

Effect of CD59-targeted multi-directional intervention on transmembrane apoptotic signal transduction of cervical cancer HeLa cells

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Summary

Objective: With GPI-anchored protein CD59 as the target, the effect of the abnormal expression of the CD59 gene on the proliferation and apoptosis of cervical cancer HeLa cells was studied to investigate the mechanism of the malignant proliferation of cervical cancer cells. **Materials and Methods:** Recombinant plasmid pSUPER-siCD59 (CD59 RNA interference) was transfected into HeLa cells via liposome transfection. The plasmid expressed green fluorescent proteins that enabled the observation of the transfection efficiency and was separately transfected. The co-transfection of a pIRES-WTCD59 (wild-type CD59) plasmid, pIRES-MCD59 (with CD59W40 active site mutation) plasmid, and pLeGFP plasmid helped the direct observation of the transfection efficiency, which was examined via an inverted fluorescence microscope at 24, 48, and 72 hours after transfection. Then, the stable transfected cell lines were screened by G418. Western blot, RT-PCR, and immunofluorescence were used to detect the gene and protein expression of CD59 in the stably transfected HeLa cell lines, and the differences were evaluated. The short peptide seal specific to CD59 was exerted on the HeLa cells. Cell proliferation was detected by MTT colorimetric assay, and apoptosis was detected by TUNEL and flow cytometry. **Results:** The proliferation activity of the HeLa cells with CD59RNAi, CD59W40 active site mutations, and the short peptide seal specific to CD59 significantly decreased. In particular, the proliferation activity of the HeLa cells treated with a high dose of the short peptide seal specific to CD59 was significantly reduced, while the proliferation activity of the HeLa cells with a high expression of CD59 was significantly enhanced. **Conclusion:** As an inhibitory regulatory protein in the terminal stage of complement regulation, CD59 can promote the proliferation of cervical cancer HeLa cells via inhibiting the dissolution effect of the complement on the cells, promoting transmembrane activation signal transduction, and inhibiting apoptotic signal transduction. Thus, this study provides a new avenue for the clinical treatment of cervical cancer.

Key words: CD59 knockout; Short-peptide seal; RNAi; HeLa cell; Proliferation; Apoptosis.

Introduction

A complement is a tightly regulated system of proteins that protects hosts from infection by microorganisms or tumor growth. Complement-mediated immune responses act in the assembly of membrane attack complexes (MACs) at the cell membrane, forming a pore that leads to osmotic lysis [1, 2]. A MAC is formed by the self-assembly of C5b, C6, C7, C8, and multiple C9 molecules. Therefore, to limit damage to host tissues, it is essential to strictly control the activation. First identified as a regulator of the complement terminal, CD59 acts by binding to the C8/C9 components, thus preventing the formation of a MAC to interfere with C9 membrane insertion, polymerization, and pore formation [3-5]. The precursor of human CD59 is a single peptide composed of 128 amino acids deduced from its cDNA sequence [6, 7]. The mature protein consists of 77 amino acids arranged in a single cysteine-rich domain composed of two antiparallel β -sheets, five protruding surface loops, and a short helix [8-10].

The CD59-anchored membrane protein is a complement regulatory protein that plays a regulatory role in the assembly of the complement MAC. It prevents the formation of MAC by binding with C8 and C9, thereby inhibiting the dissolution effect of its complement to histocytes [11-13]. Studies have revealed that CD59 is highly expressed in various tumor cells, e.g., colorectal, ovarian, and prostate cancer cells, playing an important role in escaping from the tumor cells' immune surveillance [14-16]. Its expression has also been implicated in tumorigenesis and the provision of cancer cells with protection from monoclonal antibody immunotherapy [17-19]. Thus, knowledge of the CD59 binding interface may also assist with the rational design of molecules that inhibit CD59 function and enhance cancer cells' susceptibility to antibody therapy. Previous studies on the CD59 binding interface have indicated that residues 1-41 inhibit MAC formation much more strongly than 42-77 [20], and its C8 (C5b-8) and/or C9 (C5b-9) binding site is located in the vicinity of a hydrophobic groove on the membrane distal face of the protein centered around residue

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Table 1. — Comparison of sequence between mutant CD59 and wild type CD59.

Items	37	38	39	40	41	42	43	44	45	46
Human CD59	N	K	C	W	K	F	E	H	C	N
M1	N	K	C		K	F	E	H	C	N
	N	K	W	W	W	F	E	H	C	N

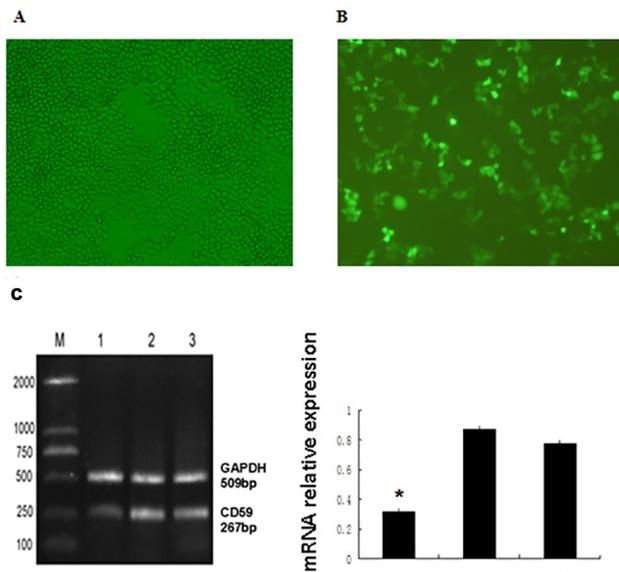


Figure 3. — Expression of GFP effected by plasmid transfection of CD59-siRNA; expression of CD59mRNA effected by recombinant plasmid transfection of pSUPER-siCD59 (1: the pSUPER-siCD59 group; 2: the normal group; 3: the pSUPER group).

siCD59 plasmid, empty pSUPER plasmid, and pLeGFP plasmid), the cells were observed under a fluorescence microscope, and the positive rate of the green fluorescent protein was calculated.

When the cells were in the logarithmic growth phase, the medium was sucked and discarded, the cells were rinsed with PBS three times, and TRIzol lysis solution was added. The cells were scraped down with a scraper, mixed with the TRIzol lysis solution, and left standing at room temperature for five minutes. Then, the mixture was transferred into a centrifuge tube and chloroform was added; the mixture was vigorously shaken for 15 s until no phase separation was observed and left standing for five minutes. Next, the mixture was centrifuged at 4 °C at 12,000 rpm for 15 min. The supernatant was transferred to another centrifuge tube; an equivalent volume of isopropanol was added, and the mixture was shaken up and down to mix, left standing at room temperature for 10 min, and centrifuged at 4 °C at 12,000 rpm for 10 min. Then, the supernatant was discarded. The cells were washed with and precipitated in 75% alcohol. The precipitate was dried at room temperature, and 50 μ L RNase-free water was added to fully dissolve the RNA precipitate. The purity of the RNA extract was de-

termined. Following this, 6 μ L of total RNA was obtained, and 1 μ L of Oligo (dT) was added until the total volume reached 12 μ L; the mixture was mixed carefully and incubated at 65 °C for five minutes in a PCR machine before being removed and immediately placed on ice. Next, 4 μ L of 5X RT buffer, 3 μ L of dNTP mixture, 1 μ L of RNase inhibitor, 3 μ L of DEPC-treated water, and 1 μ L of reverse transcriptase were successively added, and the total volume reached 25 μ L. After being shaken, the mixture was incubated in a PCR machine at 42 °C for 60 min, incubated at 70 °C for five minutes, cooled down on ice, and reserved at -80 °C. Next, 3 μ L of cDNA template was added to the reaction solution. CD59 primer sequences: up-stream, TGGACAATCACAATGGGAATC; downstream, TGCTGCCAGAAATGGAGTCAC. GAPDH primer sequences: upstream CGGGAACTGTGGCGTGAT; downstream, AGTGGGTGTCGCTGTTGAAGT. Reaction conditions of PCR: pre-denaturation at 94 °C for two minutes, denaturation at 94 °C for 30 s, annealing at 47 °C for one minute, and extension at 68 °C for two minutes. Steps 2–4 were repeated for 40 cycles, followed by extension at 68 °C for seven minutes. Next, the reaction products were mixed with ethidium bromide and underwent 1% agarose gel electrophoresis; the fluorescence signal was determined, and the ratio of CD59/GAPDH was calculated.

Cells were digested by trypsin and subcultured in 24-well bio-cytoculture plates with a cover glass at a density of 2×10^5 cells/well. The reagents were added after the HeLa cells became adherent in the short peptide seal specific to the CD59 group and cultured at 37 °C with 5% CO₂ for six or eight hours. Next, the cells were fixed with 4% paraformaldehyde and washed twice with 0.01 M of PBS (pH 7.2). Then, 3% H₂O₂ was added; the mixture stood at room temperature for 10 min and was then washed with distilled water three times. TdT and DIG-dUTP were added, and the cells were incubated in the wet box at 37 °C for two hours. The membrane was washed with 0.01 M of TBS three times. The cells were blocked with bovine serum albumin for 30 min; then, biotinylated mouse anti-digoxin antibody was added and incubated at 37 °C for 30 min. The membrane was again washed with 0.01 M of TBS three times. Afterward, horseradish peroxidase-labeled goat anti-mouse IgG antibody was added and incubated at 37 °C for 30 min; the membrane was washed with 0.01 M of TBS four times. DAB chromogenic agent was added, and the membrane was left standing at room temperature for 30 min. Then, it was washed, and the cells were slightly re-stained with hematoxylin. Next, the cells were

Table 2. — Difference of gene sequence between recombinant vector pIRES-M1CD59 and pIRES-M2CD59 coding W40W40.

M1CD59	M2CD59
251:TTTGATGCGTGTCTCATTACCAAAGCTGGG TTACAAGTGTATAACAAGTG; 301: TTGGAAGTTTGAGCAGTGCAATTTCA ACGACGTCACAACC CGCTTGAGGG primer sequences of 27bp site-directed mutagenesis, TGG deletion in coding W40	251: GATTTTGATGCGTGTCTCATTACCAAAGCT GGGTTACAAGTGTATAACAA 301: GTGGTGGTGGTTTGAGCATTGCAAT TTCAACGACGTCACA ACCCGCTTGA 33bp mismatched TGGTGGTGG instead of TGTTGGAAG

dehydrated, treated with xylene until they became transparent, and sealed before being observed under a microscope. The cells with brownish-yellow particles in the nuclei were considered positive, i.e., apoptotic.

Following this, the cells in each group were digested and transferred into a centrifuge tube and washed with 0.01 M of PBS (pH 7.2) three times. Then, 100 μ L/tube of Guava Nexin reagent was added to each group and incubated at room temperature in the dark for 20 min. The apoptotic rate was detected using flow cytometry.

The data were analyzed using the statistical software SPSS 13.0. All data were expressed as the mean \pm standard deviation.

Results

The CD59 mutant gene was successfully obtained via PCR site-directed mutagenesis, and the recombinant eukaryotic expression vector system of pIRES-M1CD59 and pIRES-M2CD59 was established using the CHO cells. While pIRES-M1CD59 had the W40 site knocked out, pIRES-M2CD59 had a mutation in the W40 site (Tables 1 and 2, Figure 1). The fluorescent dye release test revealed that anti-complement activity decreased in the pIRES-M1CD59 transfection CHO cells and that the cytolysis rate was higher in the pIRES-M1CD59 transfection CHO cells than in the pIRES-WTCD59 (wild CD59 recombinant plasmid) transfection CHO cells ($p < 0.01$). Meanwhile, the anti-complement activity was higher and the cytolysis rate lower in the pIRES-M2CD59 transfection CHO cells than in the pIRES-WTCD59 transfection CHO cells ($p < 0.05$). The mutant M1CD59 loosened its inhibitory function on the complement, while M2CD59 enhanced its inhibitory effect on the complement, suggesting that W40 is the active site of CD59 (Figure 2).

Phoenix A cells were transfected with pSUPER-siCD5, pIRES-WT CD59, and pIRES-MCD59. The green fluorescent cells were counted, and the transfection efficiency of each recombinant vector was calculated under an inverted fluorescence microscope after 48 hours. The results revealed that the transfection rates of all recombinant vectors were $> 70\%$. The RT-PCR results revealed that all cell groups presented with bands of CD59 mRNA (267 bp) and house-keeping gene GAPDH mRNA (509 bp), while the level of CD59 mRNA expression in the cells transfected

Table 3. — The expression of CD59 on mRNA level in HeLa cells.

Groups	mRNA
Untransfected group	0.7753 \pm 0.02
pSUPER group	0.8695 \pm 0.03
pSUPER-siCD59 group	0.3172 \pm 0.02
pIRES group	0.8753 \pm 0.03
pIRES-WTCD59 group	1.4511 \pm 0.03
pIRES-MCD59 group	1.5754 \pm 0.02

Data were presented as mean \pm SD, $p < 0.05$, compared with untransfected group.

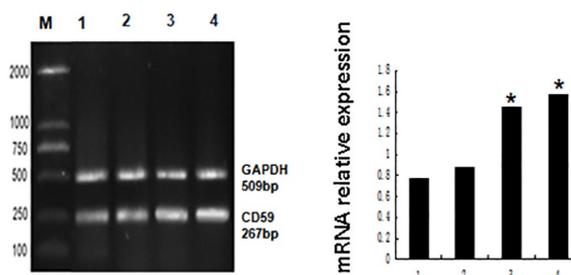


Figure 4. — Expression of CD59mRNA effected by recombinant plasmid transfection of pIRES-WT CD59 and pIRES-MCD59 (1: the pIRES group; 2. the normal group; 3. the pIRES-WTCD59 group; 4. The pIRES-MCD59 group)

with pSUPER-siCD59 (CD59-silenced recombinant plasmid) was significantly lower than in the control cells and cells transfected with pSUPER (empty vector plasmid; $p < 0.05$, Figure 3). Furthermore, the CD59 mRNA expression level in the cells transfected with pIRES-WT CD59 and pIRES-MCD59 was significantly higher compared with the control cells and cells transfected with pIRES (empty vector plasmid; $p < 0.05$, Figure 4). However, the difference in the expression level between the control cells and cells transfected with pSUPER and pIRES was not statistically significant (Table 3).

The CD59 monoclonal antibody was used to cross-link with the stimulated HeLa cells in each group. The TUNEL results revealed that typical apoptotic cells were

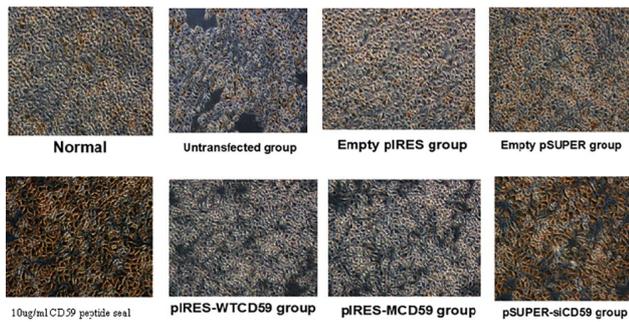


Figure 5. — High level expression of CD59 gene, silent vector transfection and TUNEL staining results of CD59 block.

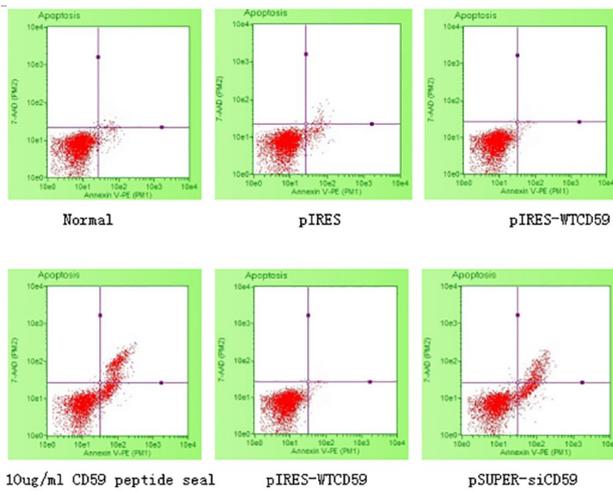


Figure 6. — High level expression of CD59 gene, silent vector transfection and testing results of TUNEL staining results of CD59 block by flow cytometry.

found in the pUPER-siCD59-transfected group (Figure 5), whose apoptosis rate was significantly higher than that of the pIRES-WTCD59-transfected, pIRES-MCD59-transfected, and control groups. After the short peptide seal specific to CD59 was exerted on the HeLa cells, the apoptosis rate significantly increased with the increase in dosage (Figures 6 and 7). The apoptosis rate of the HeLa cells exerted by the short peptide seal was higher than in the HeLa cells transfected with pSUPER-siCD59, revealing that the apoptosis-promoting effect of the short peptide seal specific to CD59 on the HeLa cells was superior to that of the CD59 interference plasmid pSUPER-siCD59 (Figure 7).

Discussion

The CD59 GPI-anchored membrane protein, also known as the CD59 complement regulatory protein, plays a key regulatory role in the assembly of MACs. It can effectively prevent the formation of a MAC by binding with complement proteins C8 and C9, thereby inhibiting the dissolution of the complement on histocytes [24, 25].

The terms RNAi, post-transcriptional gene silencing, in-

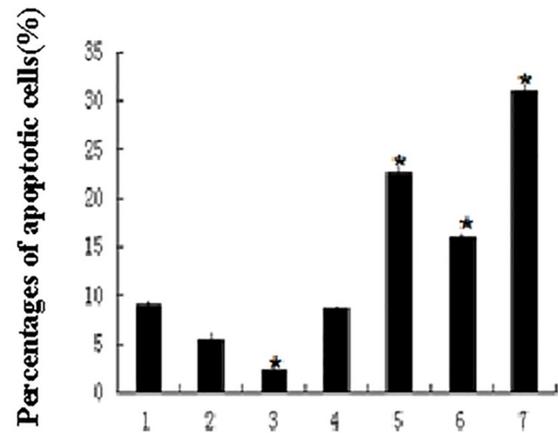


Figure 7. — High level expression of CD59 gene, silent vector transfection and comparison of cell apoptosis rate of CD59 block (1. pIRES group; 2. The control group; 3. pIRES-WTCD59 group; 4. pSUPER group; 5. pSUPER-siCD59 group; 6.1 ug/ml CD59 peptide seal group; 7. 10 ug/ml CD59 peptide seal group).

hibition, and co-inhibition are frequently found in studies of multiple pathways. In essence, they all refer to the RNAi reaction caused by impaired dsRNA and dsRNA expressed by homologous sequence mRNA. The common trait among these molecules is that they are highly conservative in their evolution and exist in a variety of organisms, such as plants, fungi, and animals [26, 27]. Moreover, RNAi has many functions, including growth regulation, gene expression silencing, and virus and transposon invasion prevention [28-30]. In this study, based on the characteristics of the expression of CD59 molecules in the cervical cancer HeLa cell line, the expression of CD59 in the HeLa cells was intervened with using the CD59RNA interference plasmid pSUPER-siCD59 and CD59 22-peptide short peptide seal HSACDLLMHPM-linker-MPHMPLDCASH. Furthermore, serving as controls, two kinds of plasmids highly expressing CD59 (wild-type CD59-pIRES-WTCD59 and CD59-pIRES-MCD59, a mutant type with a mutation at the CD59W40 active site) were transfected into HeLa cells, and the effects of these various interferences in CD59 expression on the cells' apoptosis were detected and compared. The results revealed that intervention in the CD59 expression in the HeLa cells using the CD59RNA interference plasmid and short peptide seal promoted the apoptosis of the cells to a certain extent; the effect of the short peptide seal was superior to that of the RNA interference plasmid. Although the intervention by the two plasmids that highly expressed CD59 had opposite results, the cell proliferation was faster than that in normal HeLa cells, and no apoptosis was observed. As CD59 is an inhibitor of the MACs assembled by C5b-9, when its expression is low, its inhibition effect on the formation of the MACs decreases; hence, the dissolution effect of the complement on the HeLa cells fully works, affecting cell proliferation and inducing apoptosis. The up-

regulation of CD59 expression can produce the opposite effect [31, 32]. This phenomenon may also be related to Fas-mediated cell death; Fas can produce pro-apoptotic signaling by binding with a specific antigen while being mediated by caspase-3 and bcl-2. As the protein expression of CD59 in the HeLa cell membrane decreases, the protein expression of Fas also decreases; hence, the apoptosis signal transduction it mediates decreases. This manifestation is rapid cell proliferation, and apoptotic cells are rare. However, the detailed mechanism remains to be explored.

In this study, the expression of the CD59 molecule in tumor cells and that of its products were intervened with using gene knockout, gene silencing, and a short peptide seal to observe the changes in the cells' sensitivity to the dissolution and apoptosis induction of the complement. It was confirmed that CD59 inhibited the complement's activity and apoptosis. As the active site of CD59, the mutation or deletion of W40 or the sealing of the W40-related epitope of the CD59 molecule could make these cells susceptible to being dissolved through the complement or undergoing apoptosis.

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Conflict of Interest

The authors report no conflicts of interest in this work.

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