on ice for 1 h and added to the pre-plated 293FT cells. Two days after infection, the cells were trypsinized and GFP expressing cells were analyzed by flowcytometry (Partec, Germany). Cells treated only with the diluted pseudovirions were considered as negative control. Neutralization titers were determined as the highest sera dilution of each immunized group at which the HPV16 and HPV18 pseudoviruses were neutralized (at least 50%) in comparison with cells infected with diluted pseudovirions without sera.

Statistical analysis

Comparison between mice groups for level of *in vitro* neutralization and IgG titers was made by One-way ANOVA analysis. Bonferroni's pairwise comparison test was carried out with GraphPad 6.0 (GraphPad Software, San Diego, CA). Significant differences between groups were set at *P* values less than 0.05.

RESULTS

Production of dual-type L2 fusion peptide in E. coli

Restriction analyses of the recombinant pET28a (+) plasmid harboring the gene corresponding to the dual-type fusion L2 peptide and agarose gel electrophoresis (Fig. 1) followed by DNA sequencing reactions (data not shown) confirmed the proper construction of the expression vector with the proper size of the inserted gene (375 bp). Induction of *E. coli* BL21 (Rosetta DE3) harboring the dual-type fusion L2 peptide-encoding pET28a (+) vector by IPTG resulted to the expression of a distinctive band with a molecular weight (MV) of ~14 kDa (Fig. 2A).

The observed size for this protein band in Coomassie blue-stained SDS-PAGE was comparable to the calculated size of the dual-type fusion L2 peptide for a total 128 amino acids (17-36×3 HPV16 + 17-36×3 HPV18 + 6 x His-tag and flanking regions).

Western blot analysis confirmed the induced protein band as the expected dual-type fusion L2 peptide (Fig. 2B). NI-NTA-based affinity chromatography purification of the protein indicated a homogenous band at the expected size of 14 kDa (Fig. 2C) indicating the proper expression of the C-terminal His-tag. Quantification of the endotoxin levels indicated less than 25 endotoxin units per 50 µg of the purified protein which was proper for the final aim of the immunization.

Cross reactivity of the raised antibodies in immunized mice

To assess the antibody cross-reaction of the mice immunized by the dual-type fusion L2 peptide with HPV types 16, 18, 31, and 45, the

IgG level of immunized mice were evaluated against recombinant L2 amino acids 11-200 from HPV types 16, 18, 31, and 45 by ELISA. As shown in Fig. 3, mice immunized with the dual-type fusion L2 peptide, elicited antibodies that were able to react not only with HPV16/18 (the coated 11-200 amino acids L2 peptides) with almost same titers (no significant differences) but also cross-reacted with two heterologous HPV31/45 types L2 proteins, albeit with lower levels (P = 0.001 and P < 0.0001, respectively). No cross-reactivity was observed for sera of mice immunized by PBS or CFA/IFA alone.

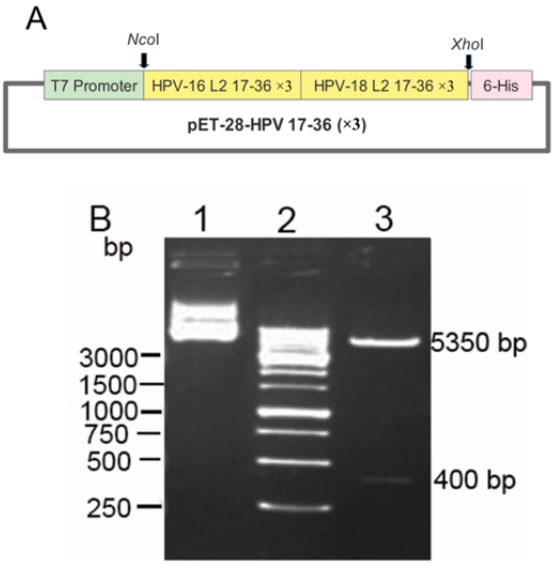

Fig. 1. Construction and characterization of a recombinant plasmid encoding the dual-type fusion peptide. (A) Schematic diagram of the recombinant pET28a harboring the dual-type L2 fusion peptide (pET-28a-L217-36 ×3). The synthesized dual-type L2 fragment was subcloned into the NcoI and XhoI sites of the pET-28a plasmid. (B) Gel electrophoresis of digested recombinant pET-28a-L217-36 ×3. The positive clones were confirmed using enzymatic digestion with the same enzymes. Lanes: 1, undigested plasmid; 2, DNA ladder; 3, digested plasmid.

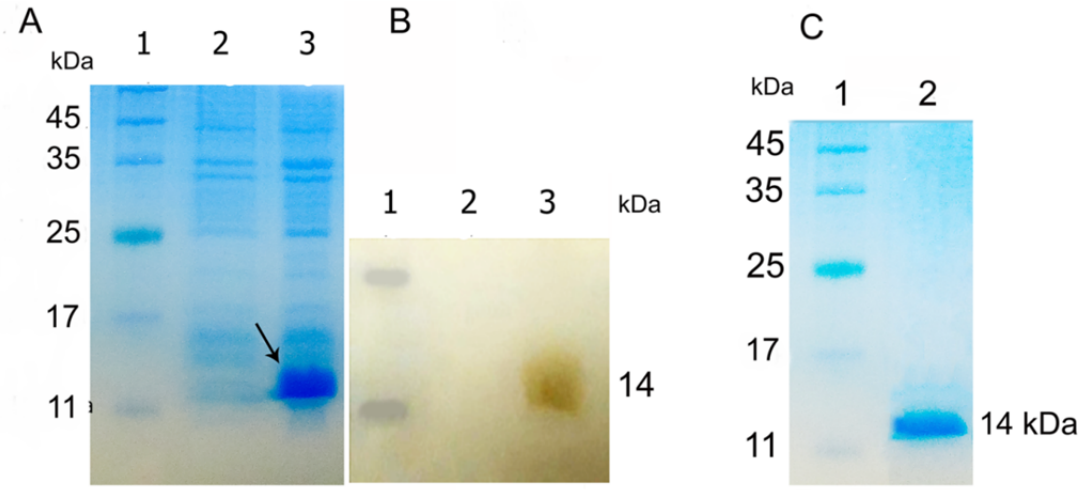

Fig. 2. SDS-PAGE and western blot analysis of the expressed L2 dual-type fusion peptide. (A) SDS-PAGE of the expressed dual-type L2 fusion peptide revealed a protein band of approximately 14 kDa (B) Western blot analysis of the expressed L2 fusion. Lanes: 1, molecular weight marker; 2 and 3, uninduced and induced cell lysates of *E. coli* harboring the pET-28a-L217-36 ×3 plasmid, respectively. (C) Ni-NTA-based affinity chromatography purified protein indicated a homogenous band at the expected size (14 kDa). Lane 1 and 2: Mw marker and purified protein respectively.

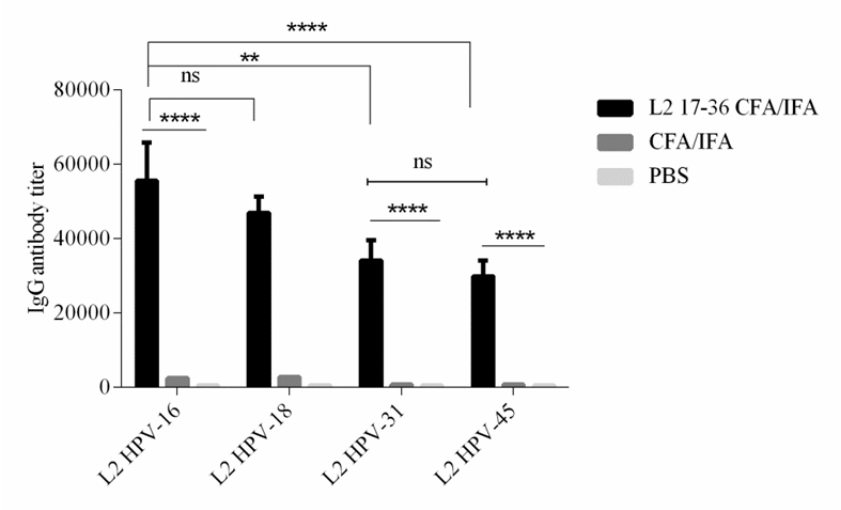

Fig. 3. Cross reactive antibody responses against L2 (amino acid 11-200). mice (5/group) were immunized three time at two weeks intervals and blood sample were collected 2 weeks after the last immunization. Serially diluted sera were tested for measuring the IgG titer using ELISA. Antibody titers were expressed as the reciprocal of the highest sera dilution at which OD 450 was 2-fold greater than that of the PBS control group. Data are expressed as means ± standard error of the means (SEM) of triplicates from 5 mice per group. ****$P < 0.0001$, **$P = 0.001$, ns; not significant.

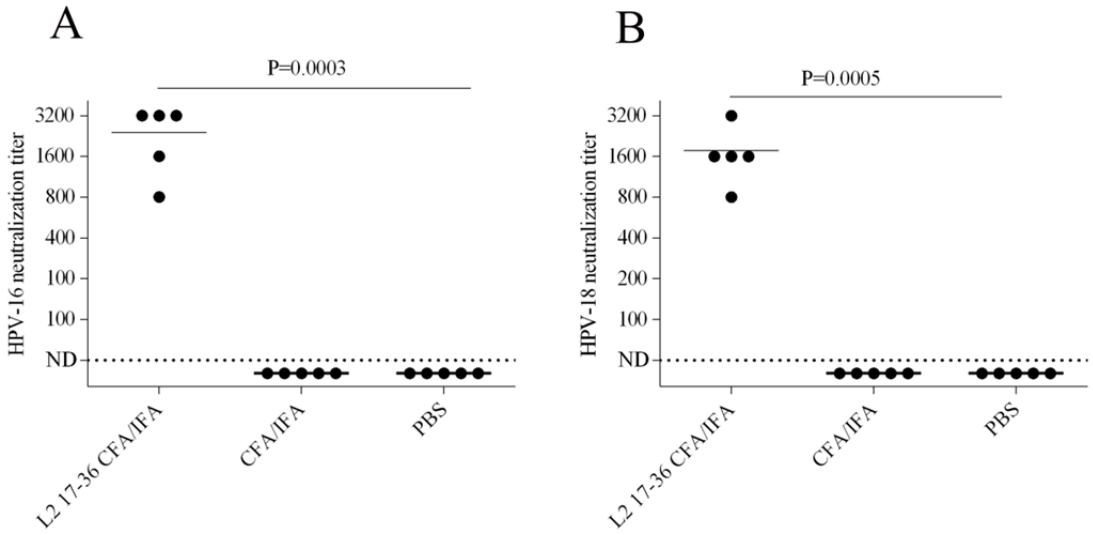

Fig. 4. *In vitro* neutralization assays of PsV-16 (A) and PsV-18 (B) by sera of mice immunized with dual-type L2 17-36 × 3 peptide. Neutralizing titers were represented as the reciprocal of the highest sera dilution at which at least 50% of the pseudovirions were neutralized compared to the positive control (pseudovirion without sera). End point titers were plotted and means represented as horizontal lines. A neutralizing titer lower than 50 was regarded as not detectable (ND).

In vitro neutralization potency of the raised antibodies in immunized mice

As shown in Fig. 4, results of the neutralization assay indicated that antibodies of the mice immunized by the dual-type fusion L2 peptide neutralized both HPV16 and HPV18 pseudovirions (PsV-16 and PsV-18), with almost same efficiencies for both types. No neutralization activity was observed for sera of mice immunized by PBS or CFA/IFA alone compared to those immunized with dual-type fusion L2 ($P = 0.0003$ and $P = 0.0005$ for HPV16 and 18, respectively).

DISCUSSION

Results of the present study indicated the possibility of proper production of a dual-type fusion L2 peptide encoding three tandem repeats of L2-RG1 epitope (amino acids

17-36) derived from HPV16 and 18 fused with the same head to tail pattern in bacterial expression system. Immunization studies in mice showed that Abs rose against this dual-type fusion L2 peptide are capable of cross-reactivity to L2-derived from HPV types 16, 18, 31, and 45 and have neutralization potencies to both HPV16 and HPV18 pseudovirions. Several prior attempts to enhance the immunogenicity of this epitope have been undertaken. These include: employing different bacterial scaffolds or bacteriophages and viruses (to display the RG1-epitope), fusion to modified IgG1 Fc fragments or lipopeptides and construction of recombinant adeno-associated virus like particle immunogen displaying L2 17-36 epitopes together with different adjuvants (2,10). Although application of these formulation and modalities enhanced the immunogenicity and spectrum of RG1 epitope, but the employed approaches were still rather complex with potentially high cost of production. However, results of the present study imply the possibility of development of a simple, low-cost L2- RG1 epitope based HPV vaccine by this dual-type fusion L2 peptide in bacterial expression system with broader spectrum (at least effective against types 16, 18, 31, 45) for low-income countries.

As shown in Fig. 1, restriction analyses and agarose gel electrophoresis of the recombinant pET28a (+) plasmid harboring the gene corresponding to the dual-type fusion L2 peptide (Fig. 1A) indicated a fragment with a size of around 375 bp (Fig. 1B) for the inserted gene corresponding to the "6 x 17-36 RG-1 epitope (17-36 × 3 HPV16 + 17-36×3 HPV18)". This observed size was equal to the initially synthesized gene (375 bp) and in accordance with prior reports on construction of the "head to tail" DNA sequences of this gene (11,12,18). Accordingly, SDS-PAGE and Western Blotting analyses (Fig. 2) indicated that the dual-type fusion L2 peptide was properly expressed in *E. coli* by appearance of a protein band of approximately 14 kDa following IPTG induction. This observed molecular weight (MW) was comparable to the calculated MW of the dual-type fusion L2 peptide for a total 128 amino acids (17-36×3 HPV16 + 17-36×3 HPV18 + 6 x His-tag and flanking regions) and is consistent with prior reports for MW of the triplet head to tail of this protein (11,12). The bacterial expression for this dual-type fusion L2 peptide was quite efficient and stable which is a very positive point compared to unstable expression of longer concatenated RG1-encoding peptides (10,19).

Results of ELISA (Fig. 3), indicated that the *E. coli*-derived "dual-type fusion L2 peptide" could elicit Abs in immunized mice with the ability to cross-react with L2 peptides representing 11-200 residues from diverse HPV types (HPV16, 18, 31, and 45). However, the observed cross re-activities were higher for the endogenous vaccine derived epitopes (HPV16/18) rather than heterologous types (HPV 31/45) (Fig. 3). This result was expected and is in accordance with prior immunization studies in mice and rabbits by a 49 kDa concatenated RG1-encoding peptides of 22 clinically relevant HPV genotypes which elicited Abs with better neutralization activity against the endogenous vaccine derived epitopes rather than heterologous types (11). Our results are also comparable to the Abs raised by immunization studies in mice by combination of RG1-epitpe and a universal T-cell helper epitope (PADRE) (20), indicating that the immunogenicity of our *E. coli*-derived "dual-type fusion L2 peptide" might be further enhanced by other supporting strategies. Of note, as it can be seen in Fig. 3, Abs raised against type 16 seems to be higher compared with type 18. Although this result was not statistically significant, but rise of higher titers of HPV16 neutralization Abs compared to that of type 18 was also reported in the natural course of infection (21).

Finally, results of neutralization assay (Fig. 4), indicated that the antibodies of the mice immunized by the dual-type fusion L2 peptide neutralized both HPV16 and HPV18 pseudovirions, with almost same efficiencies for both types. This neutralization capability against endogenous vaccine derived epitopes was expected and in accordance with prior reports indicated that immunization with "dual-type fusion L2 peptide" might properly neutralize the potential infection of HPV types 16/18 as well as their closely evolutionary

related types (31/45) (11,13), that correspond to 90% of cervical carcinomas due to the frequency of these four types in HPV-induced cancers as mentioned above (14). As indicated in Fig. 4, efficiency of neutralization for type 18 might be a little bit better (P = 0.0003) compared to type 16 (0.0005) which is in accordance with the results of cross reactivity (Fig. 3) and natural course of infection (21).

CONCLUSION

To our best of knowledge, this study presented the first report on construction of a dual-type fusion L2 peptide corresponding to the fused triplets of L2 RG-1 epitope (17-36×3 HPV16 + 17-36×3 HPV18) and its proper/stable expression in bacterial system. Our results indicated that this *E. coli*-derived peptide could raise efficient Nabs in mice capable of cross-reacting with L2 protein of HPV types 16/18/31/45 and neutralizing HPV16/18 pseudovirions. Our results imply the possibility of development of a simple, low-cost, preventive L2-based HPV vaccine in bacterial expression system with broad spectrum (at least effective against types 16, 18, 31, 45). The next steps for evaluation of the potential clinical application of this candidate vaccine might be to compare its efficacy with currently licensed L1-based HPV vaccines in animal models by addressing challenge studies and based on the results further human clinical trials might be addressed.

ACKNOWLEDGMENTS

This study was financially supported by Pasteur Institute of Iran in partial fulfillment of the Ph.D thesis of Dr. Farhad Motavalli in pharmaceutical biotechnology program. The authors thank all the members of virology lab for their helpful consultation and technical assistance. We also gratefully acknowledge Dr. Mehdi Shakouri, Dr. Shahriyar Abdoli and Dr. Mohammad Hossein Etemadzade for their technical support.

REFERENCES

1. Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. Human papillomavirus molecular biology and disease association. Rev Med Virol. 2015;1:2-23.
2. Schellenbacher C, Roden RB, Kirnbauer R. Developments in L2-based human papillomavirus (HPV) vaccines. Virus Res. 2017;231:166-175.
3. Malary M, Moosazadeh M, Hamzehgardeshi Z, Afshari M, Moghaddasifar I, Afsharimoghaddam A. The prevalence of cervical human papillomavirus infection and the most at-risk genotypes among iranian healthy women: a systematic review and meta-analysis. Int J Prev Med. 2016;7:70
4. Castle PE, Maza M. Prophylactic HPV vaccination: past, present, and future. Epidemiol Infect. 2016;3:449-468.
5. Pitisuttithum P, Velicer C, Luxembourg A. 9-Valent HPV vaccine for cancers, pre-cancers and genital warts related to HPV. Expert Rev Vaccines. 2015;14(11):1405-19.
6. Harper DM, Franco EL, Wheeler CM, Moscicki AB, Romonowski B, Roteli-Martins CM, *et al*. Sustained efficacy up to 4-5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet. 2006 ;367(9518):1247-1255.
7. Vinodhini K, Shanmughapriya S, Das BC, Natarajaseenivasan K. Prevalence and risk factors of HPV infection among women from various provinces of the world. Arch Gynecol Obstet. 2012;285(3):771-777.
8. Gambhira R, Jagu S, Karanam B, Gravitt PE, Culp TD, Christensen ND, *et al*. Protection of rabbits against challenge with rabbit papillomaviruses by immunization with the N terminus of human papillomavirus type 16 minor capsid antigen L2. J Virol. 2007;81(21):11585-11592.
9. Gambhira R, Karanam B, Jagu S, Roberts JN, Buck CB, Bossis I, *et al*. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. J Virol. 2007;81(24):13927-13931.
10. Zhang T, Liu H, Chen X, Wang Z, Wang S, Qu C, *et al*. Lipidated L2 epitope repeats fused with a single-chain antibody fragment targeting human FcgammaRI elicited cross-neutralizing antibodies against a broad spectrum of human papillomavirus types. Vaccine. 2016;34(46):5531-5539.
11. Jagu S, Karanam B, Gambhira R, Chivukula SV, Chaganti RJ, Lowy DR, *et al*. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. J Natl Cancer Inst. 2009; 101(11):782-792.
12. Rubio I, Bolchi A, Moretto N, Canali E, Gissmann L, Tommasino M, *et al*. Potent anti-HPV immune responses induced by tandem repeats of the HPV16 L2 (20-38) peptide displayed on bacterial thioredoxin. Vaccine. 2009; 27(13):1949-1956.
13. Toft L, Tolstrup M, Muller M, Sehr P, Bonde J, Storgaard M, *et al*. Comparison of the immunogenicity of Cervarix(R) and Gardasil(R) human papillomavirus vaccines for oncogenic non-vaccine serotypes HPV-31, HPV-33, and HPV-45 in HIV-infected adults. Hum Vaccin Immunother. 2014;10(5):1147-1154.

14. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, *et al*. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348(6):518-527.
15. Sullivan K. Short protocols in molecular biology, 2nd Edn. Trends in Biotechnology. 10:456.
16. Seitz H, Ribeiro-Muller L, Canali E, Bolchi A, Tommasino M, Ottonello S, *et al*. Robust in vitro and in vivo neutralization against multiple high-risk hpv types induced by a thermostable thioredoxin-l2 vaccine. Cancer Prev Res. 2015;8(10):932-941.
17. Day PM, Pang YYS, Kines RC, Thompson CD, Lowy DR, Schiller JT. A human papillomavirus (hpv) in vitro neutralization assay that recapitulates the in vitro process of infection provides a sensitive measure of hpv l2 infection-inhibiting antibodies. Clin Vaccine Immunol. 2012;19(7):1075-1082.
18. Jagu S, Karanam B, Wang JW, Zayed H, Weghofer M, Brendle SA, *et al*. Durable immunity to oncogenic human papillomaviruses elicited by adjuvanted recombinant Adeno-associated virus-like particle immunogen displaying L2 17-36 epitopes. Vaccine. 2015;33(42):5553-5563.
19. Chen X, Liu H, Zhang T, Liu Y, Xie X, Wang Z, *et al*. A vaccine of l2 epitope repeats fused with a modified igg1 fc induced cross-neutralizing antibodies and protective immunity against divergent human papillomavirus types. PLoS One. 2014;9(5).
20. Jagu S, Kwak K, Karanam B, Huh WK, Damotharan V, Chivukula SV, *et al*. Optimization of multimeric human papillomavirus L2 vaccines. PLoS One. 2013;8(1):e55538.
21. Krajden M, Karunakaran K, So S, Palefsky JM, Sharma R, Cook D, *et al*. Prevalence of human papillomavirus 16 and 18 neutralizing antibodies in prenatal women in British Columbia. Clin Vaccine Immunol. 2009;16(12):1840-1843.