Biomacromol. J., Vol. 7, No. 3, 70-76, December 2021.

Biomacromolecular Journal
www.bmmj.org

Cloning and Expression of New Polytope E6 Antigen of Human Papillomavirus in *E. coli*

M.E. Madadi[a], Z. Amini-bayat[b,*], B. Bahmani[b], N. Tafreshi[c], M. Hashemi[a] and N. Mousavi Niri[a]

[a]*Department of Genetics, Tehran Medical Sciences Branch, Islamic Azad University, Tehran, IR Iran*

[b]*Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran*

[c]*Department of Cancer Physiology, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, USA*

(Received 20 December 2020, Accepted 7 April 2021)

ABSTRACT

The method of choice for prevention and treatment of infection with Human Papilloma virus (HPV) and consequently cervical cancer is the application of prophylactic and therapeutic HPV-vaccine. The present study aimed to clone the most antigenic epitopes of the E6 antigen and to express it in E. coli at the lab-scale. Methods: The sequence of immune-bioinformatically determined epitopes of E6 was synthesized in the pGH vector. The new E6 gene was cloned into the pET28 vector by double-digestion of vector and target with NcoI and XhoI restriction enzymes. The recombinant vector was transformed into DH5α and cloned E6 gene was confirmed by colony PCR and DNA sequencing. The recombinant pET28 was then extracted from DH5α and transferred into the BL21(DE3) expression host. Expression optimization was performed using various parameters. Results: Cloning was confirmed by colony PCR and sequencing and optimized expression was performed at 25 °C, IPTG = 0.1 mM, OD600 = 1. Due to the protein production in the form of inclusion bodies and unavailability of His-Tags, the recovered protein was not confirmed by Western blot and did not purify by Ni-NAT affinity chromatography. Conclusion: This study aimed to express multi-epitope recombinant protein composed of selective E6 protein epitopes in the E. coli prokaryotic expression system to achieve an effective vaccine against HPV. The recombinant protein might be used as a therapeutic vaccine or as a platform for HPV detection.

Keywords: Human papilloma virus, Recombinant protein, Vaccine, E6, Antigen

INTRODUCTION

Human Papillomavirus (HPV) consists of a large group of more than one hundred types, which are associated with the formation of warts and cervical cancer [1]. Infection with HPV may spread through sexual and asexual contact. After breast cancer in women, cervical cancer is the second most common cancer. High-risk genotypes of HPV accounts for more than 90% of cervical cancers, of these, 16 and 18 genotypes account for more than 80% cases of cancer [2,3]. The HPV genome is composed of three distinct regions, comprising non-coding, primary and secondary control regions. The primary region encodes six non-structural proteins, E1, E2, E4, E5, E6, and E7 and the secondary region encodes two L1 and L2 capsid proteins [1,4]. The carcinogenicity of high-risk HPV genotypes is concentrated in the function of E6 and E7 proteins, which have been consistently expressed in cancer cells and their expression has been demonstrated in most cervical cancers [5,2].

Expression of E6 and E7 proteins disrupts cell differentiation and cycle and virus proliferation in epithelial cells, leading to wart formation. These proteins can induce immune responses that are detectable in immunological tests [6]. Humoral immune responses alone are not sufficient to eliminate viral infection and require an effective cellular immune response. Humoral immunity is responsible for inactivating the HPV and preventing the spread of the virus. Destruction of HPV-infected cells and the elimination of virus-induced lesions are mainly mediated by cellular immune responses. The cellular immune response to humoral immunity plays a greater role

*Corresponding author. E-mail: Amini-bayat@irost.org

in the effective response to HPV [7].

In addition to the prophylactic HPV vaccines, therapeutic vaccines are specifically used to stimulate cellular immune responses to eliminate infected cells. Many of these vaccines are based on the E6 and E7 proteins in APC antigen-presenting cells to active CD8 + and CD4 + T-cells. Unlike traditional prophylactic vaccines, therapeutic vaccines aim at principally activating the cell-mediated immune response [8]. Peptide vaccines, in general, are considered to be safe, stable, and easy to manufacture [9]. Nowadays, E6 and E7 proteins have been used as vaccine candidates against HPV and thus will be useful in inducing the immune system for various purposes. Currently, different types of therapeutic vaccines such as Virus-Like Particles (VLPs), recombinant protein/peptide vaccines, vector vaccines, and DNA vaccines expressing structural and non-structural proteins against the virus are being developed and tested for the treatment of HPV-associated diseases [9,10]. Polytope vaccines have the potential to simultaneous and specific stimulation of the immune system against multiple epitopes unique to CD4 + and CD8 + cells by focusing the immune response on conserved and important epitopes of the virus [11].

The present study aimed to optimize the expression of the multi-epitope recombinant protein composed of selective E6 protein epitopes in the *E. coli* prokaryotic expression system to achieve an effective vaccine against HPV.

MATERIALS AND METHODS

Cloning of Polytope E6 in pET28a Vector

According to the amino acid sequence of the predicted peptide-based HPV16-E6 vaccine [12], DNA encoding the peptides and spacers was artificially synthesized (Generay, Shanghai, China) with codon optimized for expression in *E. coli*. Both the synthesized gene and pET28a vector were double digested with NcoI and XhoI restriction enzymes to produce sticky ends. Briefly, in separate sterile microtubes, 25 µl of the pGH plasmid or pET28a vector were incubated with 2 µl NcoI in 4 µl tango buffer and 9 µl of sterile distilled water for 2 h at 37 °C. Five µl tango buffer and 2µl XhoI were then added to each tube and incubated at 37 °C for 2 h. Digested products were incubated at 81 °C for 21 min to inactivate both enzymes and then stored at -21 °C. The digested products were then electrophoresed on an agarose gel and purified by Gel/PCR purification mini Kit (Fermentaz) according to the manufacturer's instruction. Purified digested products with sticky ends were mixed with 1 µl T4 DNA ligase (Bio basic, USA) in an insert: vector molar ratio of 3:1 at room temperature for 1 h. A no insert reaction was used as the negative control for ligation. The ligated products were then transferred into DH5a for amplification and confirmation of the cloning of polytope HPV16-E6 in the pET28a expression vector. To this end, chemically competent DH5a cells were mixed with KCl (100 mM), $CaCl_2$ (50 mM) and the ligation product prior to heat shock at …..°C. Transformation accuracy/ability was assessed with the aid of the plasmid without the HPV16-E6 gene or negative control of the ligation. DH5a transformed cells were then cultured on LB agar containing Kanamycin (100 ng μl^{-1}). According to this selection medium, true recombinants can grow. Colony PCR, DNA sequencing, and enzymatic digestion were also applied to confirm the cloned sequence in the right direction in recombinant vectors. T7promoter and T7 terminator universal primers were used for PCR and sequencing.

Expression Analysis and Purification of the Recombinant Protein

The chemically competent *E. coli* BL21 (DE3) was prepared and transformed with 50 ng of pET28a containing the NAME THE GENEgene under a heat shock. The transformed cells were cultured on LB agar selection medium containing Kanamycin (100 ng μl^{-1}). Colonies were cultured in LB broth containing Kanamycin (100 ng μl^{-1}) overnight at 37 °C. The following day, culture medium was replaced and having reached a cell density to OD600 = 0.6-0.8, the culture was induced with IPTG (0.1 mM). Cultures were then incubated and their content were collected after 2, 4, and 16 h of induction. Samples collected at zero time were used as the negative control for expression. To determine the expression status of the E6 protein as soluble or as inclusion bodies, lysed cells were first centrifuged at 8,000 rpm for 20 min at 4 °C. The supernatant and the resulting precipitate were then stored at -21 °C and the expression status of the protein was determined by SDS-PAGE.

The pellet was lysed by sonication in chilled lysis buffer (50 mM Tris-HCl pH = 7.5). Sonication was performed

according to the following program: 0 short bursts of 10 s followed by an interval of 30 s for cooling. Finally, ultracentrifugation at 4 °C for 15 min at 14000 rpm was carried out for the removal of cell debris. The supernatant was run on 12% polyacrylamide gel and analyzed by SDS-PAGE and Western blotting for confirmation of recombinant polytope E6 expression. Protein purification of E6 was performed by Ni-NTA affinity chromatography and the purity of the purified protein was assessed using electrophoresis on a 12.5% polyacrylamide gel and subsequent Coomassie blue staining. Confirmation of the recombinant E6 production was performed by transferring the protein bands to a nitrocellulose membrane and Western blotting using 1:2000 diluted HRP-conjugated Anti His-tag Antibody in the presence of DAB substrate.

To optimize the expression, different factors such as different induction times (1, 2, 4, 16 h) were assayed to obtain the highest recombinant protein level.

RESULTS

Cloning of E6 Sequence into pET28a

Vector extraction was performed from overnight culture using a plasmid purification mini Kit (fermentaz). The plasmid was electrophoresed on an agarose gel and observed in three forms of open circular, linear and closed circular plasmids respectively (Fig. 1a). As it is shown in Fig. 1b, the electrophoresis of double-digested pET28a and synthesized E6 gene was also performed.

Ligated pET28a vector with the E6 gene was transformed into DH5a and recombinant colonies were selected in the selection medium (Fig. 2). Presence of the cloned E6 gene was confirmed with colony PCR and enzyme digestion (Fig. 3). The results of sequencing were compared with the reference nucleotide sequence by NCBI nucleotide alignment and the correct synthetic E6 gene without any variation was confirmed. ORF analysis showed that E6 coding region has been inserted in-frame so that it will be expressed next to C-terminus (6X) His-tag.

Expression of Recombinant E6 Protein in *E. coli* BL21

pET28a recombinant vector was recovered from DH5a cells after mini-prep and extraction with Plasmid DNA

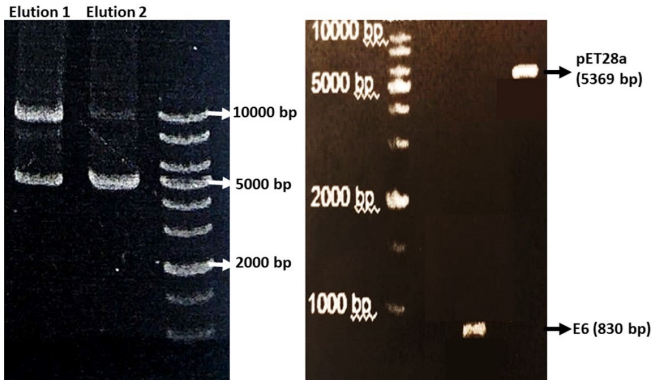

Fig. 1. (A) Extracted pET28a from DH5a cells was run in 0.7% agarose. From right to left: DNA Ladder, pET28a-elution 1, pET28a-elution 2. (B) Digested pET28a and E6 synthesized gene were run in 0.7% agarose. From left to right: DNA Ladder, E6, pET28a.

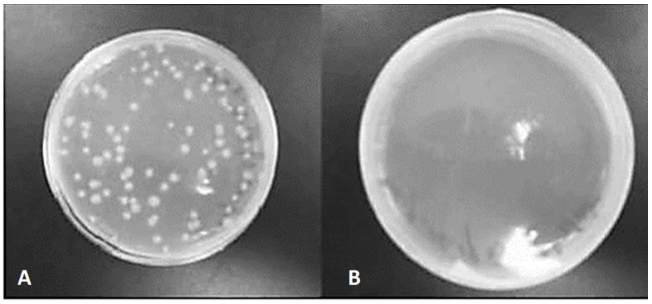

Fig. 2. Plates containing selective LB agar containing kanamycin. (A) DH5a colonies carrying pET28a recombinant plasmid; (B) Negative control plate.

Extraction mini Kit (Fermentaz) with the help of the manufacturer's instruction. Previously provided competent *E. coli* BL21 cells were transformed with the extracted vector and cultured overnight. In the following day, expression was induced with IPTG, BL21 cells were collected, and the lysed cell extract was used for protein expression analysis. Since polytope E6 recombinant protein has a molecular weight of 30 kDa, it must be seen as a distinct 30-kDa band in SDS-PAGE and Western blot. Expression of recombinant E6 protein at 25 °C, induced with 0.1 mM IPTG over times of 0 (negative control), 1 h, 2 h, 4 h, and 16 h is illustrated on SDS-PAGE (Fig. 4). As the recombinant proteins are expressed fused with His-Tag,

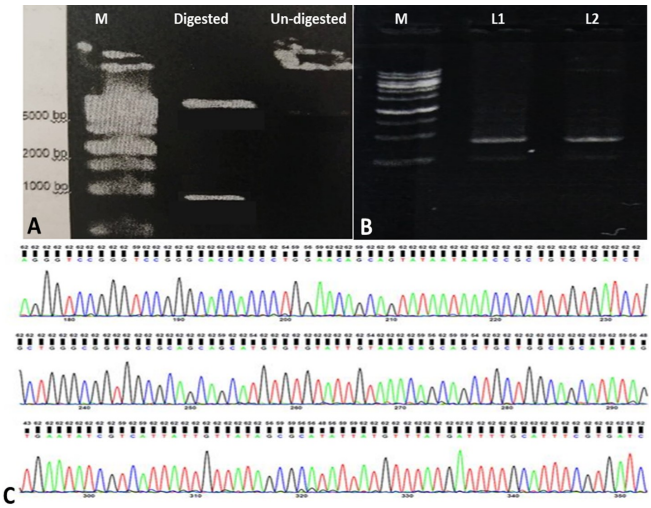

Fig. 3. Confirmation of the cloning of E7 into pET28a by (A) colony PCR, (B) enzymatic digestion, and (C) DNA sequencing. M: DNA Ladder (YT8507 includes 500, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 8000, 10000bp bands).

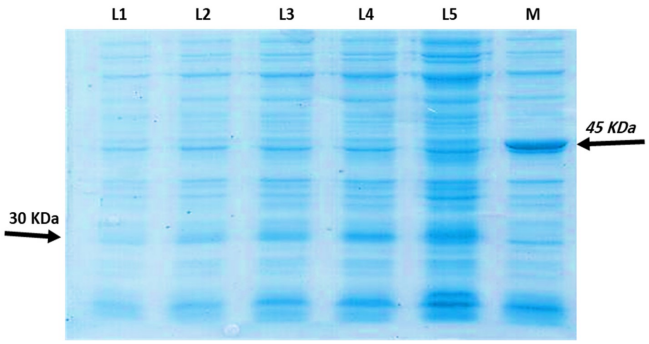

Fig. 4. A) The SDS-PAGE analysis of recombinant Polytope E6 protein on 12.5% polyacrylamide gel. Lane M: protein molecular marker (protein with known molecular weight of 45 kDa); lane 1: *E. coli* BL21 (DE3) lysate with recombinant vector before IPTG-inducing (negative control); lane 2: BL21 (DE3) crude cell lysate harboring recombinant plasmid after 1 h induction with IPTG; Lanes 3: BL21 (DE3) crude cell lysate harboring recombinant plasmid after 2 h induction with IPTG; lane 4: BL21 (DE3) crude cell lysate harboring recombinant plasmid after 4 h induction with IPTG; lane 5: BL21 (DE3) crude cell lysate harboring recombinant plasmid after 16h induction with IPTG.

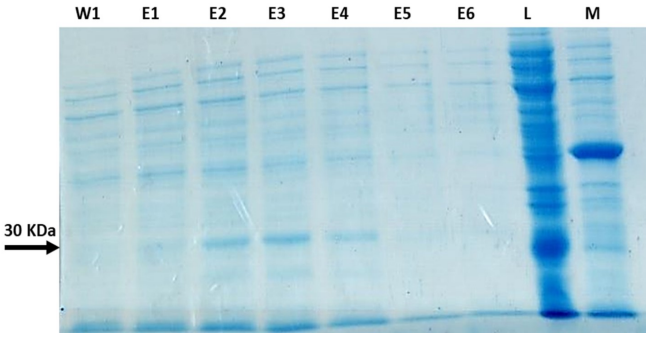

Fig. 5. Purification of E6 protein with Ni-NTA affinity chromatography. From left to right: W1, Wash1; E1, elute1; E2, elute2; E3, elute3; E4, elute4; E5, elute5; E6, elute6; L, Lysed cell extract; M, protein molecular weight marker.

Western blotting was performed using an HRP-labeled anti-His-tag monoclonal antibody on the total protein extract and recombinant E6 protein, however, no band was observed which could be due to the expression of E6 as inclusion bodies and unavailability of His-Tags. Expression optimization under different conditions revealed that the target gene was optimally expressed in OD = 1, at 25 °C, and after 16 h of induction with 0.1 mM IPTG.

Protein Purification

In the pET28 vector, the recombinant protein is expressed fused with the His-tag sequence. Therefore, a Ni-NTA affinity chromatography with a tendency to histidine was used to purify the recombinant E6 protein. The purified sample was run on SDS-PAGE gel and analyzed. In addition to the E6 protein band, other bands were also detected in the eluted fractions (Fig. 5). Our previous experiments revealed that the recombinant protein is insoluble, therefore, solubilization was performed with different buffer compositions, however, no change in the solubility of the target protein was revealed.

DISCUSSION

In the present study, *E. coli* BL21 (DE3) strain was used as the expression host and transformed with pET28a recombinant vector carrying a polytope E6 gene. The polytope E6 gene gene expression was induced using IPTG

and the recombinant protein product was analyzed by SDS-PAGE. Additionally, the expression level was tried to be optimized by changing different conditions such as the time of IPTG-induction. The highest expression was observed in *E. coli* BL21 (DE3), at 25 °C, after 16 h of 1 M IPTG induction in the logarithmic phase of the bacterial growth (OD600 = 1). Due to the expression of the target protein as inclusion bodies and unavailability of His-tags, the expression was not confirmed by Western blotting and the protein purification by Ni-NTA was poor. However, the application of solubilization buffer can help the solubility of the produced protein, consequently more protein purification by affinity chromatography.

Cervical cancer is the second most common cancer after breast cancer in women [13]. HPV is one of the most common causes involved in the development of cervical cancer [3,2]. Emerging studies have shown the expression of the E6 and E7 proteins of HPV in most cervical cancers. These proteins are able to induce immune responses that make them detectable in immunological tests. Today, E6 and E7 proteins have been used as vaccine candidates against HPV [14]. E6 is an oncoprotein in most types of HPV that is capable of immortalizing cells in cell culture. E6 has four motifs Cys-X-X-Cys in its structure, which is involved in transcriptional activity, transformation, and immortality [15,16].

Commonly approved vaccines for HPV include Gardasil and Cervarix, which contain virus particles derived from the L1 capsid protein of the HPV16-18 viruses and as like as the real HPV virus induce an antibody response [17]. These vaccines have very effective preventive potentials, however, the play no role in the elimination of the established infections and therefore has no therapeutic effect [18]. From the numerous studies that have selected the two E6 and E7 proteins as candidates, Yan *et al.* designed a DNA vaccine to evaluate cellular immune responses to E7 and E6 HPV type 16 peptides, in which their findings showed that significant cellular immune responses associated with disease recovery [14]. In another study, Li and colleagues expressed E6 and E7 proteins under induction with IPTG in the Rosseta expression host and injected into C57BL/6 mice. Their results revealed that vaccination with HPV16-E6 or -E7 protein was able to induce specific immunity against tumor growth [15]. Huang and colleagues developed a DNA vaccine encoding the heat shock protein 60 fused with E6 and E7 antigens and studied its immunization capability in mice. The results of this study showed the appropriate ability of the recombinant protein to stimulate the immune system against E6 and E7 proteins [16]. However, one of the most important vaccines has been developed by Merck et al. that is a nano-valent vaccine containing VPLs of 9 high-risk HPV types (6, 11, 16, 18, 31, 33, 45, 52, and 58), known as Gardasil approved by US Food and Drug Administration (FDA) [17].

Thus, a construct containing the immune system-inducing epitopes of the E6 gene was successfully expressed in the frame of pET28a vector in the cost-benefit *E. coli* BL21 host at the lab-scale. Confirmation of E6 protein expression by Western blotting was not possible due lack of solubility and folding of the protein and thus unavailability of histidine tags at the end of the produced E6 protein for binding of the anti his-tag antibody. Based on the negative control sample (sample collected before induction) and the positive control (purified protein with known molecular weight), the expression was confirmed. Due to the above-mentioned reasons and the formation of inclusion body, purification of the produced E6 recombinant protein was not successful using Ni-NTA affinity chromatography. As a result, different buffers were used for solubilization of target protein, however, with no acceptable results. Inclusion body formation results from an unbalanced equilibrium between protein aggregation and solubilization. So, it is possible to obtain a soluble recombinant protein by strategies that ameliorate the factors leading to inclusion body formation [19]. One is to fuse the desired protein to a fusion partner that acts as a solubility enhancer such as maltose-binding protein (MBP) and glutathione S-transferase (GST). Nonetheless, in some cases, the generation of the inclusion body can be an advantage by providing a simple method for achieving a significant one-step purification of the expressed protein, especially if the protein can be refolded easily *in vitro* [20].

Because high production of recombinant protein is one of the important considerations in vaccine candidate preparation, optimization of protein production and purification is important. There are many plasmid vectors for recombinant protein production. In this study, the pET28a vector system was used which was efficient in

producing recombinant protein. In this study, we used optimized recombinant protein expression under different conditions such as time of IPTG-induction. Although the results were indicative of optimal expression in the optimized condition, applying more approaches such as solubility enhancer tags and codon optimization might be helpful in the recovery of more recombinant protein.

CONCLUSIONS

HPV is one of the most common sexually transmitted infections and has a considerable burden worldwide. The development of prophylactic/therapeutic HPV vaccines is a significant pharmaceutical innovation with the potential to reduce/eliminate HPV-related morbidity. However, the universal use and acceptability of the HPV vaccines are still the main barriers to continue in both economically privileged and disadvantaged countries. In the present study, the cloning of a constructed polytope E6 in pET28a resulted in the considerable expression of the polytope recombinant protein. Therefore, this system is very applicable, beneficial, and cost-effective in the recombinant protein expression field of biotechnology. However, more optimization must be performed on the solubilization of the produced protein for the recovery of soluble polytope E6. Further studies are required to be performed on the expressed protein for its potentials in a platform for screening and detection of HPV infection in screening programs, stimulation of immune responses in vaccination strategies, and application in therapeutic approaches.

ACKNOWLEDGMENTS

This research was done and supported by Iranian research organization for science and technology (IROST). [No. 1012197002]

Conflict of Interest

Authors declare that there are no potential conflicts of interest in relation to the present study.

REFERENCES

[1] K.P. Braaten, M.R. Laufer, Reviews in Obstetrics and Gynecology 1 (2008) 2.

[2] A. Bolhassani, E. Mohit, S.R. Rafati, Human Vaccines 5 (2009) 10.

[3] M. Schiffman, P.E. Castle, J. Jeronimo, A.C. Rodriguez, S. Wacholder, The Lancet 370 (2007) 9590.

[4] L. Bruni, L. Barrionuevo-Rosas, G. Albero, M. Aldea, B. Serrano, S. Valencia, Human Papillomavirus and Related Diseases. The World: Ico Information Centre On Hpv And Cancer Hpv Information Centre, 2016.

[5] V. Madrid-Marina, K. Torres-Poveda, G. Lopez-Toledo, A. Garcia-Carrancá, Archives of Medical Research 40 (2009) 6.

[6] S. Bellanger, S. Blachon, F. Mechali, C. Bonne-Andrea, F. Thierry, Cell Cycle 4 (2005) 11.

[7] A. Handisurya, C. Schellenbacher, B. Reininger, F. Koszik, P. Vyhnanek, A. Heitger, R. Kirnbauer, E. Förster-Waldl, Vaccine 28 (2010) 30.

[8] B. Jabbar, S. Rafique, O.M. Salo-Ahen, A. Ali, M. Munir, M. Idrees, M.U. Mirza, M. Vanmeert, S.Z. Shah, I. Jabbar, Frontiers in Immunology 9 (2018).

[9] A. Yang, J. Jeang, K. Cheng, T. Cheng, B. Yang, T.-C. Wu, C.-F. Hung, Expert Review of Vaccines 15 (2016) 8.

[10] A. Yang, E. Farmer, T.C. Wu, C.-F. Hung, Journal of Biomedical Science 23 (2016) 75.

[11] J.A. Tine, H. Firat, A. Payne, G. Russo, S.W. Davis, J. Tartaglia, F.A. Lemonnier, P.L. Demoyen, P. Moingeon, Vaccine 23 (2005) 8.

[12] B. Bahmani, Z. Amini-bayat, M.M. Ranjbar, N. Bakhtiari, A.H. Zarnani. International Journal of Peptide Research and Therapeutics 27 (2021).

[13] N. Muñoz, F.X. Bosch, S. De Sanjosé, R. Herrero, X. Castellsagué, K.V. Shah, P.J. Snijders, C.J. Meijer, New England Journal of Medicine. 348 (2003) 6.

[14] G. Boulet, C. Horvath, D.V. Broeck, S. Sahebali, J. Bogers, The International Journal of Biochemistry & Cell Biology 39 (2007).

[15] K.J. Syrjänen, S. Syrjänen, Papillomavirus Infections in Human Pathology. Wiley New York, 2000.

[16] H.L. Howie, R.A. Katzenellenbogen, D.A. Galloway, Virology 384 (2009) 2.

[17] M. Adams, B. Jasani, A. Fiander, Vaccine 27 (2009) 25.

[18] A. Hildesheim, R. Herrero, S. Wacholder, A.C. Rodriguez, D. Solomon, M.C. Bratti, J.T. Schiller, P. Gonzalez, G. Dubin, C. Porras, Jama 298 (2007) 7.

[19] M.M. Carrio, A. Villaverde, FEBS Letters 489 (2001) 29.

[20] G.L. Rosano, E.A. Ceccarelli, Frontiers in Microbiology 5 (2014)172.