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EXPERIMENTAL STUDY

HPV16 L2 improves HPV16 L1 gene delivery as an important approach for vaccine design against cervical cancer

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ABSTRACT

BACKGROUND: High-risk human papillomavirus (HPV) infections have been associated with the development of cervical cancer. HPV16 is the most dominant high-risk types of HPV worldwide. L1 and L2 are the major and minor capsid proteins of HPV, respectively. Both proteins are able to self-assemble as a virus-like particle (VLP).
METHODS: In the current study, the human embryonic kidney cells were transfected with the plasmid DNA encoding HPV 16 L1 or L1–L2 genes and their expression was compared using different transfection reagents.
RESULTS: Our data showed that the recombinant L1–L2 DNAs were expressed in a high efficiency compared to L1 DNAs as detected by western blotting, fluorescent microscopy, and flow cytometry. In addition, Lipofectamine and Turbofect as the transfection reagents conferred more potent delivery than PEI 25 kDa indicating high toxicity of this system on HEK-293 cells. These results suggest the use of the full length of L2 as an efficient agent for overcoming the cell barriers and poor uptake of DNA *in vitro and in vivo*.
CONCLUSION: The high expression of HPV16 L1–L2 in HEK-293 cells using different delivery systems opens the way for new studies concerning to the use of L2 for DNA delivery via covalent linkage with the gene of interest *(Fig. 5, Ref. 20)*. Text in PDF *www.elis.sk.*
KEY WORDS*:* HPV, cervical cancer, L1, L2, lipid and polymeric carriers.

Introduction

Human papillomaviruses (HPV) have been known as an original agent in the progress of cervical cancer (1, 2). Among the 15 oncogenic genital HPV types, HPV16 is the most common (~50 %), followed by HPV18 (~20 %) and HPV45 (~10 %) of cervical cancer cases worldwide (3). Thus, the generation of a preventive or therapeutic HPV vaccine would be mostly helpful in these cases (1). Recently, the virus-like particles (VLPs) have been considered as the best candidate for vaccine development against HPV infections. HPV capsids are composed of 72 pentamers of the L1 major coat protein, and an unknown number of the L2 minor coat protein (4). VLPs can be produced by an expression and assembly of the L1 protein alone or its co-expression with the L2 protein (1). The major challenge for the use of L2 protein in VLP-based vaccine alone is its poor immunogenicity as compared to HPV L1 VLP. Regarding to the conserved epitopes of L2 protein, the efforts were done to enhance the L2 immunogenicity by the linkage of L2 to TLR agonists or the use of a concatenated N-terminal fragment of L2 (5). Furthermore, the addition of L2 to L1 VLPs increases the number of neutralizing antibodies as well as better yields for the L1/L2 particles than for the L1 VLPs (1, 6). Indeed, the chimeric L1–L2 capsids may have higher stability than the L1 capsids (6). The studies showed that HPV L1 alone or accompanied by L2 expressed in cultured cells could be self-assembled in the nucleus (7, 8). The recent evidence has shown that VLPs harboring both the L1 and L2 capsid proteins may be more potent for DNA delivery than VLPs composed of L1 alone (9, 10). Despite current researches on using chimeric HPV VLPs as a vaccine or DNA/peptide delivery system, there are not any full reports comparing the quality and quantity of *in vitro* L1 protein expression alone, with L1–L2 fusion protein using lipid and polymeric delivery systems. Herein, the role of L2 in increasing L1 gene delivery and subsequently its *in vitro* expression was evaluated by fluorescent microscopy, flow cytometry, and western blot analysis. Our data indicated that L2 augmented the expression of L1 protein likely by facilitating its delivery. In addition, we evaluated the efficiency of three delivery systems such as lipofectamine, Turbofect, and polyethylenimine (PEI) for L1 and L1–L2 plasmid DNA delivery, *in vitro*.

Materials and methods

Preparation of pcDNA-L1–L2

For the generation of eukaryotic expression vector harboring the L1 (pcDNA-L1), the L1 DNA, was amplified by PCR from pUF-L1 (kindly provided by Prof. Martin Muller, German Cancer Research Center) using primers designed to generate *Xba*I and *Not*I restriction sites at the 5' and 3' ends of the amplified fragments,

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respectively. The amplified L1 DNA was then cloned into the unique *Xba*I and *Not*I cloning sites of the pcDNA3.1 (Invitrogen).

Forward L1: 5'- ATTCTAGACTCGAGACCATGGGCCT-GTGGCTG-3'

Reverse L1: 5'- ATGCGGCCGC CAGCTTCCTCTTCTTCC TCTTG-3'

To make the L1–L2 fusion (pcDNA-L1–L2) (Fig. 1), the L2 gene was amplified by PCR from pUF-L2 (kindly provided by Prof. Martin Muller, German Cancer Research Center) using primers designed to generate *Not*I and *Kpn*I restriction sites at the 5' and 3' ends of the amplified fragments, respectively and then cloned into the unique cloning sites of the pcDNA-L1. We designed two L2 reverse primers with and without stop sequence for construction of pcDNA-L1–L2.

Forward L2: 5'-AAGCGGCCGCAAGGCACAAGAG-GAGC-3'

Reverse L2 (Stop): 5'-TAGGTACCTCAGGCGGCCAGGCT-CAC-3'

Reverse L2 (Non-stop): 5'-ATGGTACCAAGGCGGCCAG-GCTCAC-3'

The accuracy of these constructs (pcDNA-L1, pcDNA-L1–L2) was confirmed by DNA sequencing.

Preparation of pEGFP-L1–L2

For the generation of L1–L2-expressing plasmid (pEGFP-L1–L2) (Fig. 2), the L1–L2 was subcloned from pcDNA-L1–L2 (without stop codon) into the *Xho*I/*Kpn*I cloning sites of pEGFP-N1 expression vector (Clontech, Mountain View, CA). DNA constructs containing L1, and L1–L2 (pEGFP-L1, pEGFP-L1–L2, pcDNA-L1, and pcDNA-L1–L2) were purified in large-scale using Midi-kit (Qiagen). DNA concentrations were determined by the absorbance measured at 260 nm. The presence of the inserted L1, and L2 fragments was confirmed by PCR and restriction enzyme digestion as detected on gel electrophoresis.

In vitro protein expression of L1, and L1–L2 in HEK-293 cells

The pEGFP-N1 harboring the L1 or fused L1–L2 genes (pEGFP-L1 and pEGFP-L1–L2) were prepared in large scale with a high purity. Human HEK-293 cells were maintained in complete RPMI (Sigma) medium supplemented with 10 % foetal calf serum (FCS, Gibco) at 37 °C and 5 % CO_2 atmosphere. Then, the cells were seeded into a 12-well plate and transfected using TurboFect, Lipofectamine, and polyethylenimine (PEI) as *in vitro* transfection reagents. For transfection with PEI (cationic polymer), PEI/DNA complexes were generated by mixing LIN-PEI 25 kDa (10 μM, NrE = 10, Polysciences, Europe) with 2 μg of each pEGFP-L1, pEGFP-L1–L2, and pEGFP-N1 as a positive control in HBS buffer (HEPES buffered saline) in a final volume of 100 μl and incubated at room temperature for 15 min. HEK-293 cells were used as a negative control. PEI/DNA complexes were added to 1×10^5 HEK-293 cells in serum-free media. The medium was replaced after 6 h incubation at 37 °C with RPMI 10 %. The level of protein expression (*i.e.,* transfection efficiency) was determined by the fluorescence microscopy, flow cytometry (Partec GmbH), and western blotting at 48 h after transfection. For TurboFect (cationic polymer) or Lipofectamine 2000 (cationic lipid, invitrogen) transfections, the DNA was pre-incubated with 4 μl of reagent in a final volume of 25 μl and incubated at room temperature for 20 min to allow the DNA-Lipofectamine/TurboFect complexes to form. The complexes were then added to each well containing cells and medium. Cells were harvested 48 h post-transfection, washed, and resuspended in PBS, to determine the proportion of fluorescent cells expressing L1 or L1–L2 using a flow cytometry. The quality of protein expression was also detected by fluorescent microscopy and western blotting. Furthermore, the delivery of pcDNA-L1, and pcDNA-L1–L2 was performed by different transfection reagents as above mentioned and the protein expression was evaluated by western blot analysis.

Western blot analysis

HEK-293 cells were washed with PBS and lysed in whole-cell lysis buffer (10 % glycerol, 0.5 mM EDTA, 1 mM DTT, 2 mM natrium fluoride, 0.2 % Triton X-100 in PBS pH = 7.4) supplemented with protease inhibitor (Sigma). Proteins were separated on 12.5 % (w/v) polyacrylamide gel and transferred to nitrocellulose membrane (Millipore). The anti-HPV16 L1 monoclonal antibody (MD2H11, kindly provided by Prof. Martin Muller, German Cancer Research Center; 1 : 10000 v/v) was used to confirm L1, L1–L2 protein expression under standard procedures. The immunoreactive protein bands were visualized using peroxidase substrate named 3, 3'-diaminobenzidine (DAB, Sigma).

Statistical analysis

Statistical analysis (Student's t-test) was performed by Prism 5.0 software (GraphPad, San Diego, California, USA) to analyze the percentage of L1-GFP, and L1–L2-GFP expression using flow cytometry. The value of $p < 0.05$ was considered statistically significant. Similar results were obtained in two independent experiments.

Results

Generation of L1–L2 DNA constructs

The L1–L2 fusion gene was firstly inserted into the pcDNA3.1 (-) with stop codon at the end of L2 sequence for construction of pcDNA-L1–L2. In addition, pcDNA-L1–L2 without stop codon was designed to clone the L1–L2 fusion into the N-terminal of a mammalian expression vector; pEGFP-N1 and the obtained clones were referred to as pEGFP-L1–L2. These plasmids as well as pEGFP-N1, pEGFP-L1, and pcDNA-L1 were prepared in large scale. The genes of L1, and L1–L2 migrated as ~ 1515, and 2958 bp in agarose gel, respectively, using PCR and enzyme digestion (data not shown).

HPV16 L2 gene could increase transfection efficiency of L1 DNA detected by flow cytometry

For confirmation of the L1 and L1–L2 DNA delivery *in vitro* (pEGFP-L1, pEGFP-L1–L2), PEI 25 kDa, TurboFect, and Lipofectamine were used as the transfection reagents. GFP expression was evaluated by fluorescence microscopy and flow cytometry at

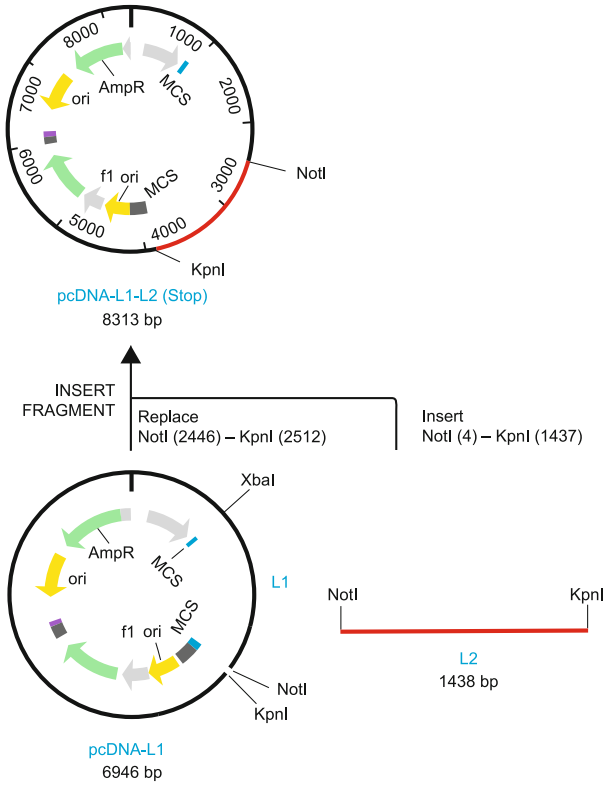

Fig. 1. Cloning of L2 into pcDNA-L1 vector.

48 h after transfecting HEK-293 cells. GFP fluorescence was observed in cells that received 2 µg of pEGFP-N1, pEGFP-L1 and pEGFP-L1–L2 vectors. The transfection efficiency using different delivery systems showed that the Turbofect and Lipofectamine were more potent than PEI 25 kDa (NrE=10). In addition, the level of GFP expression detected by pEGFP-L1–L2 delivery was significantly higher than that by pEGFP-L1 transfection ($p < 0.05$). The levels of protein expression were 89.14 %, 25.17 %, 84.91 % for pEGFP-L1–L2; 65.05 %, 10.73 %, 45.35 % for pEGFP-L1; 86.53 %, 33.76 %, 82.43 % for pEGFP-N1, using Lipofectamine, PEI, and TurboFect, respectively. The transfection efficiency of L1 and L1–L2 genes using three methods has been shown at 48 h after cell transfection by flow cytometry and fluorescent microscopy in Figs 3 and 4, respectively.

Detection of L1 and L1–L2 protein expression using western blot analysis

Western blot analysis was performed using anti-L1 monoclonal antibody to ensure the proper expression of L1 and L1–L2. The specific bands with expected size of 82 kDa, 106 kDa, and 133 kDa were detected for L1-GFP, L1–L2, and L1–L2-GFP expressed from pEGFP-L1, pcDNA-L1–L2, and pEGFP-L1–L2 vectors in the transfected cells as shown in Figure 5. Indeed, L1 or L1–L2 expression was detectable in transfected cells as compared to untransfected cell extracts by western blotting.

Discussion

The prevention of cervical cancer would need to induce immune responses against at least 7 high-risk HPV types (e.g., 16, 18, 31, 33, 45, 52, and 58), which increases the cost and complexity of prophylactic vaccines (5). The recent L1 protein-based HPV vaccines stimulate the neutralizing antibodies against infections and offer type-restricted protection (5). The HPV L2 protein is a good candidate for development of prophylactic vaccine, because L2-specific antibodies have cross-neutralizing activity against various HPV types (11, 12). L2 polypeptide vaccines could provide a broad range of protection; however, the L2 protein vaccines are poorly immunogenic compared to L1 vaccines. Thus, the chimeric VLPs showed the potential to use as a vaccine candidate for a broad spectrum of high-risk HPVs (13). Different studies demonstrated that L2 (especially the amino terminal of L2) has a potential as a protective antigen, although it does not make VLP (14, 15). Indeed, L2 confers more stability to the VLP and is also necessary for HPV infections (8).

In this study, the human embryonic kidney cells (HEK-293) were transfected with the DNA constructs expressing HPV16 L1, and HPV16 L1–L2 proteins. Our major goal was the evaluation of L1–L2 expression compared to L1, *in vitro*. Furthermore, we compared the efficiency of different cationic polymers and lipids to deliver plasmid DNAs. Recombinant L1–L2 DNAs were expressed in HEK-293 cells in a high potency, detected by fluorescent microscopy, flow cytometry, and western blotting. High GFP fluorescence was observed in the cells that received pEGFP-L1–L2

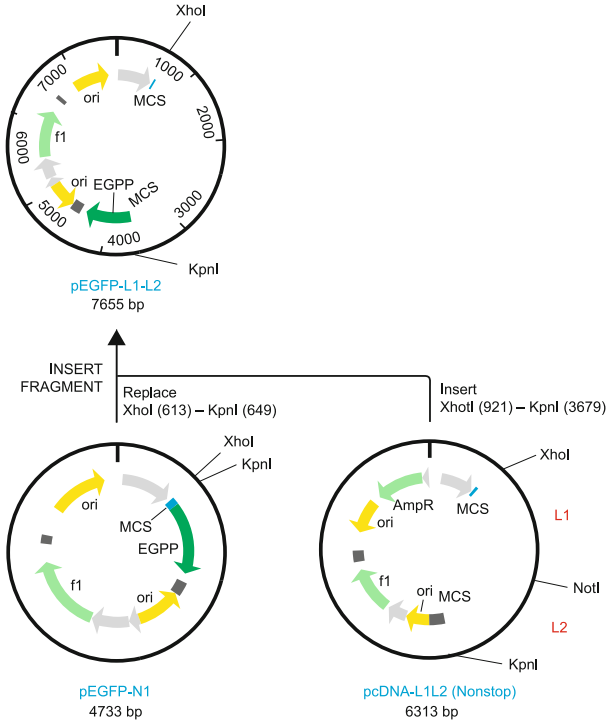

Fig. 2. Cloning of L1-L2 into pEGFP-N1 vector.

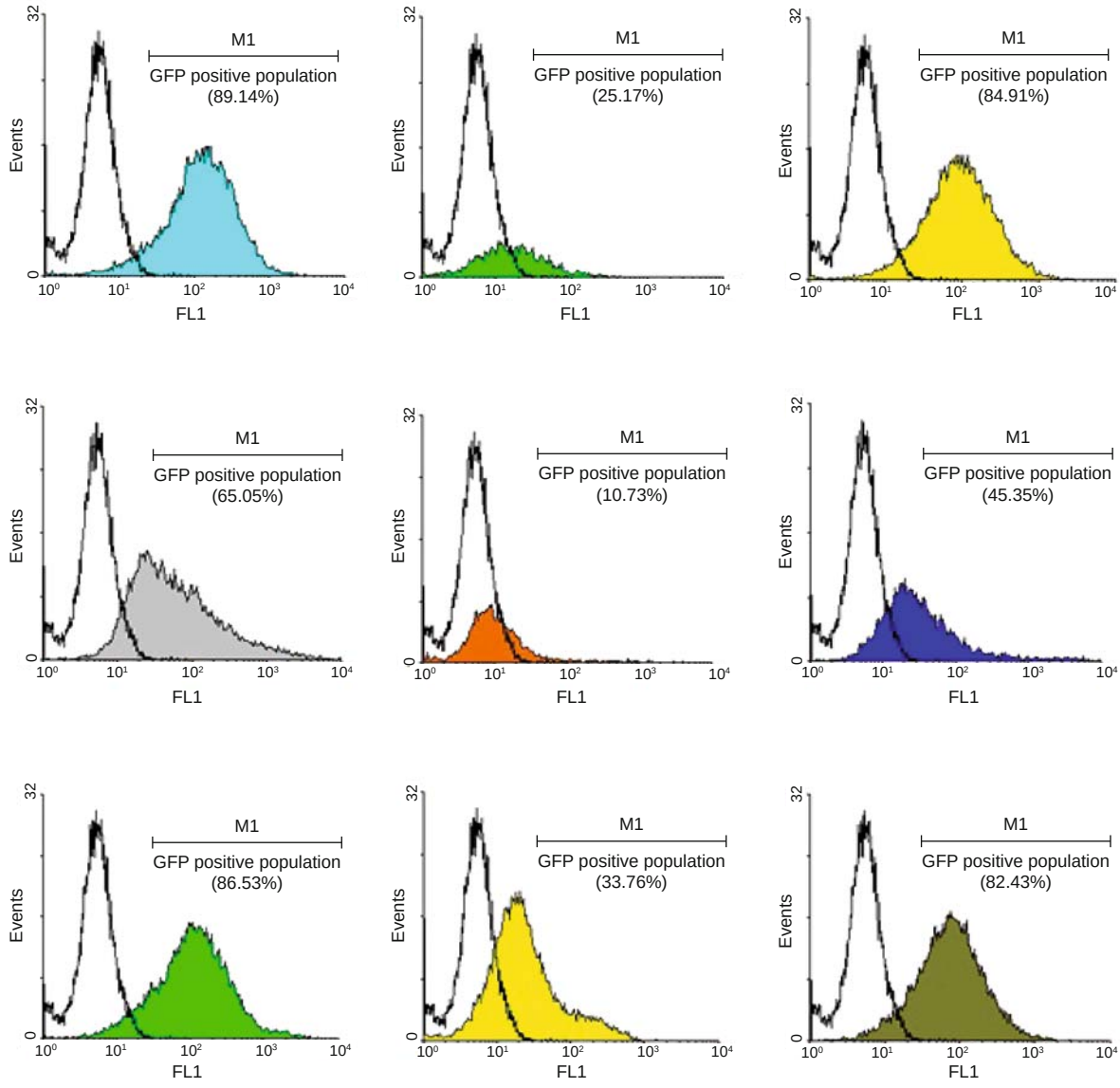

Fig. 3. The transfection efficiency of L1 and L1-L2 genes using flow cytometry analysis: Delivery of pEGFP-L1-L2 (A-C), pEGFP-L1 (D-F), and pEGFP-N1 (G-I) by Lipofectamine, PEI, and Turbofect, respectively. High GFP expression level was observed for pEGFP-L1-L2 delivered by Lipofectamine (89.14%) and then TurboFect (84.91%) as compared to pEGFP-L1 delivered by these systems (65.05%, 45.35%, respectively)

vector as compared to pEGFP-L1 vector. In addition, the transfection efficiency observed by Lipofectamine and then Turbofect was higher than that by PEI. Flow cytometry analysis indicated a clear and significant quantitative separation between L1-GFP or L1–L2-GFP expressing transfected cells and untransfected cells (negative control). L1–L2-GFP expression was detectable in 84–89 % of the transfected cells as compared to L1-GFP expression (~45–65 %) using Lipofectamine and TurboFect transfection systems. Using PEI reagent, higher expression of L1–L2-GFP protein (~25 %) was observed in comparison with L1-GFP protein (~10 %). However, PEI 25 kDa showed more toxicity on HEK-293 than two other delivery systems suggesting a lower percentage of GFP fluorescent. Furthermore, L1 or L1–L2 expression was also detectable in the transfected cell extracts compared to untransfected cells by western blotting. The dominant bands of ~82 kDa, 106 kDa, and 133 kDa were detected in transfected cells expressing L1-GFP, L1–L2, and L1–L2-GFP using Anti-L1 antibody. No such corresponding band was revealed in the untransfected cells. These results confirmed the expression of L1–L2 protein as fused to GFP or not. Regarding the obtained data, it seems to consider the L2 gene as facilitating agent of DNA delivery followed by its protein expression.

Other studies also showed that L2 is necessary for intracellular encapsidation of papillomavirus genomes. During the primary

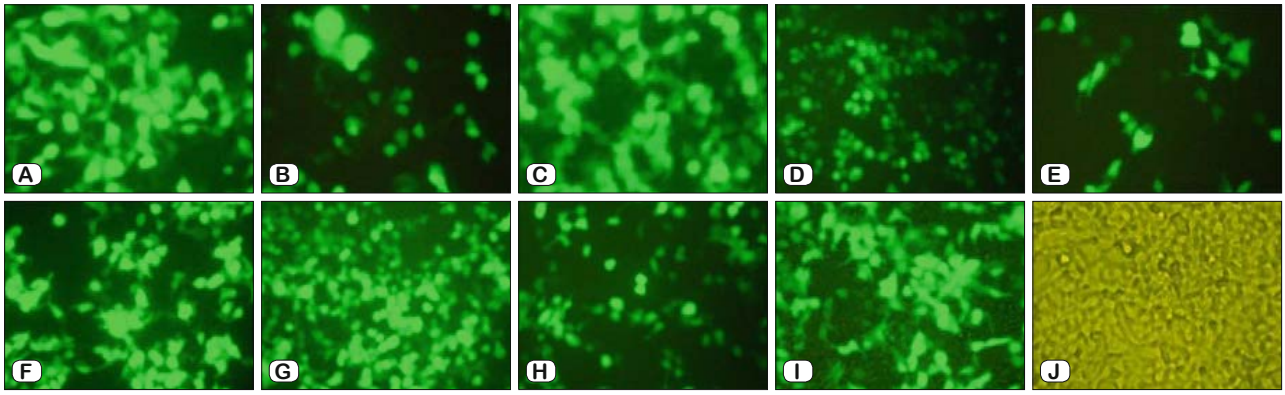

Fig. 4. The transfection efficiency of L1 and L1–L2 genes using fluorescent microscopy: Delivery of pEGFP-L1-L2 (A–C), pEGFP-L1 (D–F), and pEGFP-N1 (G–I) by Lipofectamine, PEI, and Turbofect, respectively against untransfected cells (negative control, J).

infection, the L2 protein localizes in sub-nuclear domains known as nuclear domain 10 (ND10). The targeting of L2 to ND10 may facilitate the delivery of the viral genome to ND10 for initiating viral transcription (11, 16–18). L2 has also been shown to mediate co-localization of L1 and DNA within the nucleus in promyelocytic leukemia oncogenic domains (POD) (19, 20). Moreover, it was recently indicated that VLPs containing both the L1 and L2 capsid proteins might be more efficient for DNA delivery than VLPs consisting of L1 alone (9). The studies showed that DNA co-delivered with L1 VLPs was retained within endosomes, and that efficient endosomal escape was dependent on a 23 amino acid sequence located within the C-terminal region of L2 (10). Generally, L2 may facilitate expression of co-delivered DNA not only by mediating endosomal escape, but also by mediating localization of DNA to transcription sites (10). All these experiments utilized L2 VLP associated with L1 VLP (L1/L2 VLP) for DNA delivery or VLP-based vaccination. In the current study, the full length of L2 gene fused to the full length of L1 gene without linker could increase the level of L1 expression as compared to the L1 DNA. This is the first investigation on the effects of L2 DNA as linked to L1 gene as well as the comparison of three transfection reagent. The similar results were obtained by Lipofectamine, Turbofect, and PEI for high expression of L1–L2 DNA.

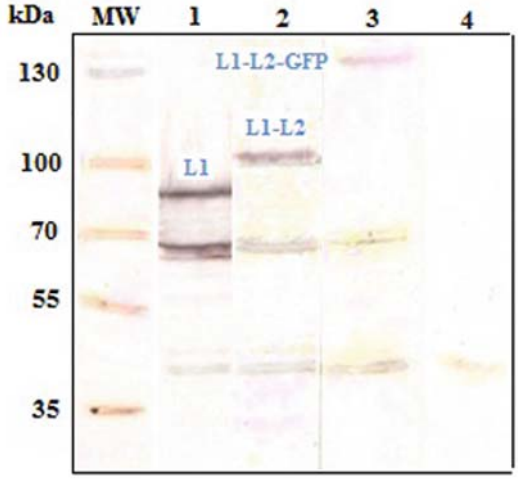

Fig. 5. Western blot analysis using an anti-L1 monoclonal antibody showed the expression of the full-length L1-GFP (line 1, 82 kDa), L1-L2 (line 2, 106 kDa), and L1-L2-GFP (line 3, 133 kDa) proteins, respectively. Any detectable band was not observed in untransfected cells as a negative control (line 4)

Conclusion

In summary, our findings support the use of L2 as an efficient DNA delivery system to overcome cell barriers and enhance protein expression for development of HPV vaccines.

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