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Research Article

Down Regulation of Phosphatidylinositol Glycan Class S (PIGS) by siRNA Sensitizes HeLa Cells to UV and H_2O_2

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Abstract

Our research group had recently found that an endoplasmic reticulum membrane protein, phosphatidylinositol glycan class S (PIGS), interacts with a protein involved in DNA repair, DNA polymerase beta (pol) (using yeast 2 hybrid system), suggesting a role of PIGS in base excision repair pathway. As an essential component of glycosylphosphatidylinositol (GPI) transamidase, PIGS helps many eukaryotic proteins to anchor on the cell surface. In the absence of PIGS, the carbonyl intermediate of GPI is not generated, and GPI biosynthesis remains incomplete. In order to further our understanding of the function of PIGS, we knocked down the gene by using siRNA technology in HeLa cells. The knocked down cell lines were tested for their sensitivity to ultraviolet (UV) radiation and hydrogen peroxide (H_2O_2) toxicity. The proliferation activity was compared by wound-healing assay. Apoptosis was studied by DNA fragmentation assay, colony forming assay, and caspase assay. Wound healing assay result indicates remarkably higher (1.5-2.7 times) proliferation rate of PIGS knocked down cells. The results from the sensitivity test to UV and H_2O_2, assessed using DNA fragmentation assay, show that PIGS siRNA cells are more sensitive than the control cells. Apoptosis of these sensitive cells is mediated by the release of cytochrome C in the cytosolic fraction followed by activation of caspase 9, 3 respectively. Therefore, in the future, RNAi technology may be used in the therapy of cervical cancer by depleting PIGS and then treating the patients with UV and H_2O_2.

Keywords: Caspase 3; Caspase 9; PIG-S gene; SiRNA; UV; H_2O_2

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1. Introduction

Cervical cancer is one of the most common cancers among women around the world. According to the estimates of the American Cancer Society, 12,170 new cases of cervical cancer are expected to be diagnosed in the United States in 2012, and more than 1/3rd of these diagnoses are expected to be fatal [1]. It was estimated that more than 270,000 women succumbed to this disease worldwide in 2010 [2]. The treatment of cervical cancer involves surgery followed by radiation and/or chemotherapy, depending upon the stage of the disease.

Glycosylphosphatidylinositol (GPI), a glycolipid that can be attached to the C-terminus of proteins, helps many eukaryotic proteins to be anchored to the cell surface. The mammalian GPI transamidase is a complex of at least four subunits, GPI anchor transamidase (GPI8), Glycosylphosphatidylinositol anchor attachment 1 protein (GAA1), PIG-S, and GPI transamidase component PIG-T (PIG-T). GPI is synthesized by sequential additions of sugars and ethanolamine phosphates to phosphatidylinositol in the endoplasmic reticulum (ER) [3, 4]. PIGS is essential component of GPI transamidase and, in the absence of PIGS, the carbonyl intermediate would not be generated. However, lack of GAA1 or PIGS may indirectly affect the signal recognition by causing a conformational change of the protein complex. Our group recently found that PIGS interacts with a dominant negative mutant of a DNA repair protein, DNA polymerase beta (polβ). In order to further understand the role of PIGS, in the present study, we down-regulated PIGS in HeLa cells by using siRNA technology. HeLa cell is a cervical cell line that was originated from a cervical cancer patient. The sensitivity towards UV and H_2O_2 treatment was measured in this report by using different methodologies.

2. Materials and methods

2.1 Preparation of PIG-S siRNA constructs

Two reverse complementary pairs of SiRNA–PIGs oligos were made (PIGS1FP: 5’-GATCCGGCAGAAGCCATGTTAGATTTCAAGAGAATCAAACATGGCTTCTGCCTCTTTTTTGGAAA-3’; PIGS1RP: 5’- AGCTTTTCCAAAAAAGAGGCAGAAGCCATGTTAGATTGT CTTCAAATCTAACATGGCTTCTGCCG-3’;PIGS2FP:5’GATCCGGACATGATGAGCTACA TTGTCAACAGAAAAGTAGCTCATCATGTCCTGTTTTTGGAAA-3’; PIGS2RP: 5’–AGCT TTTCCAAAAAACAGGACATGATGAGCTACTTTTCTGTTGACAATGTAGCTCATCATGTCCG-3’).

The two pairs of oligos were annealed as per the instruction of the supplier and ligated into Psilencer 4.1-CMV hygro kit expression vector (Ambion). Ligated products were transformed into E.coli competent cells and DNA was purified. Plasmids from antibiotic positive clones with the siRNA template inserts were purified and the sequenced with the primers suggested by the supplier. The GAPDH control insert containing plasmid, and pSilencer negative control plasmids were also made for this study.

2.2 Cell culture, Transfection and preparation of stable PIG-S siRNA cell line, Chemical treatment of cells

HeLa cell line was purchased from NCCS, India. Monolayer cells were grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% FCS, 100 U/ml of penicillin and streptomycin (100 μg/ml). The cells were cultured at 37°C in 5% CO2 atmosphere and 95% air humidity. Pure plasmid DNAs were transfected into HeLa cells using siPORT XP-1 transfection agent (Ambion). After 24 hours

positive transformants were selected by using hygromycin (200 μg/ml) by culturing for another month to make stable cell lines. This cell line has been named PIGS-siRNA. The cells were treated with UV and H_2O_2 for the desired time period for desired time.

2.3 Confirmation of down regulation of PIGS gene by RT-PCR and western blot analysis

2.3.1 RT-PCR

RNAs were isolated from PIG-S siRNA transfected cells using Trisure (BIOLINE USA Inc, MA) following the manufacturers instruction. RNAs isolated from transfected cell lines were reverse-transcribed to produce first strand cDNA synthesis and then PCR amplified using PIG-S specific primers using Bioscript (Bioline). PCR reaction was performed in total volume of 25 μl with 0.25 mM of each appropriate primer, in the presence of Tris-Cl pH 8.7, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$ (Fermentas, 10X PCR buffer), 0.25 mM of dATP, dCTP, dGTP, dTTP (Invitrogen) and 1 unit of DNA polymerase (Bioline) along with 50ng of template DNA. The initial denaturing step at 94°C for 2 min followed by 30 cycles consisting of a denaturing step for 30 sec at 94°C, an annealing temperature of 30 sec at 50°C and an extension step for 2 min at 72°C, with a final extension for at 72 ℃ for 5 mins. Following PCR amplification, the DNA products were electrophoresed on a 1.2 % agarose gel.

2.3.2 Western blot analysis

After treatments, total cell lysates were isolated from PIG-S siRNA cells and control cell lines by using lysis buffer (10mM Tris-Cl pH 8, 1mM EDTA, 400mM NaCl, 2mM β mercaptoethanol, 1mM PMSF, and protease inhibitors). Proteins (50μg) were denatured in Laemmli buffer (60mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-ME, 0.01% bromophenol blue). The proteins were separated on 12.5 % SDS-PAGE gel, transferred to nitrocellulose membrane. The antibody for PIGS gene was used to detect PIGs protein (Santacruz-cat# SC-54980; 1:500). Donkey antigoat IgG HRP conjugate was used as secondary antibody (SC-2020; 1:10,000).

2.4 Wound healing assay

Cells were seeded in 6 well plates. Confluent dishes were used to make scratch by using a sterile pipette tip (p10) [5]. Plates were then rinsed with PBS and grown in complete medium for 0 hr, 6 hrs, 12 hrs and 24 hrs. Images of the plates were captured by the Leica inverted microscope fitted with camera.

2.5 Determination of cell viability (MTT assay)

Around 104 cells/well were plated onto 96-well plates one day before treatment. The confluent cells were treated with UV (10-25J/m^2) and H_2O_2 (12.5-100 μM). Cell growth and cytotoxicity were tested using MTT [6, 7]. MTT was added to each well at a final concentration of 0.5mg/ ml after washing twice with PBS and incubated for 3 hrs at 37ºC. DMSO (100 μl/well) was added to dissolve the formazan crystal, and absorbance was measured at 550 nm. Each experiment was repeated three times. Rate of survival was calculated as following this formula Cytotoxicity (%) = (1-OD of treated cells / OD of control cells) x 100.

2.6 DNA fragmentation assay

To assess the fragmentation of cellular DNA into the characteristic apoptotic ladder, PIG-S SiRNA cells ($5x10^5$/100 mm dish) were seeded and treated with UV, H_2O_2 at 10-25 J/m^2 and 12.5 to 100 μM respectively and pelleted at 6, 12, 18 and 24 h post treatment incubation. Then cells were lysed in ice-cold cell lysis buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 0.5% Triton X-100 [8]. The lysed cells were centrifuged at 7000xg for 10 min at 4°C. The supernatant was added with 1% SDS, RNAse A (final conc. 5μg/μl) and proteinase K (final conc. 5 μg/ μl) and incubated at 56°C for 2 hrs. The DNA was precipitated with 2.5 vol ethanol after addition of ½ vol of 10M ammonium acetate, dried and dissolved in TE buffer. Then the DNAs were separated by 1.8% agarose gel electrophoresis. HeLa cells were

used as control in this experiment.

2.7 Colony forming assay

Approximately 400 cells were seeded in a 35mm dishes and treated with UV at 5-15 J/m^2 and with H_2O_2 at a conc. of 500 μM for 0.5 – 6 hrs. After treatment, the cells were washed with PBS and fresh medium was added. After 15 days, cells were stained with crystal violet. Colonies with more than 50 cells were counted.

2.8 Caspase 3, 9, and cytochrome c Assay

After treatments with UV (10-25 J/m^2) and H_2O_2 (0.5 -6 hrs; 0.5 mM), cells lysates were prepared by using lysis buffer (20mM Tris-HCl pH 8, 150mM NaCl, 1mM of EDTA, 1mM EGTA and Protease inhibitor). Proteins (50μg) were denatured with Laemmli buffer and electrophoretically separated on 12.5% gel SDS-PAGE and transferred onto nitrocellulose membrane.

The cytosolic and mitochondrial fractions were separated for cytochrome c assay. Cells were harvested after the treatment as stated above, washed with ice-cold PBS and sonicated in buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 175 mM sucrose and 12.5 mM EDTA) [9]. Cell extracts were centrifuged at 1000xg for 10 min to pellet nuclei. The supernatant was collected and centrifuged at 18,000xg for 30 min to pellet the mitochondria. The resulting supernatant was termed as the cytosolic fraction. The mitochondria were purified as described [9]. The pellet was lysed and the cytochrome c content was measured in cytosolic and mitochondrial fractions by Western blot analysis [10].The proteins (50μg) were denatured, and separated on 14 % SDS-PAGE and transferred to nitrocellulose membrane.

Nitrocellulose membrane was pre-incubated with 5% fat free milk in TBST for 1hr. Then incubated in primary antibody of caspase 3, caspase 9 (Cell signaling cat#9668, #9505; dilution 1:1000) for overnight as per manufacturer's instruction. Then membrane was rinsed with TBST and incubated afterwards with HRP conjugated secondary antibody (Bio-Rad; cat#170-6516, Caspase3-Goat antimouse IgG; 1:1000; Cell signaling, Caspase 9, #7074; 1:1000 conc.) for 1hr. After washing with 0.1% Tween 20, cleaved products were visualized by using Super Signal west pico (Pierce, IL).

3. Results

3.1 Confirmation of down regulation of PIG-S by RT-PCR and western blot analysis

PIG-S gene was knocked down by siRNA technology in HeLa cells (Figure 1). Both RT-PCR and Western blot analysis showed that PIGS gene has been knocked down. Panel A of Figure 1 shows that PIGS gene is expressed only in HeLa cells but not in PIGS-siRNA transfected cell line. β-actin was used as control for RT-PCR. Panel B of Figure 1 shows the non-expression of PIGS protein in two types of PIGS-siRNA stable cell lines. HeLa was used as control showing the expression of wild type PIGS protein. Lower panel shows the equal expression of actin gene.

3.2 Wound healing assay

In this study, HeLa cells show collective migration pattern. This typical type of migration is manifested by cell-cell contact, assembly and disassembly of cell matrix, etc. On the other hand, the PIGS-siRNA cell line showed less attachment to the surface. Therefore, we examined whether PIGS depletion in HeLa cell affects the migration behavior by this assay. The migration of cells at different time points are shown and compared with parental HeLa cell line (Figure 1C). The PIGS-siRNA transfected HeLa cells showed faster proliferation rate compared to HeLa cells (~1.5-2.7 times faster) (Figure 1C&D). In 6, 12, and 24 hrs, the PIGS-siRNA cells migrated 25.5, 42.4, and 98% only whereas 17.2, 22.6, and 35.5% HeLa cells migrated at the same time period suggesting a faster proliferation rate in this cell line.

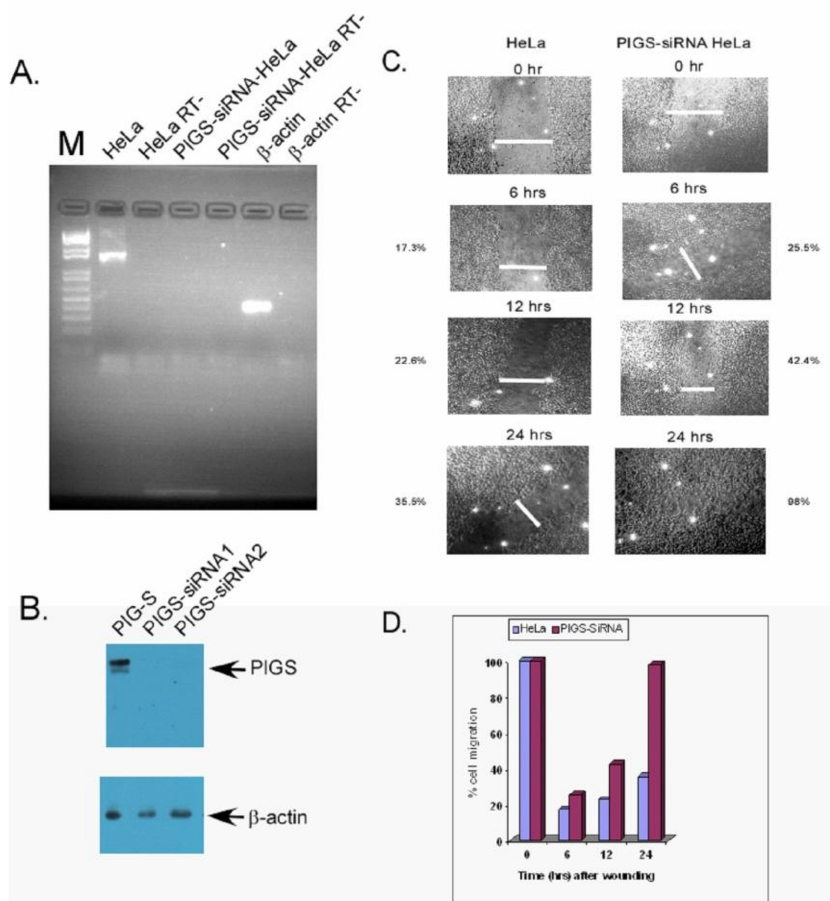

Figure 1 Analyses of expression of PIGS and wound healing assay: A&B: Study of PIGS mRNA and protein after transfection of HeLa cells by PIGS-siRNA. A. RT-PCR analysis of mRNA isolated from HeLa and PIGS-siRNA cells. B. Western blot analysis of protein lysates isolated from HeLa and PIGS-siRNA cells. C&D. Wound healing assay. C. HeLa and PIGS-siRNA cells were scratched and pictures were taken after 6, 12, 24 hrs. D. The results were plotted in graph.

3.3 Determination of cell viability (MTT assay)

Cytotoxicity of PIGS-siRNA cells were determined and compared with the control HeLa cells and vector DNA transfected HeLa cells (data not shown) by the MTT assay. Treatment of PIGS-siRNA cells with UV (5-50 J/m^2) resulted in the decrease of cell survival %. Treatment of this cell line with 15 J/m^2 resulted in the 90% cell death compared to 52% cell death noticed in HeLa control cells (Figure 2A). The results show that the PIGS-siRNA cells were more sensitive to UV irradiation than parental HeLa cell line (Figure 2A). Similar results were obtained when PIGS-siRNA cells were treated with H_2O_2 (0.01-1 mM of H_2O_2). The results show that almost 50% HeLa cells died after treatment with 0.5mM H_2O_2 is 6 hrs. Therefore, both HeLa and PIGS-siRNA cells were treated with different concentrations of H_2O_2 for 6 hrs. Treatment resulted in the death of 23, 47, 93 % cells at concentrations of 10 µM, 100 µM, and 1mM whereas the HeLa cells exhibited 9, 18, 71 cell death at the same concentrations of H_2O_2 (Figure 2B). These data indicate that down-regulation of PIGS could remarkably increase the sensitivity of HeLa cells towards UV and H_2O_2.

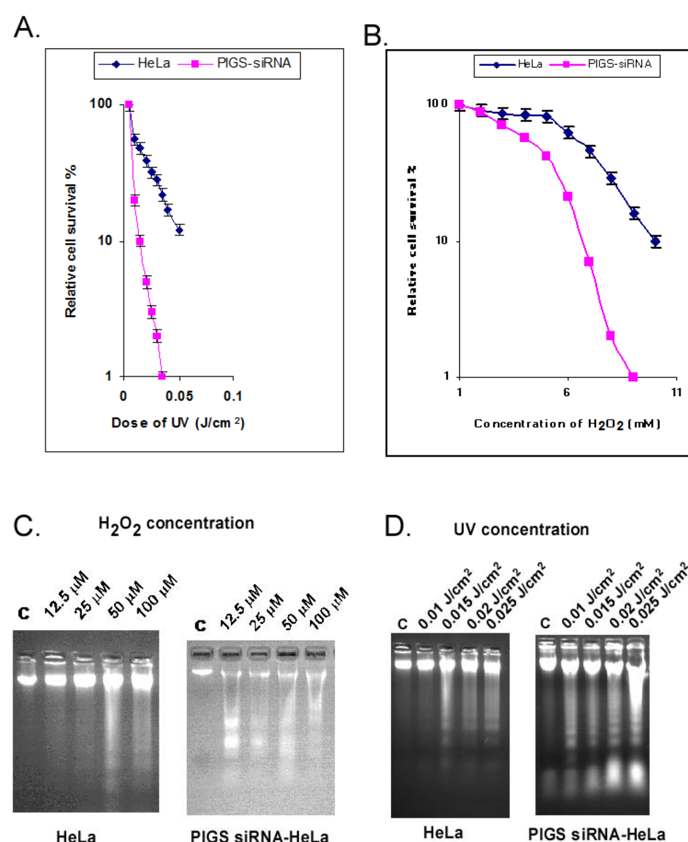

Figure 2 MTT and DNA fragmentation assay. A. MTT assay after treatment of PIGS-siRNA and HeLa cells to UV. B. MTT assay after treatment of cells to H_2O_2. C&D. DNA fragmentation assay after treatment of PIGS-siRNA and HeLa cells to H_2O_2 and UV, respectively.

3.4 DNA fragmentation assay

HeLa and PIGS-siRNA cells were treated with different concentrations (12.5-100 μM) of H_2O_2. DNA fragmentation was detected as small bands in a laddering pattern in ethidium bromide stained gel. The results show that at even 12.5 μM concentration of H_2O_2, the DNA fragmentation is prominent in PIGS-siRNA cells whereas the fragmentation was detected in 50 μM H_2O_2 treated HeLa cells (Figure 2C). In case of UV treatment, at the dose of 10 J/m^2, the DNA laddering was prominent in PIGS-siRNA cells (Figure 2D).

3.5 Colony forming assay

We tested whether exposure to UV and H_2O_2 would increase the sensitivity using colony forming assay. Figure 3B depicts the survival curves for HeLa and PIGS-siRNA cells treated with UV over a range of 1-5 J/m^2. Figure 3C represents the survival graph for HeLa and PIGS-siRNA cells treated with H_2O_2 for different time period (1-4 hrs). Figure 3A represents the results of colony forming assay.

3.6 Caspase 3, 9 activation and cytochrome c Assay

Earlier evidence demonstrated that mitochondria participate in the execution of apoptosis by the release of cytochrome C [11]. Binding of cytochrome C to Apaf-1 results in the cleavage of procaspase 9, which in turn activates caspase 3 [12]. In order to determine whether caspases are activated after cytochrome c release we measured the changes in caspase 9 and 3 activity in HeLa and PIGS-siRNA cells after H_2O_2 treatment. In the present study we noticed UV and H_2O_2 induced the activation of both caspase 3 and caspase 9 in both HeLa and PIGS-siRNA cells, but the activation rate is higher in PIGS-siRNA cells compared to HeLa cells. As shown in Figure 4A (upper panel), the activation of caspase 3 occurred at the dose of 25 J/m^2 in HeLa cell, whereas the same activation was detected in

PIGS-siRNA cells at 15 J/m^2 dose indicating the activation is an early event (Figure 4A, lower panel). UV induced activation of caspase 9 is also earlier event in PIGS-siRNA (10 J/m^2; Figure 4B, lower panel) than in HeLa cells (25 J/m^2, Figure 4B, upper panel). Both caspase 3 and 9 are activated at 12 hrs of H_2O_2 treatment (Figure 4 C&D, upper panel) whereas in case of PIGS-siRNA, the activation occurs as early as 6 hrs (Figure 4C&D, lower panel). Therefore this result suggests that hydrogen peroxide and UV are inducing apoptosis in both the cells by the mitochondrial pathway.

Cytochrome c release from mitochondria is a critical step in the apoptotic cascade as this activates downstream caspases. To examine the release of cytochrome c in H_2O_2/UV treated cells in the cytoplasm, we conducted western blot analyses in the cytosolic fractions only. These experiments demonstrate a consistent increase in cytochrome c in cytosolic fraction after treatment with hydrogen peroxide and UV (Figure 4 E). It has also been noticed that the amount of cytochrome c released in PIGS-siRNA cells within 30 mins are comparatively higher that in HeLa cells. Equal loading control is shown in the lower panel of each cell line.

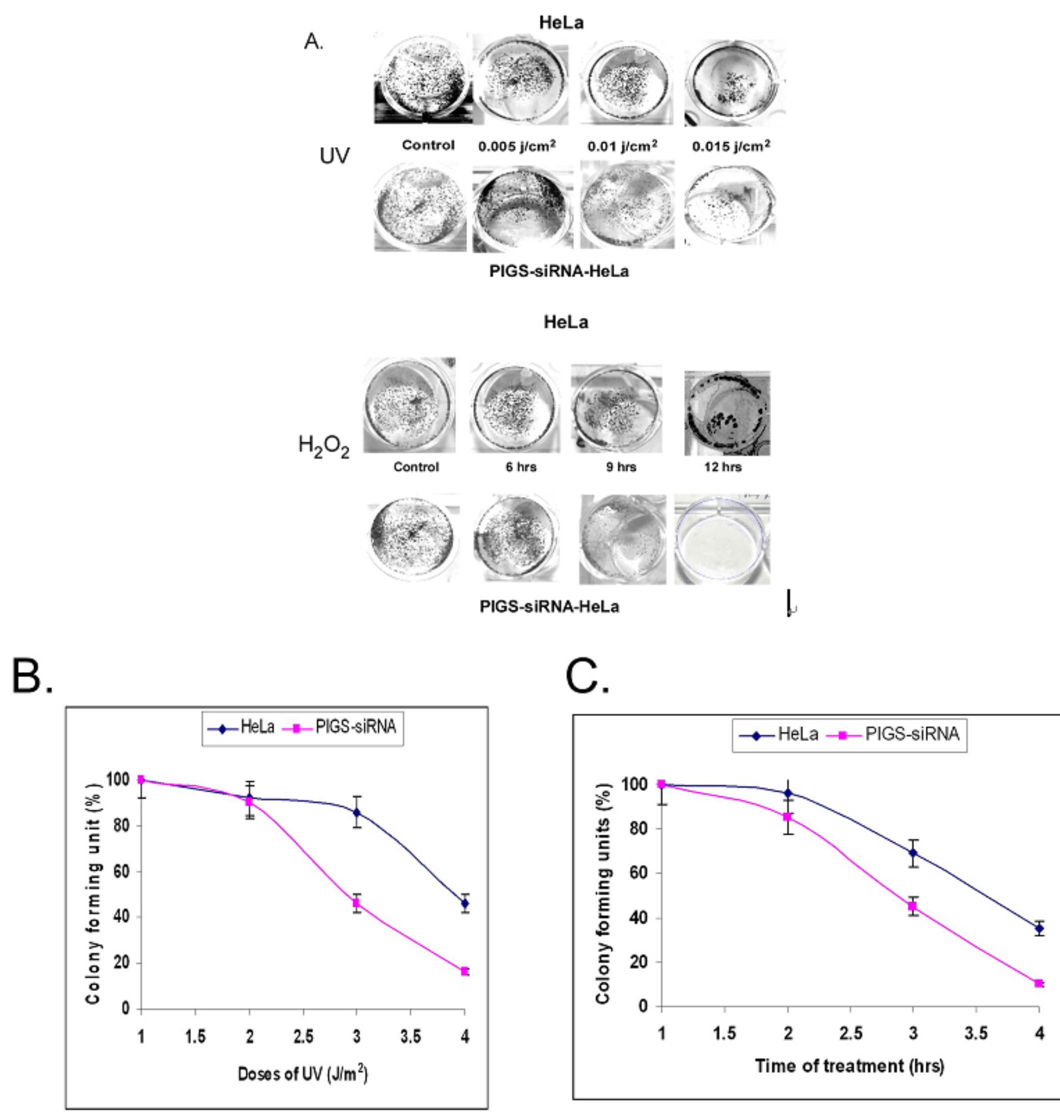

Figure 3 Colony forming assay. A. Cells treated with UV and H_2O_2. B. Graphical representation of the result after the cells were treated with UV. C. Graphical presentation of result after the cells were treated with H_2O_2.

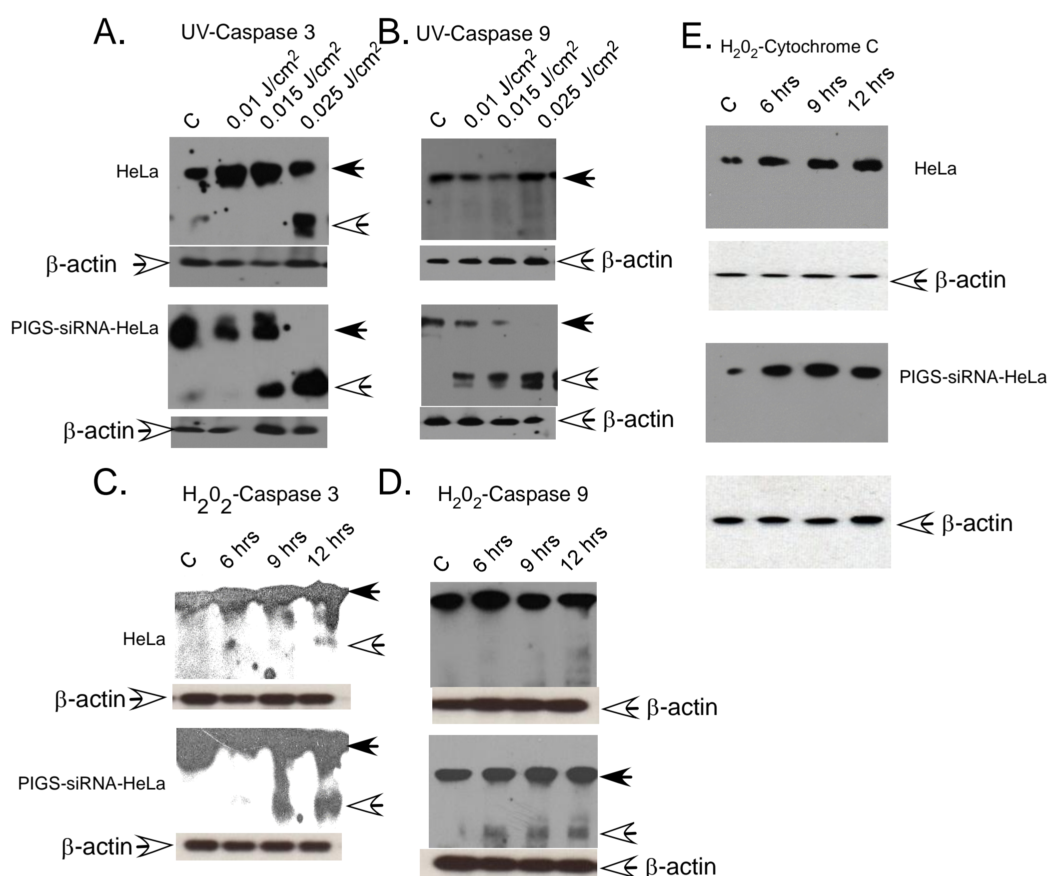

Figure 4 Caspase 3, 9 activation and cytochrome c Assay in PIGS depleted cell line. A&C: Caspase 3 activation in PIGS-siRNA cells. B&D: Caspase 9 activation in PIGS-siRNA cells. E. Cytochrome c assay: Cytosolic fraction showed the increment of cytochrome c in the cytosolic fraction and decrease of cytochrome c in the mitochondrial fraction. β actin was used as loading control.

4. Discussion

In India cervical cancer is the third largest cause of cancer mortality after cancers of the mouth & oropharynx, and oesophagus, accounting for nearly 10% of all cancer related deaths in the country [13]. Despite recent advances in the diagnosis and treatment of cervical cancer, it is still the most common cancer among women of most developing countries, constituting 34% of all women's cancers. The rate of mortality is higher in low and middle income countries [14]. Despite the fact that more than 80% of cervical cancer cases are in developing countries, only 5% of women there have ever been screened for cervical abnormalities [15]. Hence, the treatment after diagnosis is the real key for the women to survive. Therefore, in this study we tried to unveil a mechanism by which a cervical cell line, HeLa is being sensitized by the suppression of a gene, PIGS, to UV and H_2O_2.

The result in the Figure 1C (12, 24 hrs-PIGS-siRNA) indicates that the migrating PIGS-siRNA cells were detached from each other and had migrated out from the surface. Thus this finding suggests that PIGS is necessary for collective migration of HeLa cell. Therefore, PIGS seems to play an important role in cell-cell adhesion and coordinated control of cell matrix during migration of cells in HeLa cell.

The MTT assay results have shown that the PIGS-siRNA cells are more sensitive to UV as well as H_2O_2 (Figure 2A&B). In this study, the UVB-induced PIGS-siRNA cells have showed an enhanced DNA

fragmentation into mono- and oligonucleosomes than the HeLa cells. DNA laddering of PIGS-siRNA cells was apparent at 10 J/m^2 UVB irradiation (Figure 2D), no DNA laddering was visible at that concentration of UV in case of HeLa cells (Figure 2C).

Colony forming assay was performed to confirm the viability of the cells after treatment with H_2O_2 and UV. It was noted that inhibition of PIGS by siRNA didn't alter the colony forming ability in the absence of UV or H_2O_2 but did enhance the suppressive effect of UV/H_2O_2 on colony-forming ability of HeLa cells (Figure 3).

Several studies have shown that at the early stage after UV exposure, production of ROS occurs in irradiated tissues [16-19], and accumulation of ROS within UVB-treated keratinocytes may promote cell death [20]. Similarly hydrogen peroxide induced apoptosis has also been widely studied. The major effect of H_2O_2 is the generation of reactive oxygen species (ROS), which is also an important event for many chemotherapeutic agents. In most of the reports, ROS mediated apoptosis is linked to the intrinsic pathway of apoptosis [21]. The first step for the ROS mediated apoptosis is the activation of Bax, which aggregates into mitochondria and results in the release of cytochrome c. Once released into cytosol during apoptosis cytochrome c binds to Apaf thus forming a complex called apoptosome which recruits and activates procaspase 9 [11, 22]. Then caspase 9 cleaves inactive procaspase 3 to active caspase 3, thereby providing a link between the mitochondria and UV, H_2O_2 induced apoptosis in PIGS-siRNA cells.

We thus examined the effects of H_2O_2 and UV on the processing of caspase 9 and caspase 3, which are directly activated by caspase 9 [23, 24]. The activation of caspase 9 became apparent after 1/2 hour elapsed time (coinciding with the timing of profound cytochrome c release) and progressed at least up to 6 hours (Figure 4D) after H_2O_2 treatment. The most effective is the processing of the effector caspase 3 within 0.5 hr to 3 hrs. The activation of caspase 9 started at 10 J/m^2 and then the processing of caspase 3 starts at 15 J/m^2 of treatment (Fig 4A, B).

In summary, we clearly demonstrated that depletion of PIGS by siRNA technology makes HeLa cells susceptible to chemotherapeutic agent like H_2O_2, or radiation by UVB. The RNAi caused better suppression of PIGS and increased significantly more chemo and radio-sensitivities. This would represent a clinical significance if translated into clinical setting because resistance to radiation or chemo during the relapse of the disease could be potentially overcome by this approach. Hence hopefully in the near future, strategies based on RNAi will be ready for preclinical or clinical trials.

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