Site-Directed Mutagenesis of HPV16 Spliced E6 Protein and Localization Patterns

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Abstract

Infection of cervical cells by high risk human papillomaviruses (HPVs), such as type 16 and 18, is a well-known cause of cervical cancer. E6 is one of the two main HPV oncoproteins responsible for cellular transformation and immortalization. The high risk HPV16 E6 gene generates a spliced form of 43 amino acids, so called E6-I, whose precise role is not known. Cellular localization of HPV16 E6 protein containing both full and spliced forms was previously shown to be dispersed in the nucleus and the cytoplasm, whereas full-form E6 protein was preferentially localized in the nucleus. Our previous results indicated that E6-I localized equally both in the nucleus and the cytoplasm. Based on amino acid sequence homology between E6-I and the N-terminus of E6, the E6-I protein contained only a weak nuclear localization signal whereas the full-form showed three of them, a combination of which generated a strong signal. In this study, we generated E6-I variants by site-directed mutagenesis and examined their localization in a human embryonic kidney cell line, 293T, in order to determine residue(s) necessary for nuclear localization of the spliced protein. Our results indicated that a weak nuclear translocation signal present in E6-I was not sufficient for its nuclear entry since mutations in this region did not affect their localization patterns which suggested that other amino acid sequences or E6-I binding protein might be required for E6-I to enter the nucleus.

Keywords: HPV 16, spliced E6, nuclear localization

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Introduction

Cervical cancer, a malignant cancer of the cervix uteri, is one of the most common causes of cancer death in women worldwide including in Thailand. The most important risk factor in the development of cervical cancer is infection with high-risk strains of human papillomavirus (HPV), particularly, HPV16 and 18. Infection of cervical cells by HPV 16 alone accounts for more than 50% of cervical cancer incidences [1]. The low-risk viruses rarely cause lesions and normally do not develop into cancer for example HPV types 6 and 11.

E6 is one of the two main high-risk HPV oncoproteins responsible for cellular immortalization, transformation, and cancer progression [2-3]. The key role of E6 is inducing p53 degradation and inhibiting p53-mediated apoptosis through E6-associated protein (E6AP). Only E6 protein of high risk HPV can bind to core region of p53, in the presence of E6AP.

Small forms of HPV E6 protein (spliced E6 protein or E6-I) are only presented in high-risk HPV but not in the low-risk HPV. They are translated from spliced E6 mRNAs produced during splicing. In HPV16, E6-I transcripts are spliced out at nucleotide 226 and spliced back at nucleotide 409 to generate E6-I transcripts which produces a 43 amino acid protein [4]. The amino acid sequence of E6-I is identical to the N-terminus of E6 protein [5].

There are two proposals that explain the existence of the E6-I mRNA. Firstly, E6-I encoding mRNA has the much larger space between the stop codon of the E6-I ORF and the start codon of E7 ORF [6], therefore the splicing mechanism may allow more efficient translation of E7 protein (Figure 1). Secondly, E6-I protein may have some functions. Previous reports have shown that HPV18 E6-I protein inhibited E6-mediated degradation of p53 and increased in cellular level of p53 by interacting with both full-form E6 and E6AP, but not to p53 [7-9]. The high-risk HPV18 E6-I protein forms a complex with E6AP through E6AP binding site at amino acids 28-31 [7] which corresponds to amino acids 26-29 for HPV16. HPV18 E6-I protein seems to modulate E6 protein by inhibiting some of E6 functions such as p53 degradation. Moreover, the interaction between 18 full form E6 and 18 E6-I leads to the destabilization of the E6 protein, suggesting that E6-I protein can function as a negative regulator of the full-length E6 protein [10]. Moreover, it is believed that HPV18 spliced E6 protein probably exerts the anti-proliferative effect in HPV infected cells.

While functions of E6 protein are extensively studied, the function of E6-I protein is still unclear. Although E6 can bind to a number of host proteins, both the cytoplasmic and the nuclear proteins, it is primarily localized in the nucleus. N-terminal zinc finger of E6 was proposed to be involved in the nuclear localization [11]. Surprisingly, strong nuclear localization signal of E6 was observed at the C terminus, between residues 120 and 151 of the protein [12]. Later study demonstrated that three nuclear localization signals (NLS) of the full-form 16E6 protein including the NLS1 at amino acids 8-11, the NLS2 at amino acids 66-72 and the NLS3 at amino acids 115-124 were responsible for its nuclear localization and also correlate with its transcriptional regulation activity [13]. The putative NLS motif appears to exist only in oncogenic E6 proteins from high risk HPVs but not low risk HPVs. The lack of powerful NLS in low risk E6 proteins is probably the main reason for the cytoplasmic retention of these proteins [13]. Based on 16E6 protein sequence, 16E6-I protein contains only a weak NLS1 (not NLS2 and NSL3) and E6AP binding site. Identification of the NLS motif in

HPV16 E6-I protein may provide the first insight into the novel activity of this protein.

Therefore, in this study, we aim to investigate the significance of NLS1 of E6-I protein in its cellular localization patterns and also to determine amino acid residues responsible for its nuclear entry by sited-directed mutagenesis.

Materials and Methods

Plasmid construction: E6 and E6-I cDNAs of HPV16 were isolated from SiHa cervical cancer cell line and inserted in frame at the C-terminus of green fluorescence protein between *Not*I and *Xho*I sites of the expression vector pEGFP-C1 (Clontech) to yield GFP-HPV fusion protein (Figure 2). The E6 splice donor mutant expressing only full-form E6 was generated by changing G at the splice donor site to C (nucleotide 226) resulting in a V to L amino acid substitution. Eleven HPV16 E6-I variants, namely Δ8-11, Δ23-26, Δ27-30, R8A, R10A, K11A, T17A, E18A, V31A, Y32A, and K34A, were generated by site-directed mutagenesis using *PfuTurbo* DNA polymerase (Stratagene) and primers as shown in Table 1. The GFP fusion proteins containing full-forms of HPV16E6 and HPV6E6 were also generated in the same manner and used as controls. All the obtained clones were confirmed through DNA sequencing (Macrogen Inc.).

Cell culture: Human embryonic kidney cell line, 293T, was maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, 100 U/ml of penicillin G, 100 U/ml of streptomycin, at 37^{o}C in humidified atmosphere with 5% CO_2.

Transfection: Overnight cultures of 293T cells were transfected with indicated amounts of HPV16 E6-I and its variants using Lipofectamine^{TM} 2000 (Invitrogen) according to manufacturer's protocol.

Confocal fluorescence microscopy: 293T cells were seeded on glass coverslips in 35-mm culture dish and were transfected with 1 μg of HPV16 E6-I in pEGFP-C1 backbone. Twenty four h after transfection, cells on coverslips were washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. Cells were washed three times and incubated with nuclear staining TO-PRO®-3 iodide dye (Molecular Probes®; Invitrogen) with 1:500 dilutions in PBS at room temperature for 45 min in dark. After air dried, cells were mounted on slide using VECTASHIELD® Mounting Medium (Vector Laboratories) and analyzed under the Olympus FV1000 confocal microscope with two excitation laser beams of 488 nm and 633 nm. The images were taken under 60x objective oil immersion lens with 2x zoom.

Results and Discussion

Site-directed mutagenesis

The cloned 16E6-I cDNA was inserted in frame at the C-terminus of the green fluorescence protein (EGFP) gene in the expression vector pEGFP-C1 at *Not* I and *Xho*I sites as shown in Figures 2A and 2B. These allowed the expression as GFP- 16E6-I fusion proteins which could easily be followed under confocal microscope. Eleven types of site-directed mutagenesis in the 16E6-I gene were constructed and their positions and amino acid changes were illustrated in Figure 2C.

Since the amino acid sequence of E6-I was identical to the first 43 amino acids of full- form E6, the significance of these residues were then extrapolated from those reported for full-form E6. The 16E6-IΔ8-11 variant had a deletion of amino acids 8 to 11 which corresponded to nuclear localization signal 1 (NLS1) of 16E6 protein. The two variants 16E6-IΔ23-26, and Δ27-30 had

deletions of amino acids 23 to 26, and 27 to 29 respectively, both of which corresponded to binding site of an E6 associated protein. Variants containing point mutations namely 16E6-I-R8A, R10A, and K11A had altered amino acid residues in NLS1 sequence whereas 16E6-I-T17A, E18A, V31A, Y32A, and K34A contained mutations in the conserved domains of the high risk HPVs.

Nuclear localization of HPV16 E6-I and its variants

High risk HPV16 E6-I protein has previously been shown by our laboratory and others to disperse throughout the cells, but preferentially localized in the nucleus whereas its full-form E6 was found specifically in the nucleus l [13-14]. As mentioned earlier, HPV16 E6 protein was identified to contain three nuclear localization signals (NLSs); NLS1, NLS2 and NLS3, that drive the 16E6 protein into the nucleus. The HPV16 E6-I protein contains only a weak nuclear localizing signal 1 (NLS1), RPRK (amino acid 8 to 11) residing in the N-terminal region of HPV16 E6 protein. It was of our interest to investigate whether E6-I entered the nucleus using solely a weak NLS1.

We monitored the cellular distribution of all 16E6-I variants in 293T cells using confocal microscope. The results in Figure 3 corresponded to those in earlier reports that the 16E6 construct which expressed both full-form and spliced E6 proteins localized both in the nucleus and the cytoplasm, whereas only full-form E6 was predominantly detected in the nucleus. It might be possible that the detected signal in the cytoplasm of 16E6 expressing cells derived from the 16E6-I protein.

Interestingly, all 16E6-I variants (both deletions and point mutations) showed the same localization patterns as that of the wild type indicating that these positions did not significantly affect the nuclear transport of the E6-I protein. However, we have observed decreased signal in the nucleus when high dose of the wild type E6-I was used (data not shown) which implied that localization of the small form of E6 protein is dose dependent. Since the wild-type HPV16 E6-I and all variants gave similar localization patterns to that of GFP protein, the low-risk HPV6 E6 protein, which lacked of efficient NLS (NLS1 and 2 of 16E6 protein) but contained only the motif similar to very weak NLS3 of HPV16 E6 protein, and could not enter the nucleus, was used as negative control. Results from 6E6 confirmed its localization only in the cytoplasm but not in the nucleus. The studies by Tao *et al*. in 2003 using fluorescence microscope showed that NLS1 was responsible for the accumulation of 16E6-I in the nucleus since decreased but not abolished signal was observed when amino acids in NLS1 were mutated [13]. However, no data on dose effect was clearly presented.

In summary, in order to identify amino acids necessary for nuclear localization of HPV16 E6-I protein, we have constructed eleven 16E6-I variants using the advantage of GFP fusion technology to produce GFP-HPV16 E6-I fusion proteins which could be monitored under the confocal microscope. We observed that all mutations in 16E6-I did not alter their localization patterns including mutations in the nuclear localization signal suggesting that other residues in E6-I protein might be required for its nuclear transport, or else E6-I might be carried by other nuclear proteins to the nucleus

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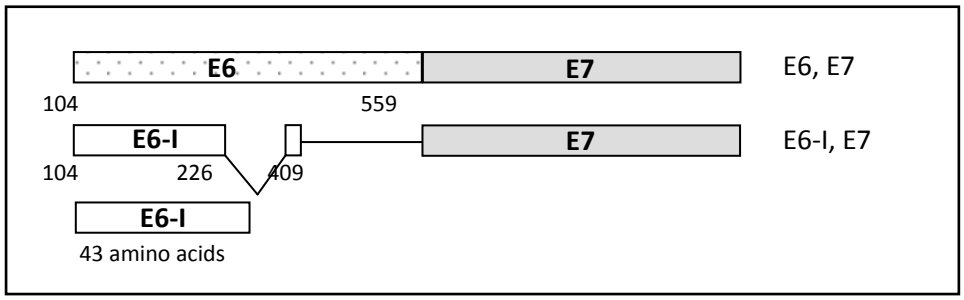

Figure 1: Schematic illustration of the HPV16 E6-I transcript, and its coding potential. HPV16 E6-I transcript was generated by using a splice donor site at nucleotide 226 and a splice acceptor site at nucleotide 409. Coding regions are indicated by boxes, excised intron fragments are indicated by thin lines.

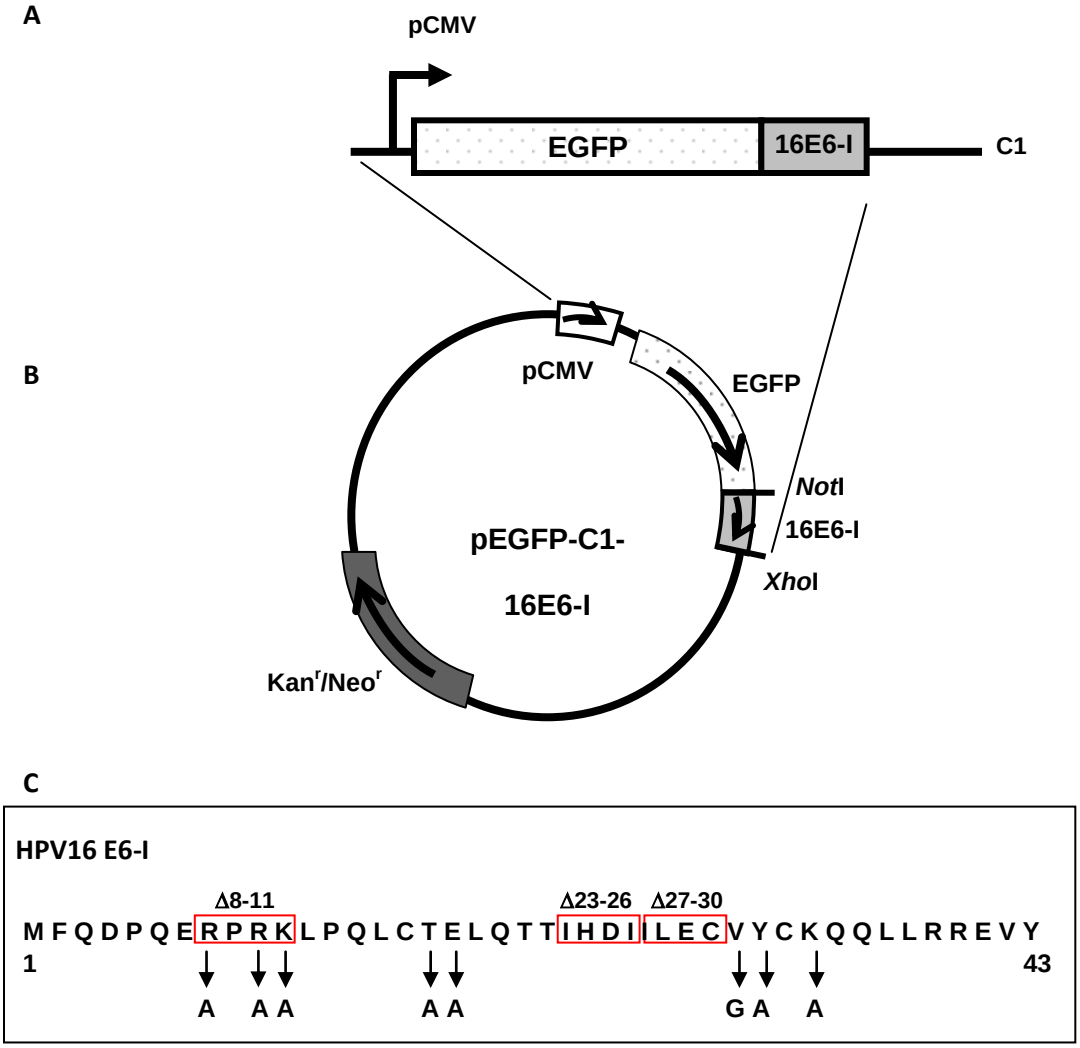

Figure 2: Diagram showing the 16E6-I coding region inserted downstream (C1) of the GFP gene (A) in the pEGFP expression vector (B). Schematic illustration depicting the positions of three deletions (Δ8-11, Δ23-26, Δ27-30) as indicated by boxes and eight point mutations in HPV16 spliced E6-I obtained from site-directed mutagenesis (R8A, R10A, K11A, T17A, E18A, V31G, Y32A, and K34A) as indicated by arrows.

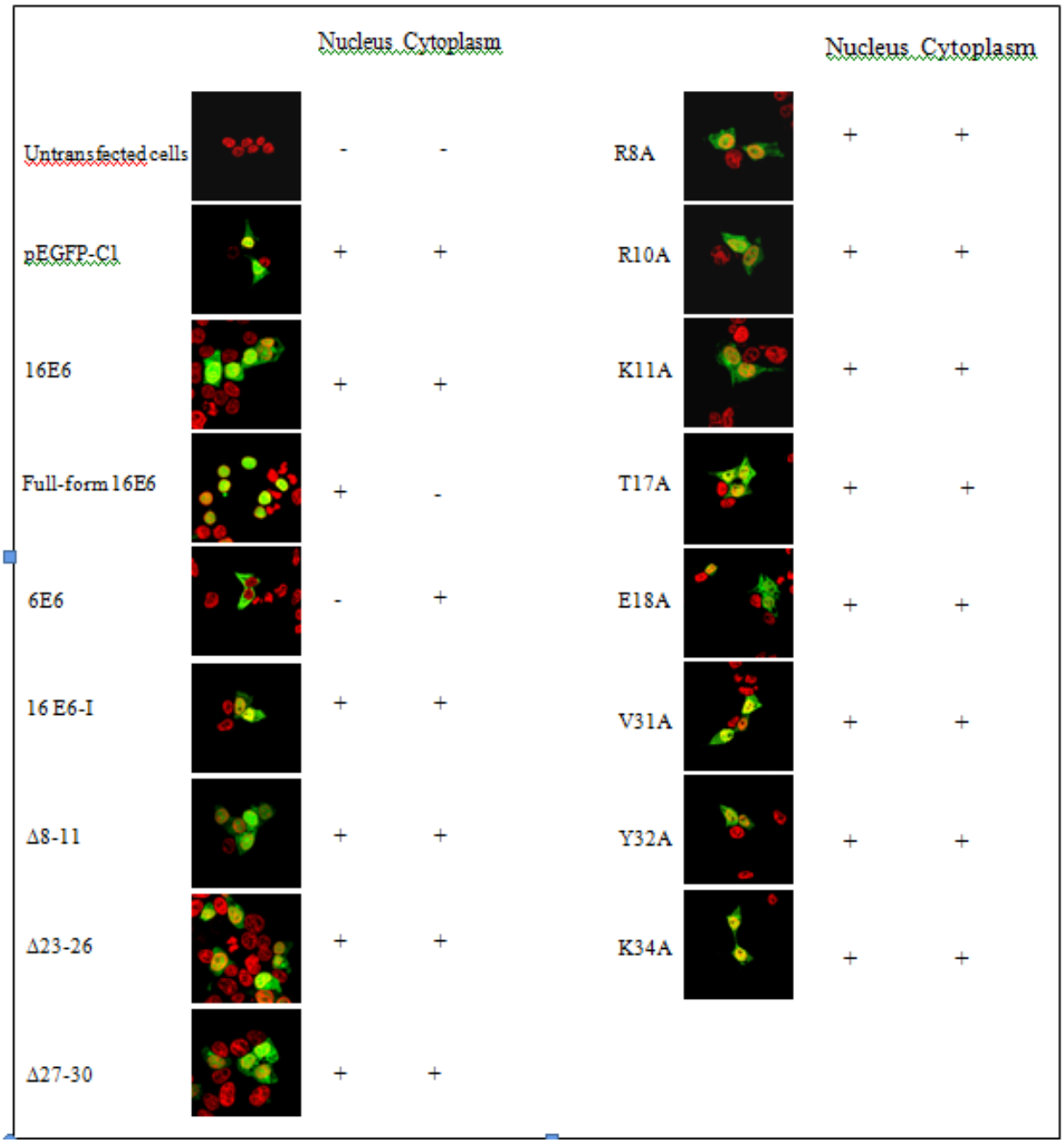

Figure 3: Cellular localization of HPV16 E6-I variants in 293T cells as monitored by confocal microscope after 24 h transfection. The fluorescence-TO-PRO®-3 merged images of 293T cells transiently transfected pEGFP-C1-16E6, pEGFP-C1-full-form16E6, pEGFP-C1-6E6, pEGFP-C1-16E6-I and pEGFP-C1-16E6-I variants (Δ8-11, Δ23-26, Δ27-30, R8A, R10A, K11A, T17A, E18A, V31G, Y32A, and K34A) are shown. The green signals show the fluorescence emission of GFP protein, the red signals show the nucleus by TO-PRO®-3 iodide dye, and the yellow signals show fluorescence emission of GFP protein in the nucleus. + or – indicates the presence or absence of nuclear or cytoplasmic localization of GFP fusion proteins. Plasmid pEGFP-C1 was used as a GFP control in transfection.

Table 1 Sequences of primers used for site-directed mutagenesis

Primer name	Sequences
Δ8-11 sense	5'-CAGGACCCACAGGAGTTACCACAGTTATGC-3'
Δ8-11 antisense	5'-CGATAACTGTGGTAACTCCTGTGGGTCCTG-3'
Δ23-26 sense	5'-CAGAGCTGCAAACAACTATATTAGAATGTGTGTACTGC-3'
Δ23-26 antisense	5'-GCAGTACACACATTCTAATATAGTTGTTTGCAGCTCTG-3'
Δ27-29 sense	5'-CAACTATACATGATATAGTGTACTGCAAGCAACAG-3'
Δ27-29 antisense	5'-CTGTTGCTTGCAGTACACTATATCATGTATAGTTG-3'
R8A sense	5'-GGACCCACAGGAGGCACCCAGAAAG-3'
R8A antisense	5'-GGGTGCCTCCTGTGGGTCCTGAAAC-3'
R10A sense	5'-GGAGCGACCCGCTAAGTTACCACAG-3'
R10A antisense	5'-TAACTTAGCGGGTCGCTCCTGTGGG-3'
K11A sense	5'-GAGCGACCCCGGGCCTTACCACAGTTATGC-3'
K11A antisense	5'-CTGTGGTAAGGCCCGGGGTCGCTCCTGTGG-3'
T17A sense	5'-AGTTATGCGCTGAGCTGCAAACAACT-3'
T17A antisense	5'-AGTTGTTTGCAGCTCAGCGCATAACT-3'
E18A sense	5'-GGAGCGACCCGCTAAGTTACCACAG-3'
E18A antisense	5'-TGTATAGTTGTTTGCAGCCCGCGTGCA-3'
V31G sense	5'-GATATAATATTAGAATGTGGGTACTGCAAGC-3'
V31G antisense	5'-GCTTGCAGTACCCACATTCTAATATTATATC-3'
Y32A sense	5'-TAGAATGTGTGGCATGCAAGCAAC-3'
Y32A antisense	5'-CTGTTGCTTGCATGCCACACATTC-3'
K34A sense	5'-GTGTGTACTGCGCGCAACAGTTACTG-3'
K34A antisense	5'-CAGTAACTGTTGCGCGCAGTACACAC-3'

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