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Constructing a Surface Display Vector Encoding E7 Protein of HPV18

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Abstract

Objectives: This study aimed the construction of a surface display vector encoding E7 protein of HPV18 to be expressed in yeast *Yarrowia lipolytica* in order to combine the advantages of both systems.

Methods: DNA from a HPV18-positive individual was applied for amplification of E7 encoding gene via nested-PCR. Both pINA1317-YLCWP110 plasmid and PCR products were double-digested using HindIII and SfiI. After purification, the double digested fragments were ligated together to create the recombinant pINA1317-YLCWP110-E7 plasmid. After transformation into *E. coli*, the positive transformants were subjected to molecular analysis. The accuracy of cloning was further assessed using Sanger sequencing.

Results: Plasmid DNA from positive transformants was analyzed by molecular methods. The E7 related band was detected in PCR, and restrictive cleavage confirmed the presence of E7 insert in the recombinant plasmid. In addition, Sanger sequencing of recombinant pINA1317-YLCWP110-E7 plasmid confirmed the accuracy of cloning; alignment of the sequencing data in gene bank verified the DNA sequence of E7 encoding gene, insert orientation, and accuracy of reading frame.

Conclusions: The constructed recombinant pINA1317-YLCWP110-E7 plasmid can be used to express the E7 protein in the yeast *Yarrowia lipolytica* to be applicable as a vaccine, molecular marker or therapeutic aspects.

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Introduction

Cervical cancer is the fourth most prevalent cancer in women worldwide with an estimated 604000 new cases in 2020. Also, an estimated 342,000 deaths from cervical cancer was reported in 2020. Approximately 90% of these deaths occur in low- and middle-income countries [1-4].

Human papillomavirus (HPV) comprises a circular double-stranded DNA genome of 8000 bp that invades epithelial tissue. The genome encodes six primary non-structural proteins [E1, E2, E4, E5, E6, E7) and two delayed structural proteins L1 and L2. To date, almost 450 HPV types have been isolated and their genome sequences were defined. Also, at least 14 high-risk (HR) types have been described as carcinogenic [5-9].

HR-HPV is significantly relevant to the pathogenesis of urogenital cancers and chronic infection with the HR types of HPV especially HPV16 and HPV18 plays a critical role in developing cervical intraepithelial neoplasia (CIN) and cancer [10, 11].

During carcinogenesis of HPV, viral onco-proteins are responsible for the early transformations in epithelial cells through inactivation of the two main tumor suppressor proteins; pRb and p53. Interfering with the function of these tumor suppressors lead to the disturbance of DNA repair and apoptosis mechanisms that promote uncontrolled cell proliferation [4, 11].

Yet, there are no precise data of HPV prevalence in Iran. But, some studies report the occurrence of HPV in a limited number of women with cervical cancer compared to the ordinary Pap smear samples. In one study in south-eastern of Iran on Pap smear samples from 2018 to 2020, 37.7% of patients were HPV positive. Among them, 62% of cases were infected with only one HPV type and 38.2% showed infection with multiple HPV genotypes. Overall, 31 HPV genotypes were reported of which 4 common genotypes were HPV6, HPV16, HPV53 and HPV51 [12]. In another study in 2019, the most common low-risk genotypes were reported to be HPV6 and HPV11, and the most high-risk genotypes were HPV16 and HPV52 [13]. Also, in 2021, a study was conducted on samples collected from 3 groups in Khorasan including Razavi and North Khorasan, South Khorasan, and tourist group. The prevalence of HPV was 48.4%, 19.9% and 33.6% in the first, second and third group respectively. The HPV6, HPV11, HPV16, and HPV514 genotypes were reported as the most common in that study [14]. Further, a study was conducted on the 2562 women of the general population living in 11 provinces of iran in 2022. The frequency of human papillomavirus and its related genotypes was obtained at 2.4% (108 out of 2562 people) with the highest prevalence in the age group of 25-34 years [15]. Also, a systematic review of 14 studies in Iran has declared the prevalence of HPV among healthy women in different provinces of Iran as 9.4%, and among them, the prevalence of HPV16 and HPV18 was reported to be 2.3% and 1.7% respectively [16].

Comprehensive control of cervical cancer includes prophylactic HPV vaccination, screening and treatment of precancerous lesions, and diagnosis and therapy of invasive cervical cancer [17]. Studied have demonstrated that immune responses to HPV vaccination are considerably stronger than immune responses to natural HPV infection [18, 19] and prophylactic HPV vaccination can be significantly effective against invasive HPV-positive cancers [20]. Over 100 countries had integrated HPV vaccines into their national immunization programs by 2020 [21].

Available prophylactic vaccines that target capsid antigens have no therapeutic effect against HPV-based transformed epithelial cells [7, 22, 23]. Due to the limited benefits of these vaccines in eliminating previous infections, new advances have been made in the production of therapeutic vaccines [18, 19]. In this regard, onco-proteins E6 and E7 of the virus have been considered to be effective on the infected cells and some vaccines have been developed based on the E6 and E7 are in clinical trials. Due to the critical role of the HPV18 in the progression of cervical cancer worldwide, the E7 protein of HPV18 was aimed at this study.

Many expression systems have been applied for the production of recombinant proteins of various origins. Prokaryotic hosts provide cost effective, simple and fast systems while lacking the complicated post-translational modifications of heterologous eukaryotic proteins [24, 25]. On the other hand, higher organisms such as mammals provide great regulatory possibilities and can fulfill the required modifications. But these higher systems require higher costs, are time consuming and possess complicated regulatory system [24, 26]. The yeast host has been currently considered as a good eukaryotic host for the production of eukaryotic recombinant proteins. This expression system is simple, fast-growing and economical that can be grown in high-density cell bioreactors and provides the necessary eukaryotic modifications. Human serum albumin, insulin and its analogues, vaccines against papillomavirus and

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hepatitis are the most important biochemical drugs produced in yeasts [27].

Many plasmids have been developed for the expression of recombinant proteins of various purposes, among them surface display plasmids have attracted a lot of interest due to the feasibility of the presentation of the synthesized protein at the surface of the cell compared to the isolation of the protein from the intracellular protein content.

A yeast surface display plasmid "pINA1317-YlCWP110" was constructed in 2008 to be expressed in *Yarrowia lipolytica.* It has a recombinant promoter that facilitates the expression in the heterologous yeast *Yarrowia lipolytica*. Having a synthetic promoter named hp4d, this plasmid has eliminated the limitations of induction by specific inducers or expression in the growth phase of yeast. The activity of hp4d promoter is similar to the earlier promoters in the fully induced state, without being limited by sources of nitrogen and carbon. Also, it shows no need to the induction by an inducer. After all, this system produces the recombinant protein in the resting phase after the growth phase. The constructed plasmid benefits from high efficiency of transformation, more stability of transformants and the improvement in the secretion of synthesized proteins. In order to present the recombinant protein on the surface of the host cell, first, 333 base pairs of genomic DNA of *Yarrowia lipolytica* encoding carboxyl terminal of YlCWP1 was added to this plasmid. The findings demonstrated that the constructed plasmid could be a great plasmid for surface display on yeast *Yarrowia lipolytica* [28]. Altogether, this study aimed to clone the E7 encoding gene fragment of the HPV18 into the pINA1317-YlCWP110 plasmid in order to benefits the advantages of both surface displayed expression of the recombinant protein and expression in yeast host simultaneously.

Materials & Methods

Primers

Primers were designed by Gene Runner 6.5.48 software using the sequence of E7 gene of the HPV type 18 available in gene bank at NCBI. Also, the sequences of restriction sites required for endonuclease digestion of this fragment was added to designed primer sequences. The accuracy and specificity of primers was evaluated by BLAST. After all, primers ordered to be constructed by Metabion (Germany). The list and sequences of PCR-primers is shown in table 1.

Table 1: The sequence of PCR primers

Name	Primer sequence (5´ 3´) →
18F	GAAACACACCACAATACCATG
18R	CGTCTGTACCTTCTGGATC
18FN	ACACAAGGGCCGTTCTGGCCATGCATGGACCTAAGGCAAC
18RN	CATTGTAAGCTTCTGCTGGGATGCACACCAC

Amplification of Gene Fragment Encoding E7 Protein of HPV Type 18 Using Nested-PCR

The DNA sequence encoding E7 protein of HPV type 18 was amplified by nested-PCR on a DNA sample extracted from a HPV-18 positive individual. Presence of extra sequences related to restriction sites in primers may result in non-specific fragments amplification. In order to avoid unwanted fragments amplification, the gene fragment encoding E7 protein of HPV-18 was amplified using nested-polymerase chain reaction, in which a large fragment encompassing E7-encoding sequences was amplified at first PCR, using 18F and 18R primers. Then, the nested (internal)-PCR was performed using the product of the first (external) reaction as template, using 18FN and 18RN primers. The first amplification reaction was performed in total volume of 20µL including 2µL of DNA template, 1µL of each 18F and 18R primers, 10µL of PCR-MasterMix (2X) (Amplicon, Denmark) and 6µL of deionized water, at 30 cycles of denaturation at 94°C for 30s, annealing at 56°C for 60s and extension at 72°C for 60s. As well, the nested-PCR was performed in total volume of 100µL including10µL of first PCR product as template, 2.5µL of each 18FN and 18RN primers, 50µL of PCR-MasterMix (2X) and 35µL deionized water at 30 cycles of denaturation at 94°C for 30s, annealing at 56.5°C for 60s and extension at 72°C for 40s in a thermocycler (Labcycler, Germany). Then, in order to eliminate unwanted PCR ingredients, the amplified DNA was purified using PlasmidMiniprep columns (GeneJET PlasmidMiniprep, Thermo Scientific, USA. https://www.thermofisher.com/order/catalog/product/k0502).

Amplification and Preparation of Plasmid pINA1317-YLCWP110

In order to obtain sufficient amounts of plasmid pINA1317-YLCWP110 for cloning, the plasmid was transformed into the *E.coli TOP10.* The kanamycin resistance gene on the plasmid made it possible to select the transformed colonies on LB[3]-kanamycin (30 µg/mL)

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plates. Then, the target transformed colonies were cultured in liquid culture medium containing kanamycin and plasmid extraction was performed using a mini-preparation kit (GeneJET PlasmidMiniprep, Thermo Scientific, USA).

Cloning of Gene Fragment Encoding E7 Protein of HPV-18 into pINA1317-YLCWP110

The first step in cloning was double-digestion of target gene (insert) and desired plasmid, pINA1317-YLCWP110, with *Hind*III and *Sfi*I restriction endonucleases (Thermo Scientific, USA). Due to the different requirements of these restriction enzymes, digestion reactions were performed consecutively. In fact, after digestion with first restriction endonuclease, the enzyme was inactivated by incubating at 65°C for 15 minutes. Then, the single-digested DNA obtained from this step was washed and purified using a DNA purification kit. As well, the second digestion reaction was performed and the restriction enzyme was heat-inactivated. Eventually, the purified double-digested DNA of insert and plasmid were ligated using T4 ligase (Thermo Scientific, USA) according to the manufacturer's order. In order to improve the ligation efficiency, the reaction mixture was extra incubated at 4°C for 24 hours. The obtained recombinant pINA1317-YLCWP110-E7 plasmid then was transformed, amplified and evaluated by several molecular methods [29].

Transformation of the Recombinant Plasmids into the *E.Coli TOP10*

The recombinant plasmids obtained in the ligation reaction were transformed into the *E.coli TOP10*. Transformed colonies were selected on LB-Kanamycin plates. The resulted colonies were cultured in liquid culture medium containing kanamycin (30 µg/mL) and subjected for plasmid DNA extraction and molecular analysis[29].

Molecular Analysis of Recombinant Plasmids

Several molecular analyses were used to examine and verify the accuracy of recombinant plasmids. Preliminarily, the presence of E7 encoding fragment in the selected colonies was shown by colony-PCR. In order to confirm the accuracy of cloning processes, more molecular analyses were performed on the so called recombinant plasmids that had been extracted from the selected colonies. Restriction analysis was performed using *Not*I restriction endonuclease. This enzyme has two cleavage sites on the plasmid and generates two fragments of different lengths.

Sanger sequencing

After confirmation of cloning by molecular analyses, the sequence of obtained recombinant pINA1317-YLCWP110-E7 plasmid was detected by Sanger sequencing method via Pishgam Company[1] to ensure the sequence accuracy and frame of the recombinant gene in the plasmid. The sequencing results were analyzed with Chromas software and BLAST tool in the gene bank.

Ethical Issues

None to be declared.

Results

Amplification of E7 encoding fragment of HPV type 18 by nested-PCR

In order to achieve the E7 encoding fragment of HPV type 18, a bigger fragment of HPV genome encompassing E6 and E7 genes was amplified. The size of PCR product would be 852 bp that demonstrated a band near 1000bp on the agarose gel (figure 1). Next, in order to amplify a purified E7 encoding gene fragment, an inner/nested-PCR was performed. The size of PCR product for this fragment was 347 bp that manifested a band between 250 bp and 500 bp on the agarose gel (figure 2).

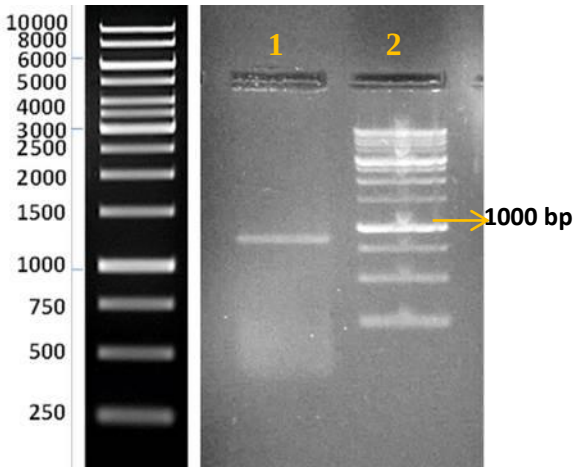

Figure 1. Amplification of a bigger fragment of HPV type 18 genome encompassing the gene fragment encoding E7 protein. 1:PCR product, 2: 1kb DNA size marker

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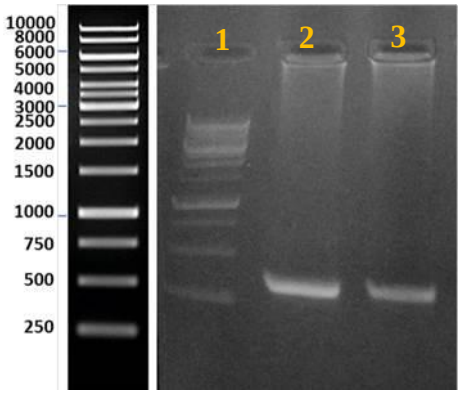

Figure 2. Amplification of gene fragment encoding E7 protein by nested-PCR. 1: 1kb DNA size marker, 2 and 3: nested-PCR products.

Isolation and double digestion of pINA1317-YLCWP110 plasmid

After isolation of the pINA1317-YLCWP110 plasmid, it was double digested using *Hind*III and *Sfi*I restriction enzymes. The PCR product also was purified and digested with the same procedure as the plasmid.

Construction of recombinant pINA1317-YLCWP110-E7 plasmid

The double digested plasmid and double digested PCR products were ligated in order to clone the E7 encoding gene into the pINA1317-YLCWP110 plasmid. After transformation of the resulted recombinant plasmid "pINA1317-YLCWP110-E7", the manifested colonies (figure 3) were evaluated by several molecular tests to verify the accuracy of cloning.

Figure 3. Advent of transformed colonies containing the recombinant plasmids resulted from the ligation reaction.

Molecular analysis of recombinant pINA1317-YLCWP110-E7 plasmid

The cloning of E7 encoding gene into pINA1317-YLCWP110 plasmid was preliminary demonstrated by colony-PCR (figure 4). Then, molecular analyzes were applied to further verify the cloning. Briefly, cleavage by *Not*I restriction enzyme revealed that the length of the digested fragment belonged to the recombinant pINA1317-YLCWP110-E7 plasmid is longer than the non-recombinant plasmid (figure 5).

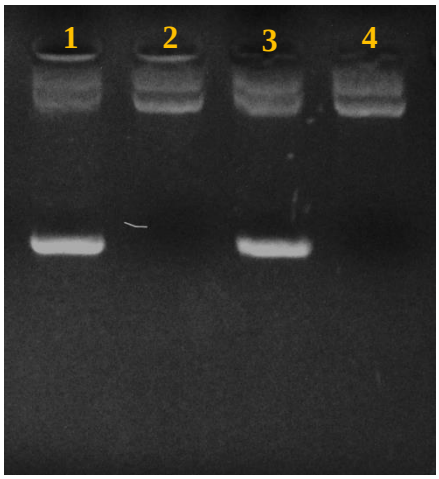

Figure 4. Identification of positive transformants containing the recombinant pINA1317-YLCWP110-E7 plasmid by colony-PCR. 1 & 3: positive transformants, 2 & 4: negative transformants.

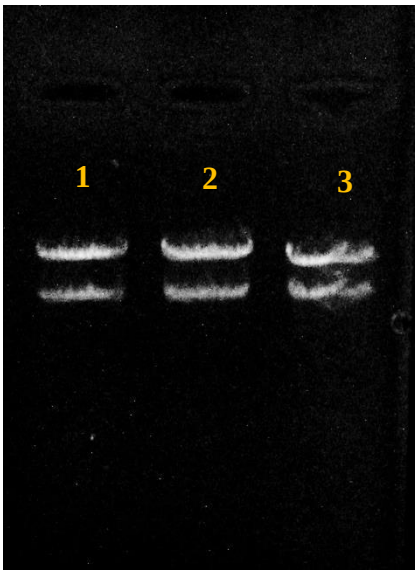

Figure 5. Digestion of recombinant pINA1317-YLCWP110-E7 plasmid by NotI enzyme. 1 & 2: Recombinant pINA1317-

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YLCWP110-E7 plasmids. 3: Original pINA1317-YLCWP110 plasmid without insert.

Sanger sequencing

After early verification of recombinant pINA1317-YLCWP110-E7 plasmid with molecular methods, the sequence of the recombinant plasmid was determined using Sanger sequencing. The results of BLAST at gene bank revealed the accuracy of the nucleotide sequence and the frame of the cloned gene fragment to be correctly expressed and displayed at the surface of the host cell (figure 6).

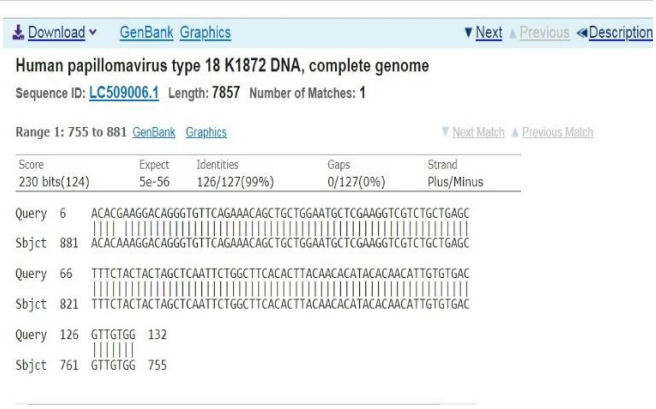

Figure 6. Alignment analysis of Sanger sequencing data of recombinant pINA1317-YLCWP110-E7 plasmid by BLAST

Discussion

High risk types of human papillomavirus are considered as the main cause of cancers in urogenital tract especially cervical cancer [11]. In this regard, HPV type18 is considered as the cause of 15.7% of cervical cancers. The oncogenic properties of human papilloma virus mainly result from the function of E6 and E7 onco-proteins, which interact with p53 and pRb tumor suppressors [1, 10, 30-32].

Currently, vaccination against high-risk types of the HPV can prevent primary infection by this virus, but can't eradicate the previous infections of the affected individuals [33, 34]. Lately, some studies have shown the applicability of recombinant E6 and E7 proteins of HPV in stimulating the immune system against the virus. Since these onco-proteins are expressed exclusively in HPV-infected cells, CINs, and cancer cells, one can benefit from the potency of recombinant E6/E7 as antigens for activation of T cell-based immune response against the HPV infected cells [32, 35] Also, recombinant E6/E7 proteins can be utilized in vaccination and screening kits. The aim of this study was construction of a E7 expressing cassette that are capable of presenting the recombinant E7 at the surface of the host cell.

Surface display plasmids play important role in the recombinant proteins technology by providing the facility of presenting the antigenic recombinant proteins at the surface of the host cell. It facilitates the purification of the recombinant proteins and prevents the agglomeration of upregulated proteins at the cytoplasm. Also, representation of the recombinant protein at the surface of the host cell makes it a good choice for presentation of antigenic recombinant proteins to the immune system or use as a vaccine. In 2014, Dan Xu et al. used the autotransporter of *Shigella IcsA* to express the HPV type 16 L1 capsid protein in *Shigella sonnei* and *Escherichia coli*. Based on their findings, they proposed this strategy for the development of a vaccine against HPV type 16 by showing surface expression of the L1 capsid protein [36]. In another study, 12–41 epitope of HPV16 L2 peptide was entered into Ad5 hexon of adenovirus. They demonstrated capsid display of HPV L2 peptide in the adenovirus 5 hexon protein and suggested a novel approach for candidate prophylactic hpv vaccine [37].

Yeast expression systems compared to other eukaryotic systems such as mammalian cells are simpler and cheaper and account as great hosts for the production of recombinant eukaryotic proteins. They benefit from the eukaryotic regulation and can fulfill post-traslational modifications that are required by some eukaryotic proteins and is absent in prokaryotic expression systems. Then, yeast expression systems account as good choice for affordable production of eukaryotic proteins [38]. Taken together, a yeast surface display plasmid was chosen in this study in order to benefit from both yeast host facilities and surface display advantages in construction of an expression vector encoding E7 oncoprotein of HPV18. This vector, after construction and verification, can be applicable as vaccine or production of the recombinant protein of interest, without the problems related to intracellular production of recombinant proteins.

Conclusion

In this study, the gene encoding E7 oncoprotein of HPV type 18 was successfully cloned into the surface display plasmid pINA1317-YLCWP110 to be expressed at the cellular surface of the yeast host "*Yarrowia lipolytica*". The positive transformants were selected by colony PCR at first. Then, the recombinant pINA1317-YLCWP110-E7

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plasmid was isolated from the positive transformants and studied by molecular analysis e.g. PCR and digestion with restriction enzymes. Cleavage by *Not*I enzyme revealed a bigger band related to the cloning of the E7 encoding gene fragment into the pINA1317-YLCWP110 plasmid. Eventually, the recombinant pINA1317-YLCWP110-E7 plasmid was sequenced by Sanger method. Alignment of the sequencing results in BLAST at the gene bank verified the accuracy of nucleotide sequence and cloning at the correct frame. Overall, the constructed recombinant pINA1317-YLCWP110-E7 plasmid in this study provides an appropriate expression vector encoding the E7 oncoprotein of the HPV18 in order to be displayed at the surface of the host *Yarrowia lipolytica.* Consequently, by combining both yeast expression system and surface display, this new expression cassette can be used as a novel vaccine strategy against human papillomavirus type 18 that combines the advantages of both systems. Additionally, using a recombinant onco-protein (E7) instead of a HPV capsid protein, allows it to be effective against previously infected or transformed cells. After verification of the expression of the E7 protein at the surface of the host cell, this vector can be applicable in the vaccination against the HPV18 and in stimulating the immune system of the affected individuals against the virus.

Abbreviations

NCBI: National Center for Biotechnology Information
BLAST: Basic Local Alignment Search Tool
LB: Luria-Bertani
PCR: Polymerase Chain Reaction
HR: high risk
CIN: Cervical Intraepithelial Neoplasia
USA: United States

Conflict of interests

The authors declare that they have no conflict of interests.

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