Efficient inhibition of intraperitoneal human ovarian cancer growth by short hairpin RNA targeting CD44

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CD44 is one member of a big glycoprotein family involved in adhesion of cells or cells and extracellular matrix (ECM). The heavily glycosylated CD44 has been proved to be a major receptor of hyaluronan and a marker of stem cells in ovarian cancer. Here, using short hairpin (shRNA) against CD44, we demonstrate that knockdown CD44 could inhibit cancer growth efficiently compared with controls. Plasmid targeting CD44 gene (pshCD44) or non-relative control sequences (pshHK) was constructed and delivered to ovarian cancer by biodegradable poly D, L-Lactide-co-glycolide acid nanoparticles (PLGANPs). Nude mice were utilized in an intraperitoneal model of ovarian carcinomatosis to assess antitumor efficacy in vivo. Antitumor efficacy was estimated by changes in tumor weights, proliferation (Ki-67), apoptosis (TUNEL) and angiogenesis (CD31 staining and alginate-encapsulated tumor beads assay) in tumor cells. As results, pshCD44 or pshHK could be effectively transfected into SKOV-3 cells by PLGANPs. Tumor weight in pshCD44/PLGANPs group was suppressed by 45% and 50% compared with those in pshHK/PLGANPs and untreated group, respectively (Ps < 0.001). Inhibition of cell proliferation, induction of apoptosis and reduction of angiogenesis in tumor cells of pshCD44/PLGANPs group also show significant difference compared with those in control groups (Ps < 0.05), respectively. These results indicate that pshCD44 delivered by PLGANPs might be a potential approach in ovarian cancer therapy, and point towards a mechanism involving the inhibition of angiogenesis, cellular proliferation and the induction of apoptosis.

Key words: CD44, shRNA, ovarian cancer, PLGA nanoparticles

Ovarian cancer cells are characterized by widespread affinity to peritoneal epithelium, which causes tumor cells diffusing freely in peritoneal cavity and leads ovarian cancer to be a dramatically aggressive tumor. Ovarian cancer ranks the fifth in causing cancer-related morbidity in females and the first leading-death cancer in gynecologic oncology [1].

A plenty of researches exploring gene targets are aimed to improve treatment of ovarian cancer. CD44 is an ideal one of these candidates, which belongs to a big transmembrane glycoprotein family widely expressed in ovarian cancer tissues [2] and play mediating ovarian carcinoma cell adhesion to peritoneal mesothelial cells [3]. In addition, CD44 was one major receptor of hyaluronan. Inhibiting interaction of CD44 and hyaluronan showed suppressions of various anti-apoptosis cell survival pathways and tumor growth [4]. Owing to a considerable CD44 isoforms generated by various splicing in tumor growth and metastasis [5], various studies had been done to target various CD44 isoforms in inhibitions of different tumor growth [6-8]. CD44 was also reported as a marker of ovarian cancer stem cells [9]. Small interfering RNA (siRNA) targeting CD44 showed efficient therapy of ovarian cancer [10]. Based on above findings, a recombinant plasmid expressing short hairpin RNA (shRNA), a relative stable and sustainable RNA interfering approach than siRNA [11], targeting CD44 gene (pshCD44) was constructed to estimate the antitumor efficacy in ovarian cancer in current study.

A safe and efficient gene carrier is crucial to gene therapy, which holds a potential promising for therapeutic approach in tumors. Owing to high efficiency of adenovirus delivery system accompanying of safty problems [12], more attentions were oriented on nonviral vectors. Nonviral delivery systems are advantageous due to their low cytotoxicity, low immuno-
genicity and improved safety profile when compared to that of viral vectors [13-15]. In recent years, poly (D,L-lactide-co-glycolide acid) (PLGA) nanoparticles (PLGANPs) have attracted considerable attention and interest due to their low toxicity, and excellent biodegradability and biocompatibility [16-18]. In addition, PLGANPs have been used in conjunction with short hairpin RNA (shRNA) to both efficiently express and knockdown genes in tumor cells with limited toxicity [19, 20]. Therefore, we chose to silence the expression of CD44 in ovarian tumors using a recombinant plasmid vector based on shRNA encapsulated with PLGANPs.

Materials and methods

**ShRNA plasmid vectors construction.** Oligonucleotide sequences CD44 (sense, 5’-TCC GCT ATG ACA CAT ATT GCT TCT TCA AGA CAA TAT GTG TCA TAC TTT TTT G-3’; antisense, 5’-AGC TCA AAA AAG TAT GAC ACA TAT TGC TTT CTT GTG AAG CAA TAT GTG TCA TAC -3’) was designed according to the design principle and an identified research [21]. HK (sense, 5’-GAT CCG ACT TCA TAA GCC GTA CGC GGA ATA CTT CAG AAA AAA CAG CTG TGC GCC GTA CGC GGA ATA TCT CAG AAA AAG CAG CTG TTT GA-5’) was designed in principle of no homology to any human gene. pshCD44 was cloned into pGenesile-2 plasmid, which holding kanamycin resistance gene. pGenesile-2 was linearized with EcoRI, ligated with annealed oligonucleotide templates CD44 into a plasmid vector using T4 DNA ligase, respectively. The resultant recombinant plasmid was digested to ensure its correctness, and the identity of CD44 genes cloned into pGenesile-2 was finally confirmed by DNA sequencing. pshHK was constructed as similar methods described above, used as one of control groups. Colonies of pshCD44 or pshHK were cultured in Luria-Bertani broth described above, used as one of control groups. Colonies of pshCD44 or pshHK were cultured in Luria-Bertani broth containing 5% CO₂ at 37°C. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin, and passaged on reaching 70%- 80% confluence at a split ratio of 1:3.

**PLGANPs preparation and characteristics.** PLGANPs encapsulating pshCD44 or pshHK were prepared similarly as previously described [19, 22]. Before large quantities of PLGANPs loaded with pshCD44 or pshHK were performed, all procedure variables had been optimized and quantized. In brief, pshCD44 or pshHK was firstly condensed via incubation with equivalent ploy-L-lysine (PLL with MW 25 kDa, Sigma) and gene-loading rate (GLR) of pshDNAs could be calculated according to the quantity of entrapped pshDNA, PLGA polymer and free pshDNA in supernatants [22]. The free pshDNA content in supernatants obtained from pshDNA/PLGANPs suspension after ultracentrifugation (Optima Optima™ L-80 XP Ultracentrifuge, Beckman Coulter, USA) at 10°C and the speed of 15,000 rpm for 30 minutes, were quantitated using UV-spectroscopy (RF-5301PC, Japan). After stained by Hochest 33258 staining solution (Sigma), the absorbance value monitoring at 455 nm was used to count the amount of pshDNA unencapsulated. All experiments were repeated triply.

**Cell culture and transfection conditions.** The human ovarian serous cystadenocarcinoma cells line SKOV3 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were cultured in Dulbeccco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin, and passaged on reaching 70%- 80% confluence at a split ratio of 1:3.

SKOV3 cell (2×10⁵) were plated in 6-well plates and cultured for 24 hours to 30%- 40% confluence. Five microgram (5 μg) of pshCD44 or pshHK was entrapped in each 100 μl PLGANPs as described before and then diluted in 1 ml DMEM medium without antibiotics and serum. After incubating 6 hours, the medium was changed to 2 ml DEME with FBS and antibiotics. Then, RNA and protein samples of cells were collected for further analyses after 60-72 hours of incubation.

**Reverse transcription polymerase chain reaction (RT-PCR).** Total RNAs were isolated from cells or tumor tissues using TRIzol reagent (Invitrogen, Grand Island, NY). The primers of CD44 were designed based on cDNA sequence using upstream primers 5’-GCT CAT ACC AGC CAT CCA AT-3’ and downstream primers 5’-GAG GTG TCT GTC TCG GTT CCA AA-3’, respectively. The predicted RT-PCR products and the best annealing temperature of CD44 was 211 bp and 55°C, respectively. Five microliters (5 μl) of each RT-PCR product was electrophoresed on 1% agarose gel. The housekeeping gene GAPDH was used as an internal control.
Western blot analysis. Cells or tumor tissues were lysed using RIPA lysis buffer (50 mM Tris-HCL, 0.25% sodium deoxycholate, 150 mM NaCl, 1% NP-40, 1 mM NaF, and 1 mM Na₂VO₄) containing 1 mM cocktail (protein inhibitor, Sigma, St. Louis, MO) and centrifuged at 12,000 rpm, 4°C, for 30 minutes. The total protein concentrations of supernatant were determined using Bio-Rad protein assay (Hercules, Calif). Equal amounts of proteins were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad, CA). After being blocked with 5% nonfat milk 2 hours, membranes were immunoblotted with mouse monoclonal antibody against CD44 (1:500 dilution, mAb, Santa Cruz Biotecnology, Santa Cruz, CA) over night at 4°C. Followed by incubating goat anti-mouse secondary (1: 3,000, ZSGB-BIO, Beijing, China) for 1 hour at 37°C. Equal loading was normalized by GAPDH (1: 1,000 dilution, ZSGB-BIO, Beijing, China) and all bands were observed with an enhanced chemiluminescence detection system (Pierce Biotech Inc., Rockford, IL, USA).

Table 1. Characteristics of two pshDNA/PLGANPs.

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<th>MD (nm)</th>
<th>PDI</th>
<th>EE (%)</th>
<th>GLR (%)</th>
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<tr>
<td>pshCD44/PLGANPs</td>
<td>250.98±13.8</td>
<td>0.20±0.00</td>
<td>85.91±2.82</td>
<td>1.72±0.12</td>
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<tr>
<td>pshHK/PLGANPs</td>
<td>226.83±3.18</td>
<td>0.18±0.02</td>
<td>89.16±3.35</td>
<td>1.83±0.07</td>
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MD: mean diameters; PDI: polydispersity index; EE: entrapment efficiency; GLR: gene-loading rate
and GLR were showed in our study than previous studies [19,
22, 24]. Ultracentrifugation was used to remove the free drugs
and polyvinyl alcohol (PVA) in the supernatant as described
previously [24].

**Down-regulation of CD44 expression in vitro.** To
evaluate the expression of CD44 after treatment with the
pshCD44/PLGANP in vitro, SKOV3 cells were collected at
60-72 hours after transfections and analyzed by RT-PCR and
western blot.

Semi-quantitative RT-PCR indicated that expression
of CD44 in pshCD44/PLGANPs group was suppressed by
61% and 62% compared with that in pshHK/PLGANPs and
untreated group (P < 0.001) (Fig. 1 A, B). There was no sig-
nificant difference between two control groups. The density of
RT-PCR bands was measured by Quantity One and normal-
ized with GAPDH (Fig. 1 A).

Suppression of CD44 at the protein level was observed
in SKOV3 after treatment with pshCD44/PLGANPs
in vitro (Fig. 1 C). Owing to random lack of 1-10 variant exons in tran-
scription level and complicated modifications in translation
level, CD44 protein appears as a wide range of varying mo-
lecular weights (80-250 kDa) due to the presence of multiple
glycoforms [25]. But to CD44 proteins reactive to the antibody
used in this work, the molecular weight is about 90 kDa. Obvi-
ously decreased expression of CD44 in pshCD44/PLGANPs
group compared with that in the control groups (Fig. 1 C).
This result was consistent with that of RT-PCR. To this end,
these findings demonstrated that CD44 expression could be
down-regulated effectively in mRNA and protein levels in vitro
by pshCD44/PLGANPs.

**pshCD44/PLGANPs inhibited tumor xenografts growth in nude mice.** To further assess the effectiveness of CD44
gene suppression in vivo, intraperitoneal tumors model were
established to evaluated inhibition of CD44 in tumor growth.
Tumor baring mice were treated with pshCD44/PLGANPs,
pshHK/PLGANPs or 0.9% NaCl every 3 days as detailed in the
materials and methods. The mice were sacrificed 3 days after
the final nanoparticles injection. All tumors were collected
and photographed.

In all mice, tumor nodules were scattered around the
pelvis. These lesions varied in size, but were larger and more
numerous in the two control groups (Fig. 2 A, B). The aver-
age weight was 1.46±0.28 g, 1.32±0.14 g and 0.73±0.18 g in
the untreated, pshHK/PLGANPs and pshCD44/PLGANPs
groups, respectively. Tumor weight in pshCD44/PLGANPs
group was reduced by 45% and 50% compared with in
pshHK/PLGANPs and 0.9% NaCl groups, respectively (Ps <
0.001) (Fig. 2 C). There are no significant differences between
two control groups (P > 0.05).

**Suppressions of CD44 genes in vivo.** Tumors from each
group were used for RT-PCR and western blot analyses. The
level of CD44 mRNA suppressed in pshCD44/PLGANPs
group by 68% and 69% when compared to pshHK/PLGANPs
and 0.9% NaCl groups (Fig. 3 A, B) (Ps < 0.001), respectively.
Western Blot confirm above results in protein level (Fig. 3 C).
In addition, immunochemical staining of CD44 in tumor
tissues further demonstrated the suppression of CD44 in the
tumors of pshCD44/PLGANPs treated mice (Fig. 3 D).

**Efficacies of cell proliferation, apoptosis and angio-
genesis by pshCD44/PLGANPs in vivo.** To explore the
mechanism of tumor suppression elicited by the pshCD44/
PLGANPs in vivo, we measured their effects on several
biological end points, including cell proliferation (Ki-67),
apoptosis (TUNEL), and antiangiogenesis.

Cell proliferation in tumor tissues was evaluated by Ki-67
staining. Minimal reduction of Ki-67 expression was observed
in pshCD44/PLGANPs group by 27.5% ± 3.7% when compared to pshHK/PLGANPs
and 0.9% NaCl groups (Fig. 4 A, B) (Ps < 0.001), respectively.
An imbalance of cell proliferation with apoptosis can
contribute to tumor growth and invasion. We therefore also
performed TUNEL staining to assay for apoptotic cells in

Figure 1. Down-regulation of CD44 expression using shRNA loaded in PLGANPs in vitro and in vivo. (A) and (B), RT-PCR on cDNA obtained from
SKOV-3 cells treated with shRNA nanoparticles against CD44. (C), Western Blot on protein abstract obtained from SKOV-3 cells treated with shRNA
nanoparticles against CD44. The housekeeper gene GAPDH used as internal control. M means marker used in RT-PCR. a, untreated (0.9% sodium
chloride) group; b, empty-vector (pshHK/PLGANPs) group; c, pshCD44/PLGANPs group. Data were expressed as means ± SD. * P < 0.05 versus the
two control groups, respectively.
Figure 2. Silencing CD44 inhibits intraperitoneal tumor xenografts growth in nude mice. (A) Intraperitoneal tumor nodules, of various sizes, were scattered in the pelvic and abdomen from each group. (B) All tumors were collected carefully and weighed immediately. a, untreated (0.9% sodium chloride) group; b, empty-vector (pshHK/PLGANPs) group; c, pshCD44/PLGANPs group. (C) Intraperitoneal tumor weight. Values are mean±SD. * $P < 0.001$ versus control groups.

Figure 3. Down-regulation of CD44 expression using shRNA loaded PLGANPs in vivo. (A) and (B), RT-PCR on cDNA obtained from tumors treated with shRNA nanoparticles against CD44. (C) Western Blot on protein abstract obtained from tumors treated with shRNA nanoparticles against CD44. (D) Immunostaining of CD44 in tumor tissue. Original magnification, ×400. The housekeeper gene GAPDH used as internal control. M means marker used in RT-PCR. a, untreated (0.9% NaCl) group; b, empty-vector (pshHK/PLGANPs) group; c, pshCD44/PLGANPs group. Data were expressed as means ± SD. * $P < 0.05$ versus the two control groups, respectively.
tumor tissues. A significant increase of apoptosis in pshCD44/PLGANPs group (16.5%±3.5%) compared with either pshHK/PLGANPs (3.3%±1.6%) or untreated group (2.1%±1.2%), respectively (Ps < 0.001) (Fig. 4 B).

Angiogenesis is important in tumorigenesis, so antiangiogenic therapies are important in the treatment of cancer. Although literature seldom demonstrates clear functions of CD44 in angiogenesis, there must be some roles of CD44 involved in. Because CD44 acts as an important adhesive factor on the surface of vascular endothelial cells, we therefore sought to evaluate the effects of suppressing CD44 protein on angiogenesis in vivo. To do so, we measured microvessel density (MVD) in endothelial cells of tumor sections by staining for the vascular marker CD31. In addition, we performed an alginate encapsulation test to evaluate vessel formation in vivo. The mean MVD was significantly decreased in tumors treated with the pshCD44/PLGANPs (MVD: 21 ± 3.6) compared with pshHK/PLGANP (MVD: 39 ± 4.5) and untreated group (MVD: 42 ± 4.4), respectively (Ps < 0.001) (Fig. 4 C). Furthermore, the inhibition of angiogenesis was evaluated by alginate encapsulation test. The alginate implants showed a significant reduction in the number of new vessels in the pshCD44/PLGANPs group, compared to the control groups. It is consistent with results of CD31 staining.

Toxicity observation. No gross abnormality was observed in treated mice of each group. Furthermore, H&E histological evaluation of the liver, lung, kidney, spleen, brain, heart, pancreas, intestine and bone marrow by two pathologists did not reveal any significant differences between treatment groups and control groups (data not show).

Discussion

By regulating the expressions of genes involved in tumorigenesis, gene therapy offers a unique method to change
the tumor and its microenvironment. shRNA has been successfully therapeutically used in many anti-cancer studies, including the treatment of ovarian cancer and was chosen in this study for its characteristics of easy preparation, relative stability and sustained expression compared to siRNA [26, 27, 28]. CD44 belongs to a large family of proteins that play an important role in cell-cell and cell-matrix adhesion and is expressed in various cells [29]. As being the primary receptor for hyaluronic acid (HA), CD44 plays an essential role in cell adhesion and cell migration [30]. CD44 is overexpressed in ovarian cancer and proved to be one marker of ovarian cancer stem cells [31]. These characteristics make CD44 to be an ideal target for shRNA gene therapy. Studies put insight to silencing CD44 gene in suppression growths of different tumors, including colon cancer, nasopharyngeal cancer and ovarian cancer [7, 8, 10]. But the transient suppression of tumor growth was shown as using siRNA targeting CD44 gene in ovarian cancer [10]. In this study, we demonstrate that silencing of CD44 with shRNA, expression stable and sustained more than siRNA, significantly inhibit the growth of human ovarian cancer xenografts in nude mice compared with controls, similar to previously reported results [10]. Other unknown functions of silencing CD44, including influence of cell proliferation, cell apoptosis and antiangiogenesis, were explored in tumor progression and metastasis.

An effective and safe delivery system is essential for the application of gene therapy. Although many viral vectors demonstrate high transduction efficacy, toxicity continues to be a concern during clinical application [30]. Over the last decade, studies were done and improvements in non-viral vectors made some excellent non-viral vectors stand out, which were proved to owe some advantages than viral vectors [13-15]. PLGA is a relatively new carrier system being employed to deliver nutrition to malignant tumors [24, 31]. As a non-viral vector, PLGA is attractive for plenty of virtues. Firstly, PLGA nanoparticles exhibit excellent biocompatibility and low cytotoxicity in vivo. Secondly, PLGA nanoparticles are slowly released in vivo, which may increase the half-life and efficacy of the drugs or genes being delivered. Finally, PLGA are harmlessly biodegraded into CO₂ and H₂O after exerting their functions in vivo [16, 18, 22]. We previously demonstrated that, PLGANPs can be used to deliver genes into tumor cells [20, 32]. In the present study, PLGANPs were used to deliver the recombinant shRNA plasmid targeting CD44 gene in SKOV-3 derived tumors of nude mice. As a result, the expression of CD44 was lessened in the tumors treated with pshCD44/PLGANPs compared with the control tumors, indicated by RT-PCR, western blot and immunohistochemistry analysis. The biologic features of PLGA have been studied extensively. Knocking down CD44 expression suppressed tumor growth in vivo, indicating that PLGANPs could deliver a large component plasmid into tumor cells successfully. No toxicity was observed in this study, suggesting that the PLGANPs should be safe in gene therapy. However, further toxicity assessment should be performed.

To elucidate the antitumor mechanism of CD44 in vivo, several biologic points of tumorigenesis were explored including cell proliferation, cell apoptosis and angiogenesis. Data from Ki67 staining and TUNEL assay suggested that silencing CD44 caused in significant reduction of cell proliferation and increase of cell apoptosis compared with control groups. Cells treated with siRNA against CD44 exhibit increased apoptosis compared with control siRNA [10]. Antitumor efficacy of CD44 silencing therapy was bases on disassembling interaction of CD44 and hyaluronic acid therefore increasing of apoptosis [30]. Using shRNA, we knocked down CD44 expression and reconfirmed those results in above studies. Angiogenesis was assessed by CD31 staining and alginate encapsulation test. A significant reduction in new vessels was observed in tumors receiving pshCD44/PLGANPs treatment, which could be one of major mechanisms in inhibition of tumor growth, but the role of CD44 is less clearly revealed. Antiangiogenesis is a complex procedure and contains of multiple steps in procedures. It is likely that induction of apoptosis in endothelial cells may be one mechanism by which CD44 knockdown can influence tumor angiogenesis. It is consistent with the result of one report that cells treated with siRNA against CD44 exhibit increased apoptosis compared with control siRNA [10]. CD44 is involved in the adhesion of endothelial cells to the vascular matrix, potentially explaining why we observed less vascularization in the pshCD44/PLGANPs treated tumors. This is consistent with a prior report that described how the multimeric signaling complex of CD44 and its related receptor tyrosine kinases are involved in angiogenesis [33]. In addition, preventing the binding of hyaluronidase to CD44 increases apoptosis [31]. However, the actual role of CD44 in angiogenesis is unclear and further study is needed to address this question. All above results indicated that antitumor effect maybe in part due to influence of antiangiogenesis, decrease of proliferation, and induction of apoptosis by knocking down of CD44.

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References


SILENCING CD44 IN OVARIAN CANCER


