Suppression of GADD45α transcription mediated by HPV16 oncoproteins and BRCA1 promotes breast cancer cell migration

Jariya Sangthong[1] Waraporn Komyod[2] and Mathurose Ponglikitmongkol[2,*]

[1] Molecular Medicine Graduate Program, Faculty of Science, Mahidol University, Bangkok, Thailand; jariya-oil0507@hotmail.com
[2] Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand; mathurose.pon@mahidol.ac.th
* Correspondence: mathurose.pon@mahidol.ac.th

Abstract: Breast cancer is the most common cancer in women worldwide. To date, many risk factors of breast cancer have been identified. BRCA1 is one of the most important proteins involved in breast cancer development. Interestingly, HPV DNAs have been increasingly detected in breast cancer specimens. However, the exact role of HPV implicated in breast cancer development is not well-examined. Growth arrest and DNA damage-inducible 45α (GADD45α) is known as a repressive gene involved in cell cycle regulation and found to be repressed in the expression in many types of cancers. In this study, we aimed to examine the effect of HPV oncoproteins and BRCA1 on GADD45α expression in breast cancer cells. MDA-MB-231 breast cancer cell line stably expressing HPV16 oncoproteins was generated. The expression of *GADD45α* and *in vitro* cell migration were analyzed in these cells. The effect of BRCA1 on *GADD45α* gene expression as determined by RT-qPCR revealed that *GADD45α* was significantly repressed in HPV16 oncoprotein expressing cells. Repression of *GADD45α* promoted cell migration in these cells as compared to cells without HPV oncoproteins. Moreover, restoration of *GADD45α* expression was observed in BRCA1 knocked-down cells in both cells with and without HPV16 oncoproteins. Thus, we demonstrated the involvement of BRCA1 in regulating *GADD45α* gene expression and that the suppression of *GADD45α* expression induced by HPV16 oncoproteins promotes breast cancer cell migration.

Keywords: HPV16; GADD45α; BRCA1; migration; breast cancer

1. Introduction

Breast cancer is the most common cancer in women worldwide. It is still a big health problem among women worldwide. In Thailand, it is the most common cancer and most leading cause of death among female cancers [1]. To date, no actual cause of breast cancer was identified. There are only risk factors have been reported. Many risk factors including genetic and non-genetic have been purposed to be associated with breast cancer development. Breast cancer susceptibility gene 1 (BRCA1) is a tumor suppressor and plays a vital role in maintenance of genomic stability. Defective of BRCA1 functions leads to involve with breast cancer development [2]. BRCA1 has three main functions in maintenance of genomic stability including DNA repair, cell cycle regulation, and regulation of gene transcription [3]. Interestingly, increasing detection of human papillomaviruses (HPVs), a causative agent of cervical cancer, in breast cancer specimens has been reported [4-7]. However, the precise role of HPVs in breast cancer development remains unclear. Growth arrest and DNA damage-inducible 45α (GADD45α) is known as a repressive gene involved in cell cycle arrest especially at G2/M phase transition [8]. It has been shown that the oncogenic stress was implicated in the function of GADD45α in breast cancer [9]. GADD45α has a negative role in cell cycle progression and it was found

to suppress the tumor growth by downregulating genes involved in tumor angiogenesis [10]. A previous study has shown that GADD45α expression was activated by BRCA1 following DNA damage [11]. Moreover, the induction of GADD45α expression was shown to be dependent on p53, a well-known target of high-risk HPVE6 oncoprotein. Thus, in this study, we aimed to determine the effect of HPV oncoproteins and BRCA1 on GADD45α gene expression in breast cancer cells.

2. Materials and Methods

Cell culture

The human breast cancer cell line MDA-MB-231 was cultured in Dulbecco's Modified Eagle Media (DMEM) (Gibco, USA) containing high D-glucose (4,500 mg/L), L-glutamine and sodium pyruvate supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), and 100 U/ml of penicillin and streptomycin antibiotics. Cells were grown in a humidified atmosphere containing 5% CO_2 at 37 ˚C incubator.

Generation of stable line expressing HPV16 oncogenes

MDA-MB-231 cells were seeded in 35 mm dishes and incubated for 24 h. Then cells were transfected with pcDNA3 expression plasmid containing with and without HPV16 oncogenes, *E6E7*. After 24 h of transfection, cells were subcultured and maintained in media containing 2 mg/mL Geneticin (G418, Gibco) as serial dilutions for selecting transfected cells. After 10 passages of establishment, stable lines were maintained in 1 mg/mL G418 and maintained in media without selective drug at least 1-2 passages prior to performing the experiments.

cDNA synthesis and reverse transcription (RT)-PCR (RT-PCR)

Total RNAs were extracted from stable lines by TRIzol reagent (Life Technologies) according to the manufacture's protocol. Then, cDNA was synthesized using reverse transcriptase based PCR method. The reaction contained SuperscriptIII reverse transcriptase enzyme (Invitrogen), RT buffer, Oligo-dT primer, and dNTP for RNA. The reaction was performed at the following condition; incubation at 42 ˚C for 1 h and 70 ˚C for 15 min. *HPV16E6E7* oncogene was detected in order to check the success of established stable lines by PCR. The primers used for amplifying *HPV16E6E7* oncogene were: forward 5′-ATGTTTCAGGACCCACAGGAGCG-3′ and reverse 5′-TGGTTTCTGAGAACAGATGGGGCAC-3′. PCR condition was as follows: pre-heating at 95 ˚C 10 min, denaturation at 95 ˚C for 30 sec, followed by annealing at 59 ˚C for 30 sec, and extension at 72 ˚C for 30 sec and reaction was done at 35 cycles. Determination of a house keeping gene, *ACTIN* was included in this experiment. The primers specific to *ACTIN* were: forward 5′-GACCTTCAACACCCCAGCCA-3′ and reverse 5′-AGGCTGGAAGAGTGCCTCAG-3′.

Reverse transcription quantitative real-time PCR (RT-qPCR)

The expression level of *GADD45α* gene was measured by RT-qPCR. Briefly, a total 10 μl reaction included 50 ng of cDNA template, GADD45α specific primers, and SYBR Green PCR mixture (TOYOBO). The PCR reaction condition was as follows: pre-heating at 95 ˚C 10 min, denaturation at 95 ˚C for 30 sec, followed by annealing at 59 ˚C for 30 sec, and extension at 72 ˚C for 30 sec and reaction was done at 40 cycles. The relative fold change of gene expression was calculated using the $2^{-\Delta\Delta}$ method. Actin was used as a house keeping gene for normalization. The primer sequences specific to GADD45α were 5′-GAGAGCAGAAGACCGAAAGGA-3′ and reverse 5′-CACAACACCACGTTATCGGG-3′.

Fluorescence immunocytochemistry

HPV16 oncoprotein (E6) expression in the stable lines was detected using fluorescence immunocytochemistry. Briefly, 50,000 cells of stable lines were seeded on sterile

cover slips (18 mm glass diameter). After 24 h of seeding, cells were gently washed and fixed with 4% paraformaldehyde for 30 min. Then cells were permeabilized using PBST (0.1% Triton X-100 in PBS). After that, cells were blocked non-specific binding using 3% BSA for 45 min. After blocking, cells were incubated with primary antibody specific to HPV16E6 protein (sc-460, Santa Cruz Biotechnology) at 4 ˚C for overnight. Then, cells were washed and incubated with Alexa Fluor 568 conjugated secondary antibody together with Hoechst 33258 for nuclear staining for 45 min at room temperature. Then, the stained cells on coverslips were mounted with anti-fade VECTASHIELD® (Vector Laboratories) and the fluorescent signals were detected by Olympus FV10i confocal microscope.

siRNA-mediated knockdown of BRCA1 gene expression

siRNA targeting *BRCA1* gene (siBRCA1) (CTM-452427) and scramble control (siCtrl) (D-001210-01) were purchased from Dharmacon. MDA-MB-231 cells were seeded into 35 mm dishes and incubated for 24 h. Then cells were transfected with either siCtrl or siBRCA1 using Lipofectamine™3000 reagent (Invitrogen) according to the manufacture's protocol. After 48 h of transfection, cells were harvested for further analysis.

Western blot analysis

To determine the levels of BRCA1 protein after knockdown experiment, western blot analysis was performed. Briefly, transfected cells were lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.25% sodium deoxycholate, 1%(v/v) NP-40, and 1 mM DTT) containing a cocktail of protease inhibitor (Amresco). Total 40 μg of protein was subjected onto 6.5% separating gel (SDS-PAGE) and then transferred to PVDF membranes (Amersham™ Hybond™) using wet transfer method. Then membranes were blocked and incubated with primary antibodies specific to either BRCA1 (sc-6954, Santa Cruz Biotechnology) or GAPDH (sc-166574, Santa Cruz Biotechnology) protein at dilution 1:250, and 1:2,000, respectively. The signal of proteins was developed by incubating with ECL substrate (ClarityTM Western ECL Substrate (Bio-Rad Laboratories) and detected gel documentation (gel doc) system (G:BOX, SYNGENE).

In vitro migration assay

MDA-MB-231 ($2x10^4$ cells) stable lines were seeded onto the upper chamber of polycarbonate Transwell® inserts (6.5 mm membrane diameter, 8 μM pore size) (Corning) and incubated for 24 h. Cells were subsequently harvested for migration assay. Briefly, the migrated cells were washed and fixed by 4% paraformaldehyde for 40 min followed by staining with 0.5% crystal violet for 1 h. Then stained cells were destained with distilled water until a clear background was observed. The migrated cells were then counted under the light microscope.

3. Results

3.1 Generation of MDA-MB-231 breast cancer cells stably expressing HPV16 oncoproteins

3.1.1 The expression of HPV16 oncogene transcripts in the established stable lines

MDA-MB-231 cells stably expressing HPV16 oncogenes were generated by transfection of plasmid containing *HPV16E6E7* oncogene using empty vector (pcDNA3) as a control. The expression of *HPV16E6E7* oncogenes was evaluated prior to performing further experiments. By RT-PCR using primers specific to *HPV16E6E7* oncogenes, the result showed the faint upper band which corresponded to the full-length *E6E7* gene (752 bp) and the thick lower band which was the spliced form of *E6E7* gene, namely *E6*E7* (616 bp). No *HPV16E6E7* oncogene transcript was observed in MDA-MB-231 cells expressing pcDNA3 empty vector [Figure 1].

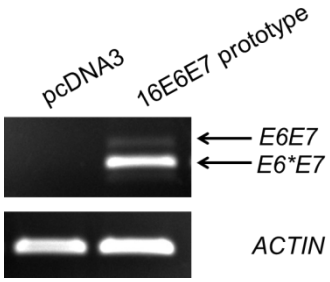

Figure 1. The expression of HPV16E6E7 oncogene in the established MDA-MB-231 stable lines. HPV16E6E7 oncogene transcripts were determined by RT-PCR. The bicistronic transcripts of E6E7 oncogenes are indicated by arrows, the full form transcript, E6E7 and the spilced form transcript, E6*E7. Cells expressing pcDNA3 empty vector was used as a negative control. Actin was used as a housekeeping gene.

3.1.2 The expression of HPV16 oncoproteins in the established stable lines

HPV E6 and E7 oncoproteins are naturally transcribed from bicistronic mRNA, we therefore determined the expression of E6 protein as a representative. The expression of HPV16E6 protein in stable lines was detected by fluorescent immunocytochemistry. The result showed E6 protein expression in MDA-MB-231 cells stably expressing HPV16 oncogene. No E6 protein staining was observed in cells expressing pcDNA3 empty vector [Figure 2]. This result confirmed the expression of HPV16 oncoproteins in the stable lines.

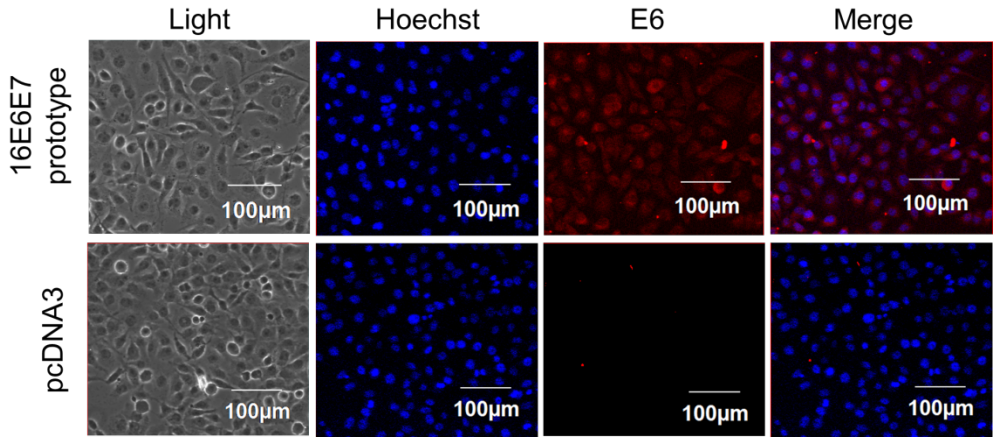

Figure 2. The expression of HPV16E6 oncoprotein in stable cell lines. MDA-MB-231 cells stably expressing HPV16E6 oncoprotein were determined by fluorescent immunocytochemistry. The first panel shows the image under the light microscope, the second panel shows nuclear staining with Hoechst dye (blue), the third panel shows E6 protein staining cells (red), and the last panel shows the merge images of nucleus and E6 staining.

3.2 GADD45α expression is repressed in MDA-MB-231 cells expressing HPV16E6E7

To determine the expression of GADD45α in MDA-MB-231 cells with and without HPV16E6E7, RT-qPCR was performed. As shown in Figure 3, GADD45α gene expression was significantly decreased in HPV16E6E7 expressing cells compared to pcDNA3 control cells. This result indicated that in the presence of HPV16E6E7 expression, GADD45α gene expression was significantly repressed.

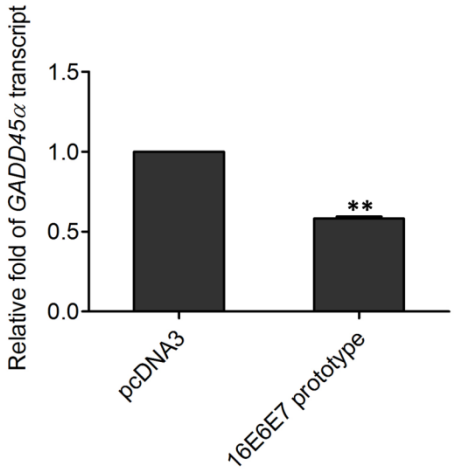

Figure 3. The effect of HPV16 oncoproteins on GADD45α gene expression. GADD45α gene expression in MDA-MB-231 cells stably expressing HPV16E6E7 was determined by qRT-PCR. The data is presented as the relative fold of GADD45α transcript normalized to housekeeping gene, *ACTIN* in relative to the control cells (pcDNA3). The data are shown as mean ± SEM from three independent experiments. ***P;*<0.01 compared to cells expressing pcDNA3 empty vector.

3.4 Downregulation of GADD45α expression promotes MDA-MB-231 cells migration induced by HPV16E6E7

To validate the role of GADD45α in growth suppression, *in vitro* migration assay was performed. The result showed that pcDNA3-control cells with higher GADD45α expression levels showed lower ability of cell migration when compared to HPV16E6E7 expressing cells [Figure 4a,b]. The numbers of migrated cells per field were counted and presented in the bar graph [Figure 4c]. This result revealed that HPV16 oncoproteins-induced cell migration was mediated by repression of GADD45α expression.

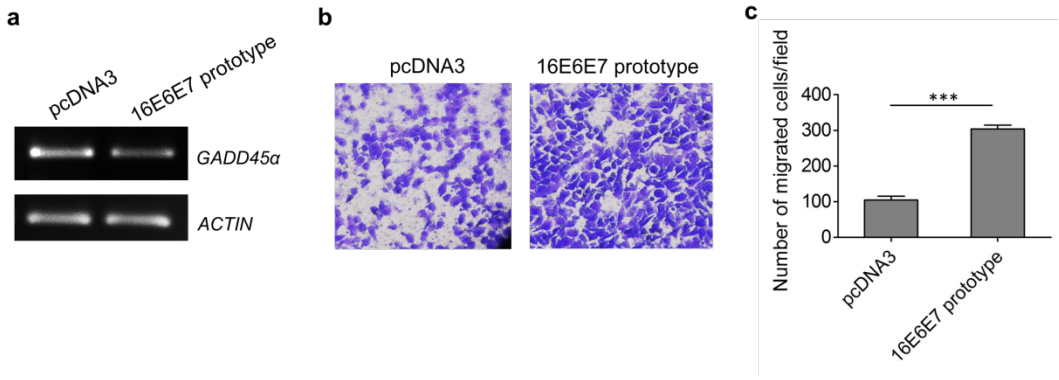

Figure 4. The Effect of GADD45α expression on the migration of breast cancer cells expressing HPV16 oncoproteins. The migration of MDA-MB-231 cells with and without HPV16E6E7 was determined by *in vitro* migration assay. (**a**) *GADD45α* gene expression in MDA-MB-231 cells stably expressing HPV16E6E7 was determined by RT-PCR (**b**) The representative images of cell migration assay of pcDNA3 control and HPV16E6E7 expressing cells are shown. (**c**) The number of migrated cells per field was counted and presented in the bar graph with mean ± SEM from three independent experiments. ***P;*<0.001 compared to cells expressing pcDNA3 empty vector.

3.3 Knockdown of BRCA1 increases GADD45α expression in breast cancer cells

To investigate the effect of BRCA1 on GADD45α gene expression in breast cancer cells with and without HPV16E6E7, siRNA-mediated BRCA1 knockdown (siBRCA1) was performed. The result showed that BRCA1 protein level was significantly decreased in cells transfected with siBRCA1 compared to the scramble control (siCtrl)-transfected cells [Figure 5a]. Then GADD45α gene expression in breast cancer cells with normal (siCtrl) and low (siBRCA1) levels of BRCA1 was determined by RT-qPCR. As shown in Figure 5b, GADD45α gene expression was increased (about 1.5-fold) in BRCA1 knocked down cells compared to that in siCtrl transfected cells regardless of the presence of HPV16E6E7. This result revealed that BRCA1 affected GADD45α by mediating repression of GADD45α expression.

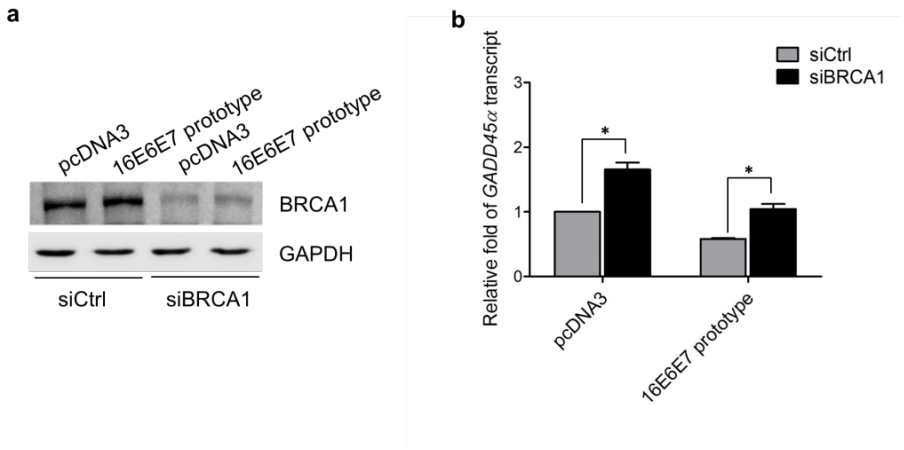

Figure 5. The effect BRCA1 on GADD45α gene expression. (**a**) The BRCA1 protein levels after siRNAs transfection was determined by western blot analysis. (**b**) The expression of GADD45α transcripts was determined by RT-qPCR and the relative fold of gene transcripts was normalized to a house keeping gene, actin, in relative to cells expressing pcDNA3 empty vector with normal BRCA1 level (siCtrl). The data are shown as a mean ±S.E.M. from three independent experiments. **P*;<0.05 compared to those of siCtrl-transfected.

4. Discussion

Deregulation of genes involved in cell cycle control leading to promotion of cell cycle progression is the major role of HPV-induced cell proliferation. GADD45α is a repressive gene (tumor suppressor gene??) that functions in cell cycle inhibition at G2/M phase [8]. In this study, we determined the effect of HPV16 oncoproteins on GADD45α gene expression and found that GADD45α was downregulated in breast cancer cells with HPV16 oncoprotein expression, suggesting the role of HPV16 oncoproteins in repression of GADD45α. Moreover, we found that downregulation of GADD45α-mediated by HPV16 oncoproteins was associated with migration ability of breast cancer cells. In consistent with a previous report, GADD45α has been shown to decrease migration and invasion ability of HepG2 cells and downregulation of GADD45α could increase migration and invasion ability of these cells [12]. Therefore, reducing GADD45α expression may associate with cancer progressiveness.

We also investigated the effect of BRCA1 on GADD45α expression and found that BRCA1 affected GADD45α expression by repressing of GADD45α expression. This result revealed the involvement of BRCA1 in controlling GADD45α level in HPV16 oncoproteins expressing cells. In contrast to our results, previous reports showed that transactivation of GADD45α expression was induced by BRCA1 [11, 13]. However, other evidence demonstrated that BRCA1 could either activate or suppress GADD45α expression

depending on the interaction with transcription factors and their binding elements [14-15]. Whether the BRCA1 knockdown affects cell migration in these HPV16E6E7 expressing cells remains to be further investigated. The limitation of this study includes the validation of this finding in breast cancer tissues to elucidate the real clinical features.

5. Conclusions

In this study, we demonstrated the effect of HPV16 oncoproteins on GADD45α expression in breast cancer cells as well as the involvement of BRCA1 in this activity. We showed that GADD45α was downregulated in HPV16 oncoproteins expressing cells and this suppression required BRCA1. Our study also revealed that suppression of GADD45α expression was associated with breast cancer cell migration.

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