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Therapeutic Effect of Glypican-3 Gene Silencing Using siRNA for Ovarian Cancer in a Murine Peritoneal Dissemination Model

Mai Hazekawa, Takuya Nishinakagawa,
Tomoyo Kawakubo-Yasukochi and Manabu Nakashima

Abstract

Ovarian cancer is known to be the most lethal gynecologic cancer. It has been reported that Glypican-3 (Gpc3) expression induces immune responses, promotes the progression in ovarian cancer. Then, we focused on this Gpc3 gene silencing, tried to prepare siRNA delivery system. In this chapter, we introduce one of the therapeutic proposals in terms of novel drug delivery system using siRNA as a targeting medicine. This chapter introduces our works about preparation of siRNA-PLGA hybrid micelles to deliver the siRNA into the ovarian cancer cells and to evaluate gene silencing effects in mice model. As a result, siRNA-PLGA hybrid micelles were shown to effectively inhibit Gpc3 expression *in vitro*. In addition, siRNA-PLGA hybrid micelles also decreased the number of tumor nodes in the mesentery *in vivo*. These results suggested that Gpc3 could be a target molecule for ovarian cancer treatment and siRNA-PLGA hybrid micelles could be an effective siRNA delivery tool even *in vivo*.

Keywords: siRNA, ovarian, Glypican-3, micelle, PLGA

1. Introduction

Epithelial ovarian carcinoma (EOC) is the most lethal gynecological malignancy. EOC accounts for about 90% of all ovarian cancers and distributed over the most common histotypes: high-grade serous (HGSC, 70%), low-grade serous (LGSC, < 5%), endometrioid (EC, 10%), mucinous (MC, 3–4%) and clear cell ovarian carcinoma (CCC, 10%) [1]. Five-year survival rates differ significantly across the histotypes, with drastically lower survival rates for serous carcinoma (SC (HGSC and LGSC), 43%) compared with EC (82%), MC (71%) and CCC (66%) in the USA. CCC is a comparatively rare tumor, depending on the geographic location. In west countries, OCCC represents <10% of all EOC. In contrast, the incidence of CCC

was reportedly 25% of EOC in Japan. The high number of patients (80%) with SC is diagnosed at advanced stages (stages III and IV). While, CCC which has the second number of patients (25%) after SC, is predominantly diagnosed at stage I (65%) [2]. Thus, CCC has different character compared with SC. Five-year survival rate at stage I for SC and CCC is same (80%). While, five-year survival rate at stage IV for SC is 40% and stage I of CCC is 25%. CCC has a very poor prognosis. One of the reasons is that CCC is associated with greater chemoresistance and a poorer prognosis compared with other EOC subtypes. Particularly for recurrent CCC, the response rate (RR) to salvage chemotherapy was extremely low. Previous studies have indicated that high L-type amino acid transporter 1 (LAT1), which belongs to system L, a Na⁺-independent carrier that transports large neutral amino acids, expression was associated with poorer prognosis and chemoresistance in CCC [3]. Furthermore, hepatocyte nuclear factor 1 β (HNF1 β) and glutaminolysis contribute for the chemoresistance to platinum-based antineoplastic agents of CCC through the intrinsically increased glutathione (GSH) bioavailability [4]. Therefore, novel and innovative strategies are required to improve outcomes for patients with CCC that is refractory to chemotherapy.

Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans. GPC3 regulates cell proliferation signals by binding growth factors such as Wnt, fibroblast growth factor, and insulin-like growth factor and plays an important role in the proliferation and differentiation of embryonic cells [5–7]. GPC3 is expressed in various fetal tissues (liver, lung, kidney, and placenta) but is not detected in normal postnatal tissue due to DNA methylation-induced epigenetic silencing [8, 9]. While, previous studies showed that GPC3 was overexpressed in several malignant tumors, including hepatocellular carcinoma (HCC), CCC and melanoma. Particularly, GPC3 is detected in $\geq 80\%$ of patients with HCC caused by hepatitis B or C [10, 11]. The function of membrane-anchored GPC3 in these cancers is unknown, but it is likely involved in the neoplastic transformation of HCC [12]. Membrane-bound GPC3 can be cleaved and secreted into the blood. Mammalian GPC family members are cleaved at GPI anchor level by endogenous GPI phospholipase D [13]. Thus, various forms of GPC3 protein are present in blood, although their functions remain unclear. Given these features, GPC3 is useful not only as a target for cancer immunotherapy but also as a novel tumor marker.

Small interfering or silencing RNA (siRNA) technologies are based on the inhibition of gene expression or translation by siRNAs targeting messenger RNA selectively [14]. Gene interference therapy using siRNA has great potential for treatment of wide variety of diseases [15], ranging from cancer [16–19] to viral infection [20, 21] and brain disorder [22, 23]. The benefit of applying this technology to cancer therapy is that siRNA can target genes which are specific for tumor cells, leaving healthy, non-tumor tissue unaffected. Despite their medical potential, the clinical translation of siRNA technologies has up to now been limited. This limited progress is due to the difficulties of delivering siRNA *in vivo*. Unprotected siRNAs are easily degraded in the bloodstream, and siRNAs alone do not translocate across cell membrane [24]. In addition, it has been reported that siRNAs can be immunogenic [25]. Therefore, safe and efficient carriers must be developed for siRNA delivery to protect siRNA from nuclease action and at the same time triggers intracellular uptake *in vivo* [26, 27].

In our previous study, we prepared slow release formulation using biodegradable polymer (poly(lactide-co-glycolide), PLGA) such as micro-/nano particles [28]. Recently, we engaged to prepare the siRNA delivery system using PLGA for anti-metastasis therapy.

In this chapter, we report the therapeutic effect of Gpc3 gene silencing in ovarian cancer, and introduce the finding about a novel siRNA delivery system of micelles for nucleic acid therapy based on our data [29].

2. Effect of anti-metastasis in ovarian cancer caused by Glypican-3 gene silencing

2.1 Role of Glypican-3 in ovarian cancer

GPC3, 55–65 kDa protein consisting of 580 amino acids, is a heparan sulfate chain proteoglycan (HSPGs) bound to cell membrane by a glycosylphosphatidylinositol (GPI) anchor. This protein is expressed in the liver and kidney of healthy fetuses but is hardly expressed in adults, except in the placenta. Loss of function mutations of GPC3 leads to Simpson-Golabi-Behmel syndrome (SGBS), a rare X-linked disorder (X chromosome, Xq26) with significant overgrowth [5], which has also been observed in GPC3-null mice [30] because the gene shows high homology between humans and mice. GPC3 is expressed ubiquitously in the embryo but is reduced in the central nervous system (CNS) in adults [31]. Thus, GPC3 is considered to be one of the factors affecting prenatal development and metabolism originally. On the other hand, GPC3 is especially overexpressed in HCC [10, 11], CCC [32, 33], melanoma [34], and lung cancer [35]. Although the precious function of GPC3 remains unclear, it has been strongly suggested that it is related to the malignant transformation, accelerating cell growth and increasing inflammatory reaction [36].

The Wnt/Frizzled/ β -catenin pathway is activated in about 50% of HCCs. Wt3a has been shown to mediate the GPC3-induced growth of HCCs via the canonical Wnt/ β -catenin pathway [6, 37]. Sulfated heparan sulfate glycosaminoglycan (HSGAG) chains of GPC3 and other HSPGs are potential substrates for desulfation at the 6-O position by human sulfate 2 (SULF2). It has been reported that SULF2 activates Wnt/ β -catenin signaling in HCC cells, and this process is GPC3-dependent and can be independent of exogenous Wnts [38]. In a previous study, a human monoclonal antibody against GPC3 inhibited Wnt3a/ β -catenin signaling in HCC cells and antitumor activity *in vivo* [39]. Furthermore, blocking the heparan sulfate chains on GPC3 with human monoclonal antibody against GPC3 also reduced c-Met activation in hepatocyte growth factor (HGF)-treated HCC cells and 3D-cultured spheroids. GPC3 is involved in HCC cell migration and motility through HS chain-mediated cooperation with the HGF/Met pathway [40].

Although the role of GPC3 in HCC has been reported little by little, the role of GPC3 in ovarian cancer, especially CCC expressed GPC3, has been remained unclear. So recurrent or persistent CCC has been reported as having a potentially chemoresistant phenotype against conventional cytotoxic agents, leading to poorer prognosis. Thus, novel treatment approaches must be adopted for CCC. With compelling evidence that EOC is an immunogenic tumor, immunotherapeutic approaches are currently being evaluated and should be optimized based on histology-specific features. Previous research also suggested that GPC3 peptide vaccinations may hold a significant impact to prolong survival of patients with refractory CCC, allowing them to maintain quality of life with no serious toxicities [41].

Based on these, we focused on knocking down of GPC3 gene therapy for ovarian cancer using siRNA which can be expected to be effective in clinical practice. Then, we evaluated the efficiency of siRNA-PLGA hybrid micelles targeted to Gpc3 on

ovarian cancer *in vitro* and examined its antitumor effects *in vivo* in a mouse peritoneal dissemination model.

2.2 Effect of anti-metastasis caused by knocking down of Gypican-3 using LPEI coating siRNA-PLGA hybrid micelles *in vivo*

The synthesis of siRNA-PLGA hybrid was described briefly as follows. PLGA was activated by DCC and NHS. Activated PLGA reacted with 3-(2-pyridyldithio) propionyl hydrazide (PDPH) as a cross-linker. After PDPH activated, PLGA (PLGA-PDPH) was used for siRNA conjugation. A thiol-modified double-strand siRNA was reacted with PLGA-PDPH, siRNA-PLGA hybrid was synthesized via a disulfide exchange reaction. The synthesized siRNA-PLGA hybrid conjugates spontaneously formed self-assembled micelles in aqueous solutions, resulting to form micelle with siRNA side facing the outer shell as shown in **Figure 1A** and **C**. Furthermore, we also prepared liner polyethylenimine (LPEI)-coated siRNA-PLGA micelles, its surface was positive charged by cationic polymer, to increase the efficiency of intracellular uptake as shown in **Figure 1D**.

Measurement of critical micelle concentration (**Figure 2**) and distribution of particle (**Figure 3**) were performed to evaluate the physical properties of micelles. The mean diameter and zeta potential of siRNA-PLGA hybrid micelles were about 110 nm and about -40 mV, respectively. The zeta potentials of siRNA-PLGA hybrid micelle were changed from negative charge to positive charge by LPEI coating.

Until now, the best agents for siRNA delivery are cationic lipids and polycations, i.e. polyelectrolytes bearing multiple positive charges to increase intracellular uptake *in vivo* [42, 43]. From these previous data, LPEI coating micelle can be expected its clinical potential *in vivo* because positive charge caused by LPEI makes micelles easy to be taken into the cell.

The GPC3 levels in HM-1 cell line, which is mouse ovarian cancer cell line, treated with siRNA-PLGA hybrid micelles were then evaluated by western blotting.

Figure 1.

(A) and (B) Structure of siRNA-PLGA hybrid and Fab'-PLGA hybrid via a cleavable disulfide linkage.
(C)–(E) Schematic diagram for siRNA-PLGA hybrid micelle structure in an aqueous environment.

Figure 2.
Critical micelle concentration (CMC) detected by measuring the relative excitation intensity ratio of pyrene at emission of 329 nm and 338 nm (I_{338}/I_{329}).

Figure 3.
Size distribution of siRNA-PLGA hybrid micelles.

As shown in **Figure 4**, siRNA-PLGA hybrid micelles significantly suppressed GPC3 expression compared with the control.

Assessment of antitumor effects of these micelles in a murine peritoneal dissemination model was performed by intraperitoneal (i.p.) injection as topical treatment. In general, topical administration is often more effective because it is easy to react since the medicine is close to the disease lesion [44]. The number of disseminated nodules and the peritoneal fluid volumes were evaluated at 15 days after injection of the HM-1 cells. As shown in **Figure 5**, the number of disseminated nodules and the volume of peritoneal fluid siRNA-PLGA hybrid micelle-treated groups were significantly low compared with the control. Next, GPC3 levels in the cell lysates of peritoneal cells collected from the peritoneal fluid were evaluated by western blotting.

As shown in **Figure 6**, the levels of IFN- γ , IL-6, and TNF- α in mice treated with uncoated and LPEI-coated siRNA-PLGA hybrid micelles were significantly suppressed compared with the control. GPC3 expression in the lymphocytes such as B cells, T cells and macrophages in the peritoneal fluid of mice, was detected by western blotting. From these results, there is a possibility that the therapeutic effect was induced by GPC3 gene knockdown of not only cancer cell but also lymphocytes in the peritoneal fluid as the additive effects.

Figure 4. Western blot analysis of GPC3 levels in HM-1 cells treated with siRNA-PLGA hybrid micelles in vitro. Data represent the mean $\pm$ SD ($n = 3$). $**p < 0.01$ versus the control group (Bonferroni test/ANOVA). Cited from Ref. [29]. Reprinted with permission from Elsevier.

Figure 5. Anti-metastasis effects of siRNA-PLGA micelles in a mouse peritoneal dissemination model. Representative images of the mesentery after laparotomy. Cited from Ref. [29]. Reprinted with permission from Elsevier.

2.3 Recognition of cancer cell using Fab'-PLGA/siRNA-PLGA hybrid mixed micelle *in vitro*

In previous study, we reported that Gpc3 knocking down using siRNA-PLGA hybrid micelle by intraperitoneal injection was effective to suppress the metastasis in peritoneal dissemination of ovarian cancer mice model [29]. However, it is

Figure 6. Effect of GPC3 knockdown caused by treatment with siRNA-PLGA micelles on the secretion of IFN- γ , IL-6, TNF- α in the peritoneal fluid in a mouse peritoneal dissemination model. Data represent the mean $\pm$ SD ($n = 5$). ** $p < 0.01$ versus the control group (Bonferroni test/ANOVA). Cited from Ref. [29]. Reprinted with permission from Elsevier.

Figure 7. Efficiency of intracellular uptake of Fab'-PLGA/Alexa 488 labeling siRNA-PLGA hybrid mixed micelles *in vitro* by flow cytometry analysis.

necessary to develop a carrier which is “targeting” and “systemically administable”. That is why, we prepared Fab'-PLGA/siRNA-PLGA mixed micelle to recognize the target cell. Fab'-PLGA hybrid was synthesized in a same method as siRNA-PLGA hybrid was synthesized. The drug design was described in **Figure 1B** and **E**.

As shown in **Figure 7**, *in vitro* experiment, intracellular uptake of siRNA using Fab'-PLGA/siRNA-PLGA mixed micelle was significantly increased compared with control. In particular, cytotoxicity was accelerated caused by treatment with Fab'-PLGA/siRNA-PLGA mixed micelle compared with siRNA-PLGA hybrid micelle. This result suggests that the characteristics of the targeting used by antibody may be expected to have an additive effect of the function of Fab' itself in addition to the increase in the intracellular uptake efficiency by cell recognition. In some antibodies, the target protein knockdown effect is dramatically obtained using Fab'-PLGA/siRNA-PLGA mixed micelle (data not shown). From these results, Fab'-PLGA/siRNA-PLGA mixed micelles are believed to be useful as one of the targeting formulations to recognize the target cell.

3. Expected side effect caused by gene therapy and limitation of assessment using animal

3.1 Off-target effects caused by RNAi

The technique of RNAi in the medical field is expected to have not only therapeutic effects for human induced by knock-down specific genes but also suffers from off-target effects. Previous study reported that algorithm or open-source desktop software was developed to design RNAi sequences to exert strong and selective suppression of target genes and predict off-target [45, 46]. However, it is difficult to predict specific side effects that appear due to off-target effects in human. Furthermore, we suggested that the details of the off-target effect are often unclear due to the fact that commercial nucleic acid medications have a short period of use. In some cases, mouse results may not be compatible with humans because off-target effects vary by its sequences though there were no noticeable side effects in our experiment *in vivo*.

3.2 Cytotoxicity of exogenous siRNA or polymer in development of formulation

Until now, some polyplex or lipoplex with high membrane permeability formulations have been used for siRNA delivery system [47, 48]. A number of polymers have been popularly utilized to form stable and nanocomplexes with its cytotoxicity problem [27, 49–53]. PEI is also probably the most frequently used polycation in gene delivery, our LPEI-coated micelles did not exhibit cytotoxic effects. The fact that no toxicity was found in our experiments at the concentrations we used was consist with previous reports [54]. The greatest feature of this micelle is that it consists of a safe polymer, PLGA. PLGA is known as one of the biodegradable polymers used in marketed medication [55]. In some cases, siRNA can be immunogenic such as virus vectors induce multiple component of the immune response, cytotoxic T-lymphocyte (CTL) response can be elicited against viral gene products of exogenous transgene products [25]. Regarding the immunogenicity of this micelle, it is unlikely that immunogenicity was shown due to the fact that cytokines in the peritoneal fluid were suppressed.

3.3 Limitation of assessment using animal

In the future as a next step, immunodeficient mice would be indispensable when we establish human model such as patient-derived xenograft (PDX) model. However, there is possibility that we cannot comprehend whether the micelle has medical potential when immunodeficient mice are used because GPC3 might be a molecule that is strongly associated with the immune system. That is why, we considered that we should further examine the usefulness of this therapy using micelles for human cancer cells based on our data using murine cell because there are different characteristics between murine and human cancer cells.

4. Conclusion

In conclusion, our results could indicate that Gpc3 gene silencing using siRNA has a possibility as an effective new therapeutic approach without side effects in ovarian cancer, especially CCC with GPC3 expression. Furthermore, this GPC3 targeting gene therapy is also useful for high GPC3 expression cancer such as HCC, melanoma and lung cancer if appropriate carrier is developed to deliver siRNA to target cancer cell by i.v. in the future.

In addition, this finding is the first study to show that siRNA-PLGA hybrid micelles can effectively deliver siRNA to cancer cells *in vivo* at a low dose with significant anti-metastatic effect on murine ovarian cancer. We expect that novel formulation with more specific effects like siRNA including drug delivery system would be developed for malignant ovarian cancer therapy in the future.

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Mai Hazekawa*, Takuya Nishinakagawa, Tomoyo Kawakubo-Yasukochi and Manabu Nakashima

Department of Immunological and Molecular Pharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan

*Address all correspondence to: mhaze@fukuoka-u.ac.jp

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