Original Article

Natural HPV58 E6 and E7 Variants Detected in Thai Breast Cancer Patients Cooperate to Induce Loss of p53 and Increase Cell Growth

Wareerat Umnajvijit BSc[1], Varasiri Pitisuphanont MSc[1], Jariya Sangthong MSc[1], Chanitra Thuwajit MD, PhD[2], Mathurose Ponglikitmongkol PhD[1]

[1] Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand

[2] Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Background: Detection of human papillomavirus (HPV) in breast cancer patients has suggested a possible contributing role of the virus in cancer progression in this population.

Objective: To investigate the presence of HPVs in Thai breast cancer patients and examine the potential activities of HPVs identified in both breast and cervical cancer cells.

Materials and Methods: Fifty-five breast cancer tissues from Thai patients were subjected to HPV detection using PCR-EIA and DNA sequencing. Detection of HPV E6 proteins in sample tissues was examined by fluorescence immunohistochemistry. Cervical and two types of breast cancer cell lines expressing HPV oncogenes were established. The separate and combination of HPV oncoproteins activity for p53 degradation and specific gene regulation were investigated using western blot analysis and qPCR. Cell proliferation was assessed by MTT assay.

Results: Twenty-two percent (10/45) of invasive breast cancers were found infected with various high-risk HPV types, with HPV58 E6D4G/E7T20IG63S being the most common variant. The percentage of HPV58 alone was approximately 50% (5/10) of all HPV positive samples. Similar potential oncogenic activity for this variant was observed in breast and cervical cancer cells. A separate analysis of single or combination of 58E6 (prototype or E6D4G) with 58E7 (prototype or E7T20IG63S) demonstrated that co-expression of 58E7T20IG63S with 58E6 (either prototype or E6D4G) significantly promoted cell proliferation compared to prototype 58E6/E7. Enhanced proliferation was mediated through elevated p53 degradation and reduced p21 expression. While p53 degradation activity was greatly diminished from E6 with D4G mutation, co-expression with E7T20IG63S cooperated to enhance degradation of p53 and promoted cell growth.

Conclusion: HPV58 E6D4G/E7T20IG63S was the most HPV oncogene variant detected in Thai breast cancer patients. This variant exhibited in promoting cell proliferation and p53 degradation. A cooperative effect was observed in combination of HPV oncoproteins.

Keywords: Human papillomavirus type 58; oncogene variant; breast cancer; Thai patients; altered cell growth

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Human papillomavirus or HPV has been a well-known cause of cervical cancer. Among almost 200 types of HPVs discovered so far, approximately 20 types, based on the epidemiological data, are considered high risk HPVs and included HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73, and 82[(1)]. In Thailand, reports from the ICO HPV Information Center in 2015 revealed that HPV16 is most frequently detected in Thai cervical cancer patients followed by HPV18 and HPV58, respectively[(2)]. A previous meta-analysis on HPV prevalence from 29 studies including a pool of 2,211 breast carcinomas revealed an overall prevalence of HPV detection in breast cancers of 23%[(3)]. Since the presence of HPV in breast cancers varied among different populations, with the highest incidence (86%) in the USA[(4)] and no incidence in samples from Europe[(5-7)], it is of interest to study the presence of HPV genome in breast cancer in Thai women. While there has been a debate about the possible role of HPVs in other tissues besides the cervix[(8)], a potential role for HPV in some human breast cancers has been suggested in several studies[(9,10)]. Examples included histopathological analysis of HPV-infected breast

Correspondence to:

Ponglikitmongkol M.

Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

Phone: +662-201-5455

Email: mathurose.pon@mahidol.ac.th

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cancers in Australian patients. This study observed similar oncogenic characteristics to those seen in HPV-associated cervical cancer, as well as the detection of putative koilocytes, which is a characteristic of HPV-infected cells in breast cancer[9,11]. In a report by Yasmeen et al, it was demonstrated that HPV16 E6/E7 oncoproteins could upregulate Id-1 expression and promote invasion and migration of breast cancer cells both in vitro and in vivo[12]. These findings raised the possibility that HPVs may be involved in the pathogenesis of a subset of breast cancers.

Objective

In the present study, the authors investigated the presence of HPV in invasive breast cancer of Thai patients. The p53 degradation activity of the most commonly found variant of HPV58 in comparison with its prototype in breast and cervical cancer cells was also evaluated.

Materials and Methods

Samples

Fifty-five formalin-fixed and paraffin-embedded cancerous tissues consisting of 45 invasive ductal breast carcinomas, one from ductal carcinoma in situ (DCIS), and nine from benign breast tissues collected between 2013 and 2015 were obtained from Siriraj Hospital, Mahidol University. The present study was reviewed and approved by the Ethics Committee of Siriraj Hospital (Si662/2011).

DNA extraction and HPV typing

Each paraffin-embedded section was deparaffinized with xylene before genomic DNA extraction with QIAamp® DNA FFPE Tissue kit according to the manufacturer's instructions (QIAGEN). The quality of DNA was assured by positive amplification of β-globin sequences. HPV detection was first screened by polymerase chain reaction-enzyme immunoassay (PCR-EIA) method[13]. DNA sequencing of E6 and E7 oncogenes from HPV-positive clones was subsequently performed to confirm the HPV genotypes (Macrogen Inc.).

Cloning of HPV oncogenes and establishment of stable cell lines

E6 and E7 ORFs from HPV-positive samples were PCR-amplified using specific primers for each HPV type: 6E6F 5'-ATGCCTCCACGTCTGCAACG-3', 6E6R 5'-AATTCTAGGCAGCACGCGCA-3', 16E6F 5'-ATGTTTCAGGACCCACAGGAG-3', 16E6R 5'-GCTCTAGATTATGGAATCTTTGCTTTTTGTCC-3', 16E7F 5'-CTGATCTCTACTGTTATGAGC-3', 16E7R 5'-GGTTTCTGAGAACAGATGGGG-3', 31E6F 5'-GCGAATTCCTGTCCATACCGATGGCGCG-3', 31E6R 5'-GCCTCGAGCGCATATCTGATGTTATACTTGGG-3', 33E6F 5'-GCGGATCCGCAAACATTTTGCAGTAAGG-3', 33E6R 5'-GCCTCGAGCGTTTTTACACGTCACAGTGC-3', 39E6F 5'-GCGAATTCCTGTCCATACCGATGGCGCG-3', 39E6R 5'-GCCTCGAGCGCATATCTGATGTTATACTTGGG-3', 45E6F 5'-ATGGCGCGCTTTGACGATC-3', 45E6R 5'-AACATGTATTACACTGCCCTCGG-3', 58E6F 5'-AAGGATCCCTGCAGGACTATGTTCCAGG-3', 58E6R 5'-AAGAATTCGGTTGTTTCCTCTCATGGCG-3', 58E7F 5'-CGGGATCCCCTGTAACAACGCCATGAGAGG-3', 58E7R 5'-GCCTCGAGCCATTGCAGATGGTGTTTATTGC-3', 66E6F 5'-CTGGAATTCTCCATGGATTCCATATTCAGC-3', and 66E6R 5'-GTTCTCGAGTTACCATGCATGGTTATACT-3'. The same sets of primers were used to amplify E6 and E7 for mutation analysis. After obtaining identical DNA sequences (Macrogen Inc.) from at least two clones each, the sequences were compared with the reference prototype sequences available from the GenBank using ClustalW from http://www.genome.jp/tools/clustalw/ to verify variations. The PCR products were subcloned into the expression vector pcDNA3 (Invitrogen) and transfected into three different HPV-negative cell lines; C33A cervical carcinoma (ATCC:HTB31), MCF7 (ATCC:HTB22) and MDA-MB231 (ATCC:HTB26) breast ductal carcinomas to establish the stable lines expressing different viral oncoproteins: HPV16 E6/E7 prototype, HPV58 E6/E7 prototype, HPV58 E6D4G/E7, HPV58 E6/E7T20IG63S, HPV58 E6D4G/E7T20IG63S, HPV58 E6 prototype, HPV58 E6D4G, HPV58 E7 prototype and HPV58 E7T20IG63S. Both HPV16 E6/E7 and HPV58 E6/E7 prototypes were prepared by site-directed mutagenesis of the isolated clones based on the reference DNA sequences (HPV16: accession no. K02718.1 and HPV58: accession no. D90400.1). These cells were separately cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. After 24 h of incubation in 5% CO_2 at 37°C, cells were transfected with 1.5 µg of HPV oncogene expression plasmids using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's protocol and selection of stable cell lines were conducted as previously described[14].

Real-time reverse transcription (RT)-PCR

Total RNA was isolated using the Illustra™

Table 1. A summary of HPV detection and typing in breast tissues of Thai patients

Types of breast tissues	Percentage of HPV detection (no. of positive sample/total)	HPV type (no. of sample detected)
Invasive ductal carcinoma	22% (10/45)	HPV6 (1[a]), HPV16 (1), HPV31 (1), HPV33 (1[a]), HPV39 (1), HPV45 (1), HPV58 (5[a]), HPV66 (1)
Fibroadenoma	0% (0/7)	-
Harmatoma	0% (0/2)	-
Ductal carcinoma in situ	0% (0/1)	-

[a] Two samples of invasive ductal carcinomas contained mixed infection, one with HPV6 and HPV58 and the other with HPV33 and HPV58

RNAspin Mini kit (GE Healthcare) and converted into cDNA using SuperScript® III RNaseH RT kit (Invitrogen). Real-time PCR protocol was performed with THUNDERBIRD® SYBR® qPCR Mix (TOYOBO) as described previously[15]. The primers used for amplification of HPRT gene were HPRT-F 5'-TGTGATGAAGGAGATGGGAGG-3' and HPRT-R 5'-AAGCTTGCGACCTTGACCATCT-3'.

Western blot analysis

For protein detection, stable cells expressing different types of HPV oncogenes were lyzed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with Protease inhibitor cocktail (Amresco) and resolved by SDS-PAGE. After transferring, the polyvinylidene fluoride (PVDF) membrane was incubated with the specific primary antibodies at 4°C overnight, followed by HRP-conjugated secondary antibody for two hours at room temperature. The detection was performed with a Clarity™ Western ECL Substrate (Bio-Rad Laboratories) having GAPDH as a loading control. Details of antibodies used were sc-166574 (1:2,000) for GAPDH, sc-126 (1:1,000) for p53, sc-74562 (1:500) for pRb, AP124P (1:5,000) for HRP anti-mouse IgG and AP132P (1:5,000) for HRP anti-rabbit IgG.

Cell proliferation using MTT assay

Five-thousand cells in 100 µl of cultured medium were seeded into each well of 96-well plate in triplicate. MTT assays were performed after incubation at four different time points: 24, 48, 72 and 96 hours, as described previously[15]. Cell proliferation was assessed as the percentage of cell viability after normalizing MTT conversion against the control cells.

Fluorescence immunohistochemistry

The paraffin-embedded sections were heated at 70°C for two hours, deparaffinized with xylene, and rehydrated using the series of decreasing concentrations of ethanol and water, respectively. The antigen unmasking was performed in citrate buffer (pH 6.0) at 95°C for 5 minutes and endogenous peroxidase was blocked with 3% H_2O_2 for 10 minutes. Permeabilization of tissue sections were performed by immersing in TBST (TBS, 0.1% Triton X-100). After blocking in 3% BSA-TBST, staining was performed by incubating the sections with specific primary antibodies at 4°C overnight followed by Alexa Fluor 647 conjugated secondary antibody using Hoechst 33258 (1:1,000 dilution, Life Technologies) for nuclear staining. Finally, the sections were mounted with VECTASHIELD® (Vector Laboratories) and the fluorescent signals were detected by Olympus FV10i confocal microscope. Details of antibodies used were bs-10201R (1:2,000) for 58E6, sc-126 (1:100) for p53, A-21235 (1:400) for Alexa Fluor 647 goat-anti-mouse and A-21245 (1:400) for Alexa Fluor 647 goat-anti-rabbit.

Statistical analysis

Data were reported as mean ± standard error of mean (SEM) from at least three independent experiments. Statistical analysis of the data was performed using GraphPad Prism5, and significance of difference was examined with Tukey's multiple comparison test (one-way ANOVA) or Bonferroni posttests (two-way ANOVA). Confidence interval at 95% was considered statistically significant.

Results

HPV detection and genotyping

Eighteen percent (10 of 55) of samples from breast tissues of a Thai cohort were HPV-positive. However, this percentage was increased to 22% when only invasive ductal carcinomas were considered (45 samples). No HPV DNA was detected in seven fibroadenoma, two harmatomas, and one DCIS samples (Table 1). The major type of HPV detected was HPV58. Other HPV types were less often observed, and the following types were found in

Table 2. Variations of base and amino acid residues in E6 and E7 oncoproteins detected in HPV58-positive invasive ductal carcinomas

Invasive ductal carcinomas	Staging	HPV58 E6 variant: Base change* (amino acid change)	HPV58 E7 variant: Base change* (amino acid change)
BR1	IIB (T2N1M0)	A120G (D4G)	ND[a]
BR2	IIB (T2N1M0)	A120G (D4G)	C632T/G760A (T20I/G63S)
BR4	IA (T1N0M0)	A120G (D4G)	C632T/G760A (T20I/G63S)
BR9	IA (T1N0M0)	A120G (D4G)	C632T/G760A (T20I/G63S)
BR38	IV (T2N0M1)	A120G (D4G)	C632T/G760A (T20I/G63S)

[a] Not determined due to lack of DNA, * Position of base changes were based on +1 transcription start site

similar numbers of HPV6, HPV16, HPV31, HPV33, HPV39, HPV45 and HPV66 (Table 1). Most HPV types were present as single infections, except two samples that contained double infections, one between HPV6 and HPV58 and the other between HPV33 and HPV58. The most prevalent HPV type in cervical cancer in Asian populations as well as identified from breast cancers in the present study was HPV58. The authors amplified the E6 and E7 oncogenes from HPV58 and investigated their genetic polymorphisms. DNA sequencing results for the five HPV58 positive isolates revealed the same novel missense mutation compared to the reference prototype HPV58 (GenBank no. D90400.1). The HPV58 mutations were at residue 4 of E6, an aspartic acid to glycine (D4G) substitution, and a double mutation in E7 at residues 20 and 63, resulting in substitutions of threonine to isoleucine (T20I) and glycine to serine (G63S) (Table 2). It is interesting that the HPV58 E6D4G/E7T20IG63S variant was also identified in cervical cancer tissues of Thai patients (unpublished data).

Expression of HPV58 E6 protein in breast tissues

The authors examined whether HPV58 E6 genes were expressed in breast cancer tissues by performing fluorescence immunohistochemistry in four HPV58-positive and two HPV-negative invasive ductal carcinomas. All HPV-positive samples showed E6 staining, mostly in the nucleus with only minor signals in the cytoplasm, as shown in Figure 1A. Results in MDA-MB231 cells transfected with HPV58 E6/E7, used as positive control and pcDNA3 as a negative control, also confirmed the major nuclear localization of HPV58 E6. These results revealed both in vivo and in vitro expression of the HPV58 E6 proteins. The fluorescent signals of E6 quantified as percentages of the positive area in HPV58 positive invasive ductal carcinomas and MDA-MB231 expressing HPV58 E6/E7 cells versus their corresponding controls were significant ($p<0.001$ and $p<0.01$, respectively) (Figure 1B).

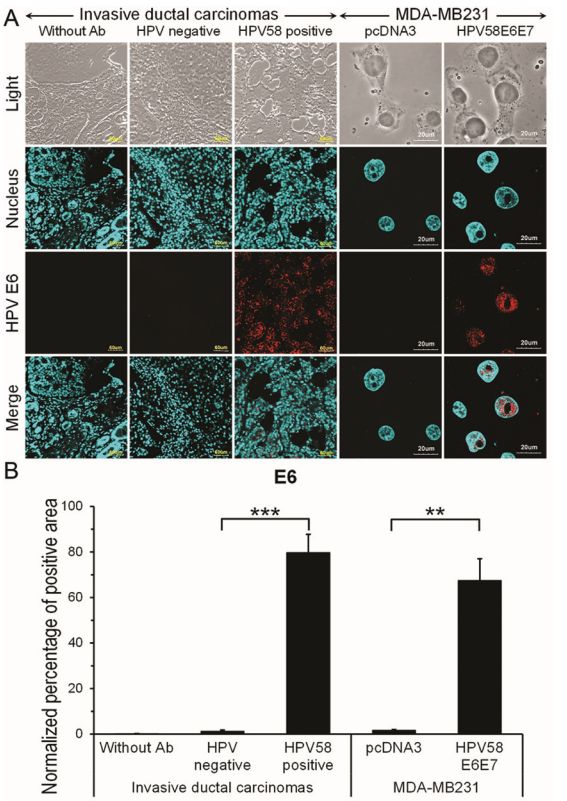

Figure 1. E6 protein expression in HPV58 positive sample as verified by immunohistochemical staining shown in A for picture and B for graph. (A) A representative picture of HPV58 positive invasive ductal carcinoma and the control MDA-MB231 cells stably expressing the HPV58E6E7 variant showed E6 staining mostly in the nucleus. Nuclei were stained by Hoechst. (B) A graph presenting normalized percentages of positive areas of both HPV58 positive invasive ductal carcinoma and MDA-MB231 cells expressing HPV58E6E7 is shown (** $p<0.01$, *** $p<0.001$).

The p53 protein degradation activity of HPV58 E6/E7 variant

To understand the roles of the HPV58 E6D4G/E7T20IG63S variant in cellular protein degradation, the authors investigated p53 degradation activity by comparing p53 degradation activities of HPV58 E6

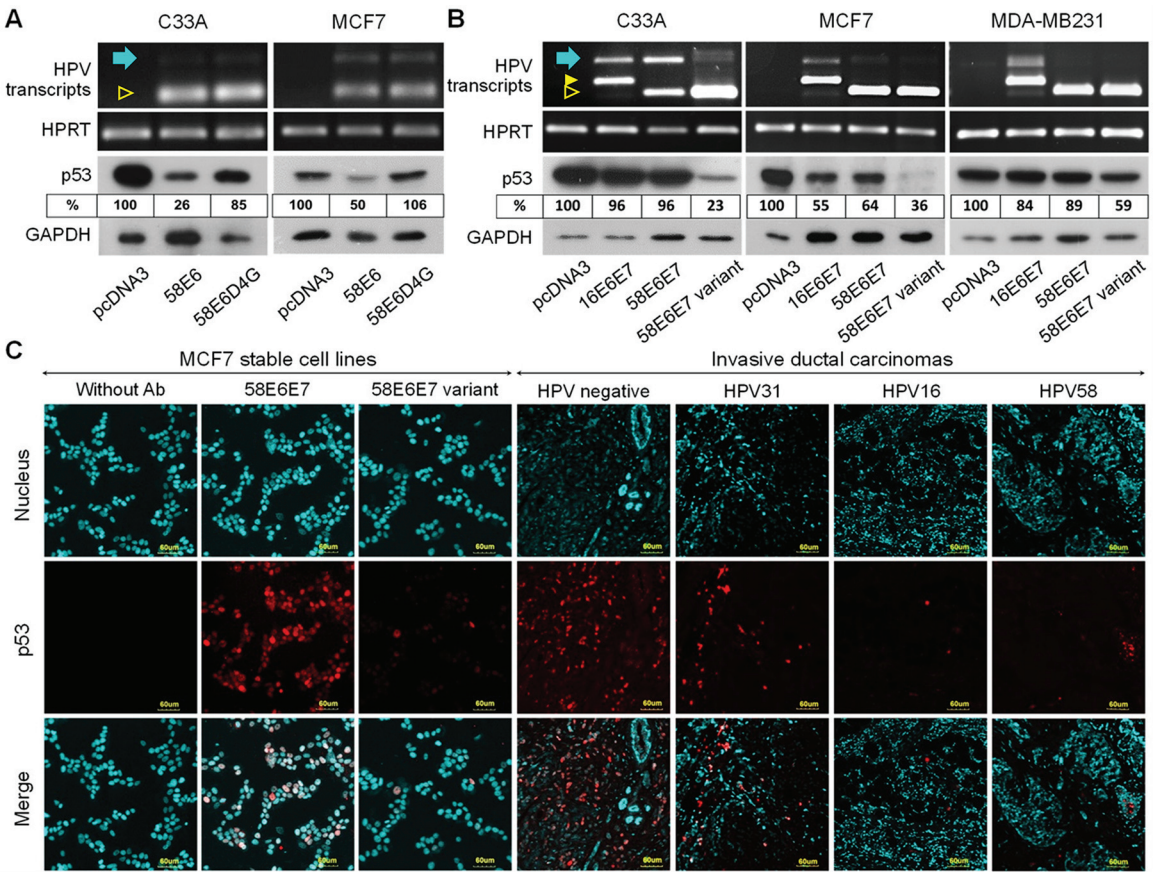

Figure 2. The p53 degradation activities of HPV58E6E7 oncoproteins as assessed by western blot analysis. The activity shown as% of control pcDNA3 (set as 100%) of (A) 58E6D4G was compared to 58E6 prototype in C33A and MCF7 cells and of (B) 58E6E7 variant as compared to prototype 16E6E7 and 58E6E7 stably expressing cells (C33A, MCF7 and MDA-MB231). GAPDH was a loading control. Expression of HPV E6/E7 transcripts with HPRT control in each cell was also indicated (the arrow for the unspliced transcripts, the filled arrow-head and unfilled arrow-head for HPV16 and 58 spliced transcripts, respectively). (C) The p53 levels in various HPV-infected clinical breast tissues and in MCF7 cells expressing either 58E6E7 prototype or variant were also demonstrated by fluorescence immunohistochemistry and analyzed as the normalized percentage of positive areas. Sample without HPV or antibody was used as a negative control.

proteins (prototype and D4G variant) in C33A and MCF7 cells stably expressing these oncoproteins by western blot analysis. The authors also observed that the D4G substitution in HPV58 E6 caused a significant decrease in p53 degradation activities (average% p53 shown in boxes) in both cervical and breast cancer cells (Figure 2A). Increased p53 levels were observed in cells expressing HPV58 E6/E7. However, the authors unexpectedly noted that cells expressing the HPV58 E6D4G/E7T20IG63S variant showed a remarkable loss of p53 (average% p53 shown in boxes) compared to prototype oncoproteins in all three cell lines, and this effect was more evident in C33A and MCF7 cells ($p<0.001$) (Figure 2B). Expression of HPV E6/E7 oncogenes was verified by RT-PCR and shown as HPV transcripts. All C33A, MCF-7 and MDA-MB231 cells were supposed to show both unspliced and spliced forms of HPV E6/E7 transcripts. However, the ratios for the spliced and unspliced forms were different in these cell lines. In both MCF-7 and MDA-MB231 cells, the spliced E6E7 transcripts were the major forms while the unspliced transcripts were hardly observed.

The effects of the HPV58 variant on p53 levels had been confirmed in MCF7 cells by immunocytochemical staining (Figure 2C). This analysis showed that signals for p53 were more apparent in cells with prototype HPV58 E6/E7 compared to those with the variant. The main subcellular localization of p53 was in the nucleus. Ductal carcinomas infected with different HPVs (HPV16, HPV31 and HPV58) exhibited low amounts

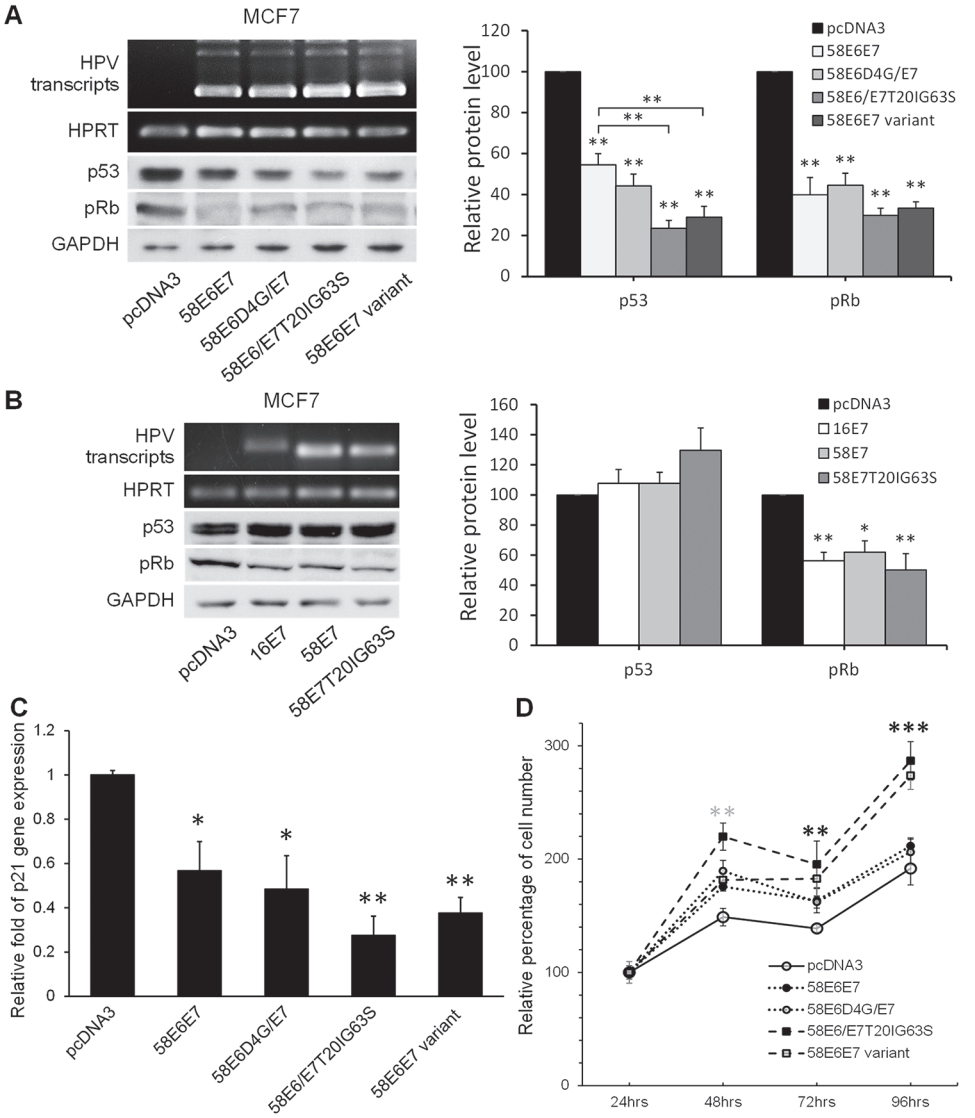

Figure 3. Effects of different combinations of HPV58E6 and E7 on cell cycle proteins. MCF7 cells stably expressing (A) combinations of E6 and E7 oncoproteins (58E6E7, 58E6D4G/E7, 58E6/E7T20IG63S, and 58E6E7 variant) and (B) of E7 alone (16E7, 58E7 and 58E7T20IG63S) were assessed for p53 and pRb levels using Western blot analysis. GAPDH was a loading control. Relative protein levels were presented in bar graphs as compared to those of pcDNA3 control set as 100%. Expression of HPV E6/E7 transcripts were monitored using HPRT as an internal control. MCF7 cells stably expressing different combinations of 58E6 and E7 were assessed for (C) p21 gene expression using real-time PCR and (D) cell proliferation using MTT assay (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

of p53 staining, with p53 signals rarely observed in HPV16- and HPV58-infected tissues, although p53 staining was more evident in HPV31-infected and HPV-negative tissues. These results indicated that E6 and E7 variants of HPV58 were involved in controlling p53 levels.

Effects of different combinations of HPV58 E6 and E7 on cell cycle proteins

To further explore the combined effects of E6 and E7, cells stably expressing different HPV58 oncogene variations were used to monitor p53, pRb and p21 levels. The present study selected to monitor the effects of E7 variants on p53 and pRb levels in MCF7 cells, which contain wild-type p53 (C33A and MDA-MB231 had mutant p53)[16,17]. Cells with E6/E7T20IG63S, representing a natural variant most often detected among HPV58 found in cervical cancer of Chinese population[18], and E6D4G/E7T20IG63S, a natural variant detected in the present study showed

much lower levels of p53 compared to control ($p<0.01$) (Figure 3A). It was possible that E7T20IG63S might play a role in controlling the cellular level of p53. However, the effect of this E7 variant was not seen when present in the absence of E6 while p53 levels in cells expressing E7 variant, E7 prototype and control pcDNA3 were not significantly different (Figure 3B). This strongly suggested that the interaction between E6 and E7 oncoproteins was important for controlling p53 levels. The pRb levels in cells with 58E7 alone, either the prototype or variant, were similar (Figure 3B). Expression of combinations of E6 and E7 clearly showed loss of pRb, compared to pcDNA3 control; however, the difference between cells containing E7 prototype and variant was subtle and not significant (Figure 3A). These results indicated that variations in HPV oncoproteins can confer specific effects on host proteins.

Since the loss of p53 might affect cell growth through transcriptional inhibition of its target gene p21, the authors also monitored the level of p21 transcripts as well as the growth rate in MCF7 cells containing four different combinations of HPV58 E6 and E7. As shown in Figure 3C, all cells expressing HPV58 E6/E7 showed significantly lower levels of p21 transcripts with the lowest being in E6/E7T20IG63S expressing cells. Consistent with this data, cells with E6/E7T20IG63S and E6D4G/E7T20IG63S displayed faster growth rates compared to cells containing prototype E7 either in combination with prototype E6 or E6D4G (Figure 3D). This indicated that the growth rate of MCF7 cells could be increased by HPV58 oncoproteins, and the increased rate depended on types of oncoprotein within cells. The growth advantage of E6/E7T20IG63S and E6D4G/E7T20IG63S expressing cells, both with low p53 levels, suggested that the E7 variant of HPV58 mainly affected host growth rate through modulation of cell cycle checkpoint proteins.

Discussion

The present study has shown for the first time the presence of HPV DNA sequences (~22%) in a set of invasive ductal carcinomas from Thai patients. In addition, the authors investigated the biological significance of the most commonly found variant of HPV58. Variations in all five HPV58 detected, relative to the HPV58 prototype (GenBank accession no. D90400.1) consisted of one amino acid change in E6 and two in E7. These HPV58 were designated as 58E6D4G/E7T20IG63S or simple HPV58 variant. The natural D4G 58E6 variant was first reported in the present study. The 58E7T20IG63S variant has been previously observed in Chinese cervical cancer patients and shown to be associated with the severity of cancer using epidemiological data[18]. The authors examined the protein degradation activities as well as the effects on cell proliferation of the HPV58 variant in comparison with those of prototypes for both breast and cervical cancer cells.

The tumor suppressor protein p53 acts primarily as a transcription factor involved in cell cycle control, apoptosis, and DNA repair processes. The level of p53 is normally regulated by the ubiquitin ligase MDM2[19]. In the presence of HPV oncoproteins, E6 promotes degradation whereas E7 stabilizes p53[20]. Promoting degradation of p53 is the most well-known role for high-risk HPV16 E6 proteins. HPV16 E6 interacts with p53 through amino acid residues from three sub-interfaces: sub-interface I in the N-terminus and α1 helix, sub-interface II mainly in α2 helix and sub-interface III at the C-terminus of E6, following binding with the E6AP protein[21]. However, mutations of amino acid residues at the N-terminus of E6 outside these sub-interfaces have also been shown to affect p53 degradation activity. These mutations included the artificially constructed D4G variation in 16E6, which showed loss of p53 binding and p53 degradation in vitro[22]. This result supports the finding that the natural occurring D4G variation identified in 58E6 disrupted its p53 protein degradation activity in both C33A cervical cancer and MCF7 breast cancer cells. The ability to degrade p53 of HPV E6 depends on various factors. Another important factor is the position and type of mutation in p53 protein. It has previously been demonstrated that mutant p53 proteins associate with HPV16 E6 could be targeted for degradation, whereas, those that cannot complex with E6 were not degraded[23]. Mutation at codon 273 (changing from Arg to Cys) of p53 protein detected in C33A cells was involved in transcriptional activity but not in E6 association.

Surprisingly, the authors found that cells expressing 58E6D4G when present together with 58E7T20IG63S showed a significant decrease in p53 protein levels comparing to control cells. The authors suggest that this E7 variant was involved in reducing p53 levels. However, this remarkable change in p53 levels was not observed in cells expressing E7 (either prototype or E7T20IG63S) in the absence of E6. To the contrary, the E7 protein alone from either HPV16 or 58 was previously shown to stabilize and increase total p53 protein levels, although the mechanism remains unclear[20,24]. However,

the increased p53 abundance did not equate with enhanced transcriptional activity[20], thus, the effect of E7 on p53 expression appears to be complex.

E6 and E7 are always expressed together; however, genes regulated by E7 can be distinct from those regulated when both E6 and E7 are present. It is of interest that much lower p53 levels were detected in the presence of both oncoproteins compared to when E7 was expressed alone. These results indicate cooperative control of p53 levels by E6 and E7. However, the authors observed that in the presence of both E6 and E7, cells with the 58E7T20IG63S variant had lower p53 abundance compared to cells expressing the 58E7 prototype, regardless of E6 type. This may indicate a higher oncogenicity for the 58E7T20IG63S variant compared to 58E7 prototype. Consistent with the present study results, the 58E6/E7T20IG63S variant has been found to be predominant (26.4% of all HPV58 isolates) in cervical cancer in Chinese patients and was shown to be associated with the severity of cancer (p_{trend}<0.001 by the exact test for trend)[18]. The significance of the T20I substitution in E7 has been proposed to be due to the proximity of T20 to the pRb binding site (L-X-C-X-G), which might affect pRb degradation. In contrast, the G63S substitution in E7 was postulated to create an additional phosphorylation site for casein kinase II[18]. The mechanism underlying 58E7-induced loss of p53 and whether this effect is specific to HPV58 needs further investigation.

As expected, low p53 levels in cells expressing E6 and E7 variants have been shown to be correlated with the decreased p21 expression. Cells expressing 58E6/E7T20IG63S and 58E6D4G/E7T20IG63S had very low p53 and p21 expression, although their growth rates were clearly higher than cells with the 58E6E7 prototype. This indicates that the T20IG63S variation in 58E7 might play an important role in promoting cell growth. HPV58 E7 has been shown to alter the cell cycle by down-regulating pRb and p130, thus extending the lifespan of primary human keratinocyte[24]. However, the present study could not detect a significant difference in pRb levels between E7 prototype and variant expressing cells. Therefore, other mechanisms for the E7T20IG63S variant in promoting cell growth, such as enhancing p53 degradation, cannot be excluded.

Consistent with these study findings, the cooperativity between HPV E6 and HPV E7 has previously been reported. HPV16 E6 was shown to cooperate with HPV16 E7 in mediating the degradation of the PDZ protein, NA+/H+ exchange regulatory factor 1 (NHERF-1). E6 is found to associate with NHERF-1 and promotes its degradation while E7 promotes accumulation of phosphorylated NHERF-1, which is a preferential form of E6[25]. In addition, these two HPV oncoproteins were also shown to cooperatively upregulate the expression of matrix metalloproteinases types 2 (MMP-2) and type 14 (MT1-MMP)[12] and to induce immortalization of human keratinocytes[26].

Conclusion

In summary, the naturally occurring E6D4G/E7T20IG63S oncogene variant of HPV58 is the predominant form found in breast cancer of Thai patients. Cells expressing this HPV58 variant exhibited a significant increase in cell proliferation. A cooperative effect was seen with co-expression of 58E6D4G and 58E7T20IG63S that resulted in an increased degradation of p53, lower p21 expression, and a higher growth rate compared to prototype oncoproteins.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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