Article

CD59 Silencing *via* Retrovirus-Mediated RNA Interference Enhanced Complement-Mediated Cell Damage in Ovary Cancer

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CD59, belonging to membrane complement regulatory proteins (mCRPs), inhibits the cytolytic activity of complement and is over-expressed in solid cancers, including ovary cancer. The aim of the present study was to construct recombinant retrovirus encoding shRNA targeted human CD59 and infect A2780 cells in order to investigate the relationship between decreased CD59 expression and tumorigenesis of ovary cancer. siCD59 and siCD59-C were successfully constructed and identified by PCR, restriction endonuclease analyses and DNA sequencing, respectively. The siCD59 was able to efficiently infect A2780 cells, which was confirmed by Western blotting. When incubated with fresh normal human serum (8%, v/v) for 1 h at 37°C, the cell viability was decreased and cell damage was increased in siCD59 infected A2780 cells compared to siCD59-C infected cells. This led to the activation of caspase-3. The apoptosis in siCD59 infected cells was shown with hypercondensed nuclei using Hoechst staining. Meanwhile, the weight of ovary tumor graft in nude mice was significantly decreased in siCD59 group compared to that of siCD59-C group. And the expression of CD59 protein in tumor tissue in siCD59 group was significantly decreased. These results suggested that CD59 silencing in ovary cancer cells *via* retrovirus-mediated RNAi can enhance complement-mediated cell damage, inhibiting growth of ovary cancer. CD59 might be a potential target for gene therapy in ovary cancer. *Cellular & Molecular Immunology*. 2009;6(1):61-66.

Key Words: CD59, complement, RNA interference, gene therapy, ovary cancer

Introduction

Complement is an important component of innate immune system against infections as well as growth of tumors (1). CD59, belonging to membrane complement regulatory proteins (mCRPs), blocks the assembly of membrane attack complex (MAC), thereby inhibiting the cytolytic activity of complement (2, 3). Studies have demonstrated that expression of CD59 is higher in most solid malignancies than in normal tissue, which helps the malignant cells escape the immunologic surveillance and complement-mediated cytolysis, and limits the effect of complement-fixing monoclonal antibodies (4-7). Immune escape of tumor cells is the main cause that leads to the failure of immunotherapy. Blocking of CD59 function on tumor cell surface may allow effective complement-mediated clearance of tumor cells and prospectively improve the effect of complement-activating anti-tumor antibodies (8, 9). Different techniques have been reported for suppressing the expression as well as inhibiting the function of CD59, such as targeting CD59 by using neutralizing mAbs, antisense oligodeoxynucleotides or synthetic siRNA (10), or using strategy affecting the regulators of CD59 expression (11). However, the effects of CD59 depletion on tumor cell growth are so far not well defined. The present study was aimed to set up a strategy of silencing CD59 expression in an ovary cancer cell line with retrovirus-mediated siRNA, and to further investigate the impact of CD59 depletion on cell viability and tumor growth in both cultured ovary cell line and in ovary cancer cell graft implanted in nude mice. The results demonstrated that retrovirus-mediated siRNA almost completely suppressed CD59 protein expression in an ovary cancer cell line, which resulted in, with the help of complement, reduced cell viability, and increased apoptosis in cultured ovary cell, as well as suppression of tumorigenesis in nude mice. These results suggested that CD59 silencing can enhance complement-mediated cell damage, inhibit growth of ovary cancer. CD59 might serve as a potential target for gene

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therapy in ovary cancer treatment.

Materials and Methods

Materials

The pSUPER retro neo+gfp vector was kindly provided by Dr. Bing Luo of Department of Microbiology, Medical College of Qingdao University. Human ovary cancer cell line (A2780) was purchased from Chinese Academy of Science. *Bgl* II, *Hind* III, *Eco*R I and T4 DNA ligase were from Fermentas. E.Z.N.A. Gel Extraction kit was from OMEGA. RPMI 1640 and DMEM was purchased from GIBCO (Gaithersburg, MD, USA); Lipofectamine 2000 was from Invitrogen™ Life Technologies. The mouse anti-human CD59 antibody was purchased from BD PharMingen. LDH kit was from Nanjing Institute of Jiancheng Biological Engineering. Fresh normal human serum (NHS) was obtained from the blood of healthy volunteers in the laboratory. Eighteen female nude mice, 5-6 weeks old, provided by the Vital River Animal Facility of Beijing, were bred under specific pathogen-free conditions and kept at a constant humidity and temperature. Other chemicals and reagents available were from local commercial sources.

Construction of small interfering RNA targeted CD59 expression vector

The pSUPER vector was digested by *Bgl* II and *Hind* III restriction enzyme and annealed oligos, siCD59: 5'-GATCC CCTGAGCTAACGTACTACTGCttcaagagaGCAGTAGTAC GTTAGCTCATTTTTA-3', siCD59-C: 5'-GATCCCCAGAC TTGACTCCTGTCGAAttcaagagaTCTGAACTGAGGACA GCTTTTTTTA-3' were ligated with this vector. The recombinants were identified by PCR, restriction endonuclease analyses and DNA sequencing, respectively.

Cell line and retrovirus infection

A packaging cell line Phoenix A and human ovary cancer cell line A2780 were cultured in DMEM and RPMI 1640 supplemented with 10% FBS (GIBCO, USA) and penicillin/ streptomycin, incubating in a humid wet (37°C, 5% CO_2) incubator. For the production of retroviruses, Phoenix A was transfected with this recombinant using lipofectamin 2000 according to the manufacturer's instructions. Culture supernatants were collected after 48 h. A2780 cells were infected with siCD59 and siCD59-C respectively in the presence of 8 μg/ml polybrene, selected by continuous growth in G418（400 mg/L）to isolate stable clones.

Western blotting

Cells were lysed directly on the culture dishes using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% NaN_3, 1% Triton X-100, 1 mM PMSF and 1 μg/ml aprotinin, 1 μg/ml leupeptin). The protein concentration was determined by the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). A hundred micrograms of total protein were subjected to SDS-PAGE (12% and 5%) and then transferred to PVDF membranes. After overnight blocking with 5% non-fat milk at 4°C, the membranes were incubated with mouse anti-human CD59 antibody (1:400) for 1 h at 37°C. Anti- mouse secondary antibodies conjugated to horseradish peroxidase were used at a dilution of 1:5,000. The protein was visualized using ECL Western blotting detection reagents and then was analyzed through scanning densito- metry by Tanon Image System. β-actin was detected by rabbit anti-β-actin polyclonal antibody (1:200) according to similar procedure to ensure sample protein loading. These experiments were repeated three times with similar results.

MTT assay for cell viability

Cells were treated with complement inactivated (56°C, 30 min) rabbit-anti-A2780 antibody (1:400) for 30 min at 37°C and 8% NHS for 60 min at 37°C. After incubation in MTT (5 mg/ml) for 4 h at 37°C, cell viability was measured by colorimetric assay (TECAN, Austria).

LDH release assay

Cell treatment was the same as above. Triton X-100 (0.1%) in RPMI 1640 was used as the 100% lysis control and RPMI 1640 alone was used for the 0% lysis control. Following incubation, 40 μl of sample supernatant was taken for LDH assay. To each well 100 μl solution C was added. Just before analysis, 10 μl solution B was added. The absorbance at 440 nm was calculated, reflecting the activity of LDH present, and the following equation applied: LDH leakage rate (%) = (NHS lysis - 0% lysis)/(100% lysis - 0% lysis) × 100%.

Active caspase-3 assay

Active caspase-3 assay was measured according to the manufacturer's protocol (BD Biosciences PharMingen, USA). Briefly, cell treatment was the same as above. After washed twice with cold PBS, cells were resuspended in Citofix/Cytoperm™ solution at a concen- tration of 1×10^6 cells/0.5 ml. After incubation for 20 min, cells were washed with Perm/Washing buffer twice, and then incubated in Perm/Wash buffer with antibody (1:5). After washed once with Perm/Wash buffer, cells were resuspended with 0.5 ml Perm/Wash buffer and analyzed by flow cytometry. The percentage of positive cells reacting with the antibody was examined with CellQuest Software.

Hoechst 33258 staining

Nuclear morphology was studied by using Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, Jiangsu, China) (12). Apoptotic cells were defined on the basis of nuclear morphology changes, such as chromatin condensation and fragmentation. The total number of condensed cells was counted manually by researchers blinded to the treatment schedule using unbiased stereology and a fluorescence Olympus microscope (Olympus, Japan). For each well, we delineated a 400 μm^2 frame and counted all condensed and normal nuclei with at least 10 different fields in one well. Average sum of condensed and normal nuclei was calculated per well. The data were expressed as a percentage of condensed nuclei number to the total number.

Xenograft studies

A2780 cells were washed twice with antibiotic-free and serum-free RPMI 1640 and finally resuspended at a density of 5×10^6 cells in 0.2 ml normal saline (NS). The cell suspension was injected *s.c.* into nude mice (9 mice a group, 2 groups), which were sacrificed after 6 weeks. Tumors were excised and weighed, fixed for 24 h in 10% formalin, and washed with PBS. The tumor specimens were then subjected to paraffin followed by the immunohistochemistry with anti-CD59 antibody (1:400) as previously described (13). Approximately 1,000 cells were counted in each immunohistochemical section. Results were presented as mean of 9 sections.

Statistical analysis

Data were presented as mean ± SD. Student's *t* test was used to analyze the data. A level of $p < 0.05$ was considered as statistically significant.

Results

Construction and identification of expression vector

The empty vector was modified by deleting the *Bgl* II/*Hind* III (2424/1441 site) fragment of about 983 bp to generate lineate vector digested by *Bgl* II and *Hind* III restriction enzyme. Subsequently, the annealed oligos with an identical sequence to human CD59 were inserted downstream of the H1 promoter, using 5 thymidines as the terminal signal. We designed a couple primer to identify the recombinant vector, the expanded production was 395 bp which comprised the inserted 60 bp oligonucleotides and the bilateral sequence of the pSUPER. The primers were as follows: pSUPER-a: 5'-CCT TTA TCC AGC CCT CAC TC-3', pSUPER-s: 5'-AGA CTG CCT TGG GAA AAG CG-3'. We took the empty and recombinant vector as the template to amplify. The positive clone was 395 bp, and the negative clone was 1,318 bp (395 - 60 + 983) (Figure 1A). Furthermore, the empty and recombinant vector was digested by *Eco*R I (2645 site) and *Hind* III restriction enzyme, the removed fragment of the empty vector was 1,204 bp (2645 - 1441), the fragment of the recombinant vector was 281 bp (1204 - 983 + 60) (Figure 1B). At last, the recombinant vector was identified by DNA sequencing.

CD59-siRNA efficiently suppressed CD59 expression

Recombinant retroviruses siCD59 and siCD59-C both contained green fluorescent protein (GFP) reporter gene, allowing us to measure infection frequency of infected A2780 cells. Seventy-two hours after infection, the infection efficiency reached more than 70% in siCD59 and siCD59-C infected cells (Figure 2). Decreased CD59 expression was confirmed by Western blotting. Data showed that CD59 protein levels were decreased significantly in siCD59 infected cells compared to siCD59-C infected cells (Figure 3).

Cell viability was reduced and cell damage was increased when treated with complement

Fresh NHS (8%, v/v) was added to the culture medium at 37°C for 1 h. Cell viability was significantly reduced and cell damage was significantly increased in siCD59 infected cells compared with the siCD59-C group (Table 1).

Cell apoptosis analysis

To verify whether the down-regulation of CD59 expression could induce cell apoptosis, flow cytometry and Hoechst 33258 staining were employed to determine caspase-3 activity and nuclear morphology. Caspase-3 activity in

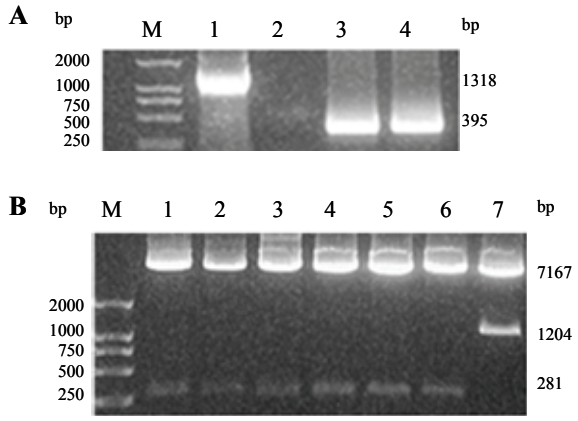

Figure 1. Identification of recombinant vectors. (A) PCR identification of recombinant vectors. Lane M, DL2000 DNA marker; Lane 1, empty vector; Lane 2, negative control; Lane 3, siCD59; Lane 4, siCD59-C. (B) Identification of enzyme digestion of recombinant vectors. Lane M, DL2000 DNA marker; Lanes 1-3, siCD59; Lanes 4-6, siCD59-C; Lane 7, empty vector.

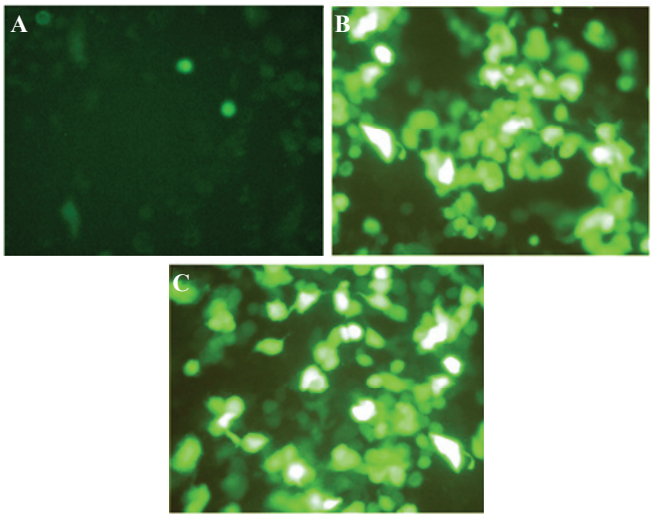

Figure 2. Fluorescence microscope images of A2780 cells 72 h after infection (200×). (A) A2780 cells without infection. (B) A2780 cells infected by siCD59. (C) A2780 cells infected by siCD59-C.

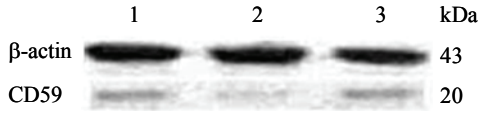

Figure 3. Expression of CD59 protein in infected A2780 cells by Western blotting analysis. The protein levels of siCD59 were much lower than that of siCD59-C infected cells. Lane 1, A2780 cells without infection; Lane 2, A2780 cells infected by siCD59; Lane 3, A2780 cells infected by siCD59-C.

Table1. Cell viability was reduced and cell damage was increased with NHS incubation (8%)

Groups	MTT (% of None)	LDH release rate
Untreated (None)	100.00 ± 2.52	12.8 ± 2.6%
NHS	88.98 ± 3.58	22.5 ± 6.6%
siCD59-C/NHS	98.66 ± 5.35	15.7 ± 3.8%
siCD59/NHS	66.57 ± 3.24*	78.8 ± 8.7%*

Data were presented as mean ± S.E.M., *p < 0.05, compared with siCD59-C/NHS.

siCD59 group was significantly increased after exposure to complement compared with siCD59-C group (Figure 4). In the siCD59-C group after complement treatment, nuclei showed regular contours, which were round and large in size. However, the nuclei of siCD59 group appeared hyper-condensed (brightly stained) and fragmentations of chromatin (Figure 5).

CD59-siRNA inhibited in vivo cancer cell growth

The effects of CD59 suppression on *in vivo* tumor growth were conducted with A2780 cells, stably expressing either siCD59 or siCD59-C, injected into nude mice. At the end of the experiment, all mice developed detectable tumors. The tumor weight (0.27 ± 0.092) in siCD59 group was significantly lower than that in siCD59-C group (1.57 ± 0.176) (t = 6.21, p < 0.01) (Figure 6).

CD59 protein expression of tumor tissue decreased in siCD59 group

CD59 protein expression in tumor tissue was examined by immunohistochemical staining using anti-CD59 antibody. The results showed that the number of positively stained cells in siCD59 group (296 ± 70) was significantly lower than that in siCD59-C group (814 ± 112) (t = 11.77, p < 0.001) (Figure 7).

Discussion

Studies have demonstrated that expression of CD59 in many tumors is significantly elevated, which interferes with tumor clearance (14, 15). It is therefore proposed that decreased CD59 expression could enhance tumor cell susceptibility to complement-mediated cytolysis and suppress tumor growth

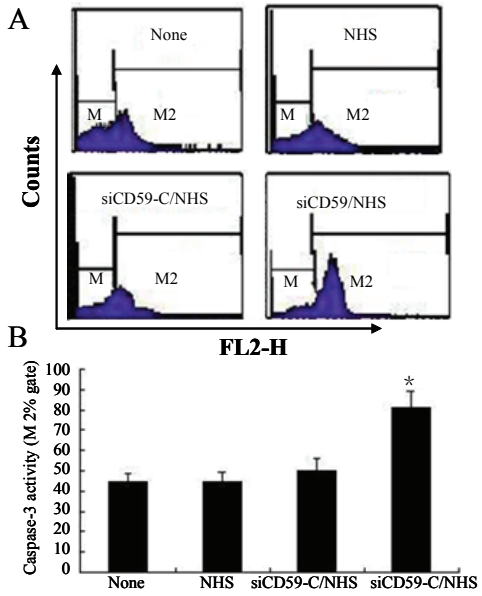

Figure 4. Caspase-3 activity was increased in siCD59 infected cells after NHS incubation. Infected cells were treated with 8% NHS for 1 h and activated caspase-3 levels were detected by flow cytometry. (A) Representatives of the flow cytometric assay. (B) Statistical analysis of caspase-3activity in different groups, *p < 0.05, compared with siCD59-C infected group.

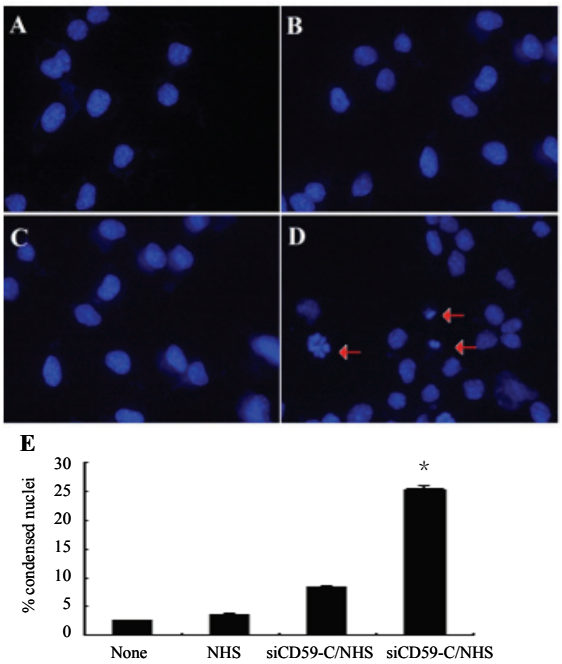

Figure 5. Morphological changes were observed in siCD59 infected cells after NHS incubation. (A) Untreated cells. (B-D) Representative photographs of Hoechst staining of NHS, siCD59-C/NHS, siCD59/NHS groups of A2780 cells. The nuclei of siCD59 infected cells appeared hypercondensed (brightly stained) and fragmentation of chromatin. Magnification, ×400. (E) The statistical analysis of condensed nuclei in different groups, *p < 0.05, compared with siCD59-C infected group.

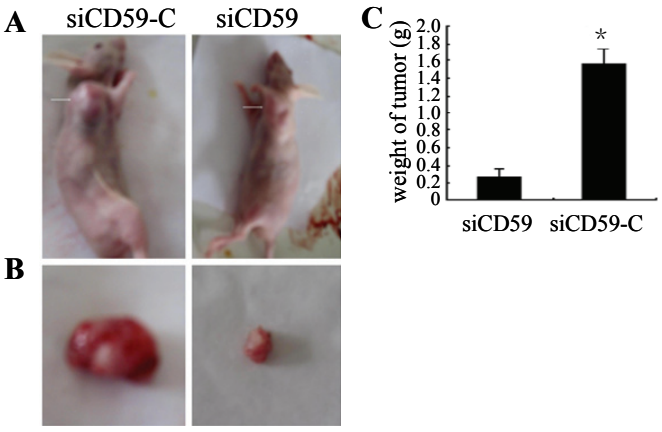

Figure 6. siCD59 decreased tumorigenicity in nude mice. Nude mice were injected 1 × 10^6 cells suspended in NS. (A) Representative tumor formations at 6 weeks after injection. (B) Representative appearance of tumor mass exercised from nude mice at 6 weeks after injection. (C) The statistical analysis of mean tumor weight at 6 weeks after injection (*p < 0.01, compared with siCD59-C infected group).

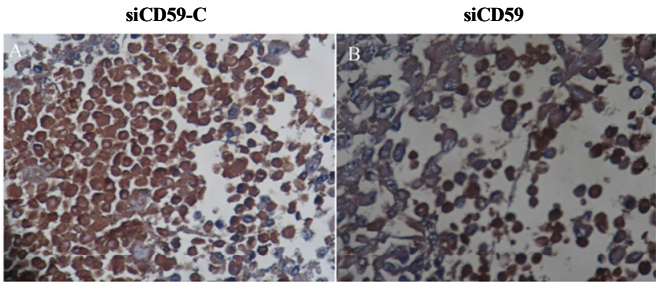

Figure 7. CD59 protein expression of tumor tissue was decreased in siCD59 group. Tumor tissues from nude mice were stained with anti-CD59 antibody followed by DAB staining (brown) and hematoxylin staining (blue). (A) Representative stains of tissue slice in siCD59-C group. (B) Representative stains of tissue slice in siCD59 group.

in vivo and *in vitro*. Our previous study has shown that down-regulation of CD59 expression led to increased cytolysis and induction of HeLa cell apoptosis *in vitro* (16, 17). Accumulating evidence highlighted the potential of CD59 as therapeutic target of cancers. We here reported that specific silencing of CD59 by retrovirus-mediated RNAi decreased cell viability, increased cell damage and apoptosis induced by complement in cultured ovary cancer cells. And ovary tumor growth implanted into nude mice was inhibited.

RNA interference has recently emerged as a genetic tool for silencing gene expression. In the present study, we successfully constructed the recombinant retrovirus encoding human CD59 with pSUPER RNAi system. Because the retroviruses induced RNAi can be integrated into the genomes of both dividing cells and nondividing cells to achieve stable, long-term expression of siRNAs, it has been wildly used (18, 19). After its infection of A2780 cells by siCD59, a significant decrease in CD59 protein expression was observed, which was in agreement with other studies (10, 11).

After exposure to human complement (NHS, 8%, v/v), the target A2780 cells were incubated with inactivated rabbit-anti-A2780 antibody for measuring their sensitivity to MAC-mediated cytolysis. When infected by siCD59, the cell viability was decreased significantly, the activity of caspase-3 was increased, and nuclear morphology changed, which showed significant cell apoptosis in ovary cancer cell. To investigate the inhibitory role of siCD59 *in vivo*, siCD59 infected cells were injected into athymic nude mice. Results showed that the average tumor weight was significantly lower in the siCD59 infected group than that in the siCD59-C group, and the rate of tumor inhibition was about 80%, which suggested that CD59 gene silencing could markedly inhibit the growth of cancer *in vivo*. The silencing of CD59 expression in tumor tissue was confirmed by immunohistochemical staining which showed significantly less CD59 protein expression in siCD59 group than in siCD59-C group. These observations suggested a main role of CD59 in induction of tumor growth, and indicated the siRNA sequences targeting CD59 could be a potential target for gene therapy.

There have been evidence that complement influences apoptosis at two distinct levels: complement determines the fate of the cell, and helps phagocytes with the disposal of the corpses of apoptotic cells (20-22). Evidence from animal models also showed that MAC could trigger apoptosis (23-25). Korty demonstrated the signaling capacity of CD59 in experiments where human CD59 was cross-linked with specific monoclonal antibodies, and shown to generate a calcium flux. Too much calcium can damage chondriosome and the damaged chondriosome releases cytochrome C which activates caspases and induces apoptosis (26).

In conclusion, the present study demonstrated that pSUPER RNAi system was a powerful and efficient system for gene silencing. Suppression of CD59 expression enhanced complement-mediated cytolysis of ovary cancer cells, CD59 may serve as a candidate targeting gene in gene therapy for human carcinomas such as ovarian, prostate carcinoma, cervical carcinoma, etc.

Acknowledgements

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