undergoing intensive investigation by many researchers [5]. GCV is converted into a monophosphate form by phosphorylation, which can be further converted into a triphosphate form by endogenous mammalian enzymes. Triphosphate-GCV changes to a toxic nucleotide analogue, which inhibits DNA replication [6].

Various vectors for gene transfer have been developed and investigated both in vitro and in vivo. However, it is difficult to find a single method that meets all the conditions for an ideal gene transfer and vector expression. There are many concerns regarding the safety of vectors and the complicated pharmacodynamics involved which must be resolved. Viral vectors like retroviruses and adenoviruses, which have high transgene expression efficiency, have been investigated for a long time [7]. Unfortunately, they have many defects. The limitations of viral gene therapies relate to the residual viral elements within the viral vectors that can be immunogenic, cytopathic or recombigenic [8]. Traditional nonviral chemical and physical methods such as calcium phosphate precipitation [9], administration of DEAE dextran [10] and electroporation [11] have relatively low efficiency. Furthermore, they are not well suited for in vivo use and are difficult to use for transfection of a large quantity of cells.

Cationic liposomes are nonviral vectors [12], and new derivatives of cationic lipid are being developed at a fast pace. Cationic liposomes are superior to viral vectors in terms of reproducibility, simplicity and safety of use. In addition, they do not invoke an immune response or protooncogene activation [13,14]. However, they are inferior to viral vectors in transfection efficiency. Most commercially available cationic liposomes were invented for in vitro rather than in vivo use, so they have low transfection efficiency under serum-containing conditions and are currently unsuitable for clinical use.

We previously developed and reported a series of new cationic liposomes containing *O,O'*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6-14) (Fig. 1), which have transgene activities in serum-containing medium for disseminated intraperitoneal tumors [15]. In this report, we describe the use of one of these novel cationic liposomes, composed of DC-6-14 and dioleoylphosphatidylethanolamine (DOPE) in a molar ratio of 5:2, for gene therapy. Furthermore, we describe its transgene expression efficiency and anti-tumor effect under serum-containing conditions. This was done in vitro but we also show significant prolongation of survival in vivo in comparison with commercially available cationic liposomes.

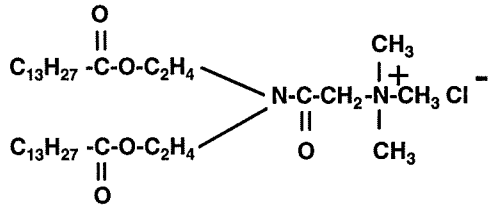

Fig. 1. Chemical structure of cationic lipid DC-6-14, *O,O'*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl) diethanolamine chloride. A powerful positive charge derived from trimethylammonium gives the affinity for DNA.

2. Materials and methods

2.1. Cell lines

Six human cancer cell lines (HRA, ES-2, mEIIL, KF, SW626 and SKOV-3) were used in vitro, and two cell lines (HRA and mEIIL) were used in vivo. The HRA and KF cells were both derived from serous cystadenocarcinoma of the ovary [16,17] and were kindly provided by Dr. Yoshihiro Kikuchi (National Defense Medical College, Tokorozawa, Japan). The ES-2, SW626 and SKOV-3 cells were derived from human ovarian carcinoma [18–20] and were purchased from the American Type Culture Collection. The mEIIL (metastatic Estrogen Independent Ishikawa Line) cells were human endometrial adenocarcinoma cells [21], which were derived from the Ishikawa line [22] and were kindly provided by Dr. Hideki Sakamoto (Nihon University, Tokyo, Japan). All six cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, Immuno-Biological Laboratories, Fujioka, Japan) supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA).

2.2. Preparation of novel cationic liposomes

We prepared cationic liposomes labeled GTE-319 for gene transfer into cancer cells. DC-6-14, a positively charged lipid was purchased from Sogo Pharmaceutical Co., Ltd. (Tokyo, Japan). A powerful positive charge derived from trimethylammonium gives the affinity for DNA. Dioleoylphosphatidylethanolamine (DOPE), a neutral helper lipid and essential to the formation of stable liposomes [23], was obtained from Nippon Oil and Fats (Tokyo, Japan). We prepared positively charged liposomes as mixtures with neutral helper lipids. A molar ratio of 5:2 was used for DC6-14/DOPE.

Liposomes were prepared as freeze-dried empty liposomes using a method described previously [15]. Briefly, DC6-14 and DOPE were dissolved in a chloroform–methanol mixture (4:1, v/v), and the solvent was removed in a rotary evaporator. After the lipids were mixed with 9% sucrose aqueous solution, they were hydrated at 60–70 °C and extruded through a polyvinylidenedifluoride membrane filter with 0.22 μm pore size by using a Liponizer LP-90 (Nomura Micro Science, Kanagawa, Japan). The dispersion was pipetted into glass vials (0.5 or 2 ml portion each) and lyophilized in a freeze-drier (Virtis, NY, USA). The dried liposomes were then reconstituted with 0.5 or 2 ml of distilled water by a gentle mixing prior to transfection.

As a control, we used the commercially available cationic liposomes DMRIE-C and LipofectAMINE (Life Technologies, Inc., Rockville, MD, USA).

2.3. Construction of expression plasmids

Dr. Jun-ichi Miyazaki (Osaka University, Osaka) generously provided the expression plasmid pCAG-lacZ. The pCAG vector expresses an inserted DNA by its CAG promoter, which consists of the cytomegalovirus immediate early enhancer sequence and the chicken β-actin/rabbit β-globin hybrid promoter [24]. The HSV-tk gene was derived from plasmid pHSV-106 (Life

Technologies, Inc.) [25] by polymerase chain reaction (PCR) amplification using the primers 5′-GCC-CGA-ATT-CTA-GAA-GCG-CGT-ATG-GCT-TCG-3′ (sense) and 5′-CCG-CGA-ATT-CCC-GTG-TTT-CAG-TTA-GCC-TCC-3′ (anti-sense). The PCR products were purified by a Microspin S-400HR column (Pharmacia Biotech, Tokyo, Japan) after digestion with *Eco*RI. The lacZ gene of the pCAG-lacZ was removed by digestion with EcoRI. We designed the pCAG-HSVtk plasmid, which was constructed by replacing the lacZ gene in the pCAG-lacZ with the HSV-tk cDNA fragment.

2.4. In vitro transfer of the lacZ gene and X-Gal staining

Cells were seeded on 6-well plates (Corning-Coaster, Cambridge, MA, USA) and incubated to reach about 30–50% confluence at 37 °C in 5% CO_2. One hundred microliters of serum-free DMEM containing 1 μg of the lacZ gene was mixed with 100 μl of serum-free DMEM containing an appropriate amount of GTE-319 suspensions (10 nmol of DC-6-14), DMRIE-C suspensions (10 nmol of DMLIE: 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) or LipofectAMINE suspensions (5 nmol of DOSPA: 2.3-dioleyloxy-*N*-[2 (spermine carboxyamido) ethyl]-*N*,*N*-dimethyl-1-propanaminiumtrifluoroacetate) with mild agitation in polystyrene tubes and incubated for 15 min at room temperature. Prior to transfection, 800 μl of DMEM containing 12.5% FBS (final conc. 10% FBS) were added. Transfection was also performed under serum-free conditions. Cells were washed with serum-free medium and added to 1 ml of liposomes/plasmid complexes. After 5-h incubation at 37 °C, the old medium was removed, then cells were washed, added to serum-containing medium and cultured for 48 h. To evaluate transfection efficiency, 5-bromo-4-chloro-3-iodolyl β-D-galactoside (X-Gal, Life Technologies, Inc.) staining assay was performed as previously described elsewhere [26]. Percentages of lacZ expression cells were determined by counting at least 1000 cells with a microscope.

2.5. In vitro sensitivity to GCV

To evaluate the effect of the HSV-tk gene transfection with GTE-319 and GCV administration in vitro, the cells were seeded on 96-well plates (Greiner Labortechnik GmbH, Frickenhausen, Germany) at $1–3\times10^3$ cells per 80 μl of DMEM containing 12.5% FBS and incubated for 24 h. An equal volume of the lacZ gene or the HSV-tk gene (1–10 μg/ml in serum-free DMEM) and GTE-319 suspensions (cationic lipid 10–100 nmol/ in serum-free DMEM) were mixed and incubated for 15 min at room temperature. Twenty microliters of liposomes/plasmid complexes was added to each well. After 5 h of incubation at 37 °C, the old medium was aspirated with a 23-gauge needle, and 200 μl of DMEM containing 10% FBS with 0, 0.001, 0.01, 0.1, 1, 10, 100 and 1000 μg/ml of GCV were added to each well. For evaluation of the anti-tumor effect in vitro, MTT assay was performed [27] after 96–144-h incubation. The IC50 value was determined by extrapolation and detailed graphically. The therapeutic index was determined by comparing the IC50 values of the lacZ gene-treated cells and HSV-tk gene-treated cells (lacZ IC50/HSV-tk IC50).

2.6. Animals

Five-week-old female CD-1 nu/nu athymic nude mice weighing 20–30 g were purchased from Charles River Japan (Yokohama, Japan) and maintained in a specific pathogen-free environment. When necessary, the animals were killed or their ascites was aspirated from their peritoneal cavities.

2.7. In vivo transfer of the lacZ gene and X-Gal staining

Viable HRA cells, 6×10^7 in 1 ml of DMEM containing 10% FBS, were inoculated into intraperitoneal cavities of nude mice with a 26-gauge needle. Two days after HRA cell inoculation, the pCAG-lacZ was transferred into cancer cells in the peritoneal cavity. Five hundred microliters of serum-free DMEM containing 20μg of the pCAG-lacZ was mixed with 500 μl of serum-free DMEM containing 200 nmol of GTE-319 suspension and incubated for 2 min at room temperature, and then mice were injected with 1 ml of the liposomes/plasmid complexes. Twenty-four hours later, we injected saline into the intraperitoneal cavity and ascites were aspirated from the mice intraperitoneal cavities. To remove red blood cells, the pellets were suspended in 0.15 M NH_4Cl–1 mM KCL–0.1 mM Na_2EDTA, pH 7.4 after centrifugation at 1000 rpm for 5 min. Then 3×10^5 HRA cells were seeded onto 6-well plates and incubated for 24 h. X-Gal staining was performed the same as in the in vitro experiment.

2.8. Intraperitoneal transfection of the HSV-tk and the lacZ gene to the nude mice and GCV administration

Viable HRA cells, 3×10^5, and 5×10^6 mEIIL cells in 1 ml of DMEM containing 10% FBS were inoculated into the peritoneal cavities of nude mice with a 26-gauge needle. The mice were

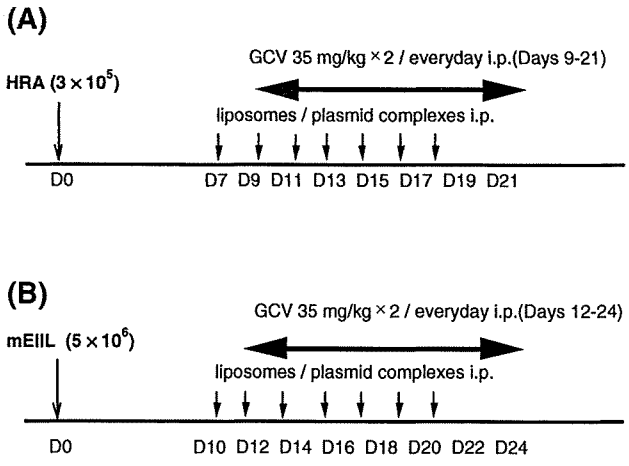

Fig. 2. Protocol of the transfection and GCV treatment of malignant cells disseminated in the mouse peritoneal cavity. (A) After HRA cells were inoculated on day 0, liposomes/plasmid complexes were injected i.p. every 2 days from day 7 to day 19 and GCV was injected i.p. twice a day from day 9 to day 21. (B) After mEIIL cells were inoculated on day 0, liposomes/plasmid complexes were injected i.p. every 2 days from day 12 to day 24 and GCV was injected i.p. twice a day from day 14 to day 26.

Table 1
In vitro transfection efficiency cells in the presence or absence of serum

	Transfection efficiency (%)					
	Serum (−)			Serum (+)		
Cell line	GTE-319	DMRIE-C	LipofectAMINE	GTE-319	DMRIE-C	LipofectAMINE
HRA	20.9	9.6	11.8	9.1	1.3	4.7
mEIIL	11.0	12.6	20.5	4.4	4.0	0.6
ES-2	17.3	17.7	26.0	13.1	10.1	3.3
KF	12.9	9.4	16.1	9.4	0.6	0.1
SKOV-3	17.4	13.1	5.4	1.9	0.2	0.2
SW626	16.8	9.5	17.9	18.1	4.2	0.2

divided into two groups: the HSV-tk treatment group and the lacZ treatment group. Seven days after HRA cell inoculation (Fig. 2A) and 10 days after mEIIL cell inoculation (Fig. 2B), gene therapy was started. Twenty micrograms of plasmid in 500 μl of serum-free DMEM and 200 nmol of cationic lipid in 500 μl of serum-free DMEM were gently mixed. One milliliter of liposomes/plasmid complexes was injected i.p. every 2 days seven times. Intraperitoneal injections of GCV (35 mg/kg) were performed twice a day for 13 days starting 2 days after the first injection of liposomes/plasmid complexes. The animals' body weight was measured every 3 days during GCV treatment, and animals were observed daily until death. Results are represented by the Kaplan–Meier method. The Wilcoxson method was used for statistical analysis.

3. Results

3.1. Transfection efficiencies of the lacZ gene in vitro

Liposomes/plasmid complexes were transferred into the cells in the absence or presence of serum 24 h after the cell had been seeded to a 6-well plate. Forty-eight hours after transfection of the lacZ gene in the absence or presence of 10% FBS, we performed X-Gal staining. Under a microscope, the lacZ gene expression cells appeared to be made of blue cytoplasm. We evaluated transfection efficiency by counting the percentages of blue-dyed cells, and at least 1000 cells were counted from each cell line. The results of the above experiment are shown in Table 1, and data represent the means of three experiments.

In the absence of serum, both commercially available cationic liposomes and GTE-319 showed comparable high expression efficiency: 11.0–20.9% in GTE-319, 9.4–17.7% in DMRIE-C and 5.4–26.0% in LipofectAMINE in all six cell lines. In the presence of 10% serum, the transfection efficiency of GTE-319 was more effective (1.9–18.1% efficiency) than that of commercially available cationic liposome reagents, which showed a marked decrease of transfection efficiency: 1.3–10.1% in DMRIE-C and 0.1–4.7% in LipofectAMINE.

3.2. In vitro sensitivity to GCV

In the case of the cells transferred with pCAG-HSVtk plasmid in the presence of 10% FBS, the IC50 values of GCV for HRA, mEIIL, ES-2, KF, SKOV-3 and SW626 cells were 0.025, 1.3, 0.062, 0.284, 3.8 and 2.05 μg/ml, respectively. In contrast, in the case of the pCAG-lacZ-transfected cells, the IC50 values of GCV for HRA, mEIIL, ES-2, KF, SKOV-3 and SW626 cells were 16, 62, 6.2, 25.5, 490 and 96 μg/ml, respectively. This is summarized in Table 2. The cells transfected with the HSV-tk gene had 640, 48, 100, 90, 129 and 47, respectively, times as much sensitivity as the cells transfected with the lacZ gene. What is evident from the table is that suicidal gene therapy by the HSV-tk/GCV system using our novel cationic liposomes was an effective method for treatment of malignant cells in vitro.

3.3. Transfection efficiencies of the lacZ gene in vivo

The methods detailed in Materials and methods determined the percentages of transfection efficiency. The percentage of lacZ-positive cells in HRA was 4.4±1.0% (n=3). Transfection efficiency in vivo was about half of the efficiency achieved in vitro.

3.4. Prolongation of survival of animals with intraperitoneal injection of the HSV-tk gene and GCV

HRA ascitic mice (n=12) were followed for 70 days, and the mean survival of the HSV-tk gene/GCV-treated group and the lacZ gene/GCV-treated group was 51.6 and 35.6 days, respectively. Kaplan–Meier survival curves are shown in Fig. 3A. The mEIIL ascitic mice (n=8) were followed for 85 days, and the mean survival of the HSV-tk gene/GCV-treated group and the lacZ gene/GCV-treated group were 76.9 and 62.1 days, respectively (Fig. 3B). In both cell lines, the mean survival of the HSV-tk treatment group was significantly

Table 2
In vitro sensitivity of cancer cells introduced by the HSV-tk gene to GCV

Cell line	HSV-tk	lacZ	Therapeutic index
	IC50 (μg/ml)		
HRA	0.025	16	640
mEIIL	1.3	62	48
ES-2	0.062	6.2	100
KF	0.284	25.5	90
SKOV-3	3.8	490	129
SW626	2.05	96	47

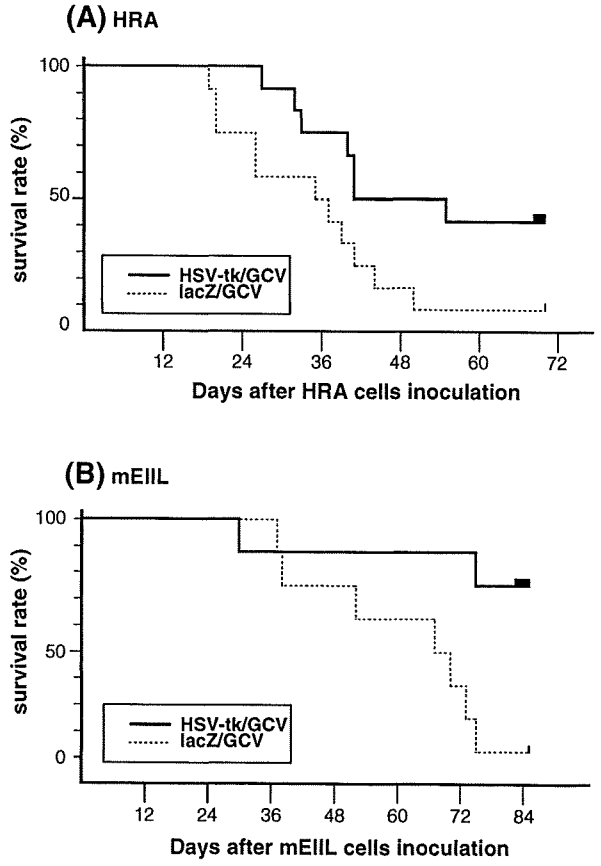

Fig. 3. Kaplan–Meier analysis of the survival of animals with HSV-tk gene/GCV (continuous line), with the lacZ/GCV (interrupted line) and without treatment (dotted line). (A) Inoculated HRA cells ($n=12$). The survival rate of the HSV-tk treatment group was significantly high compared with the lacZ-treated group ($p<0.05$). (B) Inoculated mEIIL cells ($n=8$). The rate was higher in the HSV-tk treatment group than in the lacZ treatment group ($p<0.05$).

longer than that of the lacZ treatment group ($p<0.05$, Wilcoxson test).

4. Discussion

Recently, several nonviral delivery and expression systems of exogenous genes into culture cells in vitro and different internal organs in vivo have been developed [8,28]. One of these is the cationic lipid-based system, which utilizes cationic liposomes. This system meets the requirements for efficiency, safety and repetition [23]. Cationic liposomes have numerous advantages when compared to other nonviral vectors; the technique is simple, highly reproducible and more efficient than some commonly used techniques [12]. Several clinical trials for gene therapy using cationic liposomes are ongoing with human genetic diseases and cancer. Initial results from these trials are encouraging [29].

In general, cationic liposomal transfection efficiency is reduced in the presence of serum [12,14,23]. Serum contains various types of charged molecules, which may bind to liposomes. Thereby this modification renders structural and physical characteristics of the cells sensitive to nucleases. For this reason, new cationic liposomes, which have an ideal formulation and good transgene efficiency under serum-containing conditions, have been developed for clinical use [15,30].

We developed cationic liposomes based on DC-6-14 for efficient gene transfer into peritoneally disseminated tumors. DC-6-14 has unique properties for gene delivery into human cancer cells in serum-containing medium. In the present study, the lacZ gene was transferred to human cancer cells with GTE-319 based on DC-6-14 and commercially available cationic liposomes in serum-free or serum-containing medium in vitro.

The transfection efficiency of GTE-319 was similar to other commercially available cationic liposomes for all cancer cells in the serum-free condition (Table 1). In the experiment on transgene expression through commercially available liposomes, the transfection efficiency was reduced by the presence of serum. By contrast, transgene expression using GTE-319 was maintained in serum-containing medium. Thus, GTE-319 was 1.1- to 15.7-fold more effective than DMRIE-C and 1.9- to 94.0-fold more effective than LipofectAMINE in all six cell lines in the presence of 10% serum (Table 1). These results showed that these cationic liposomes demonstrated a consistently superior transfection efficiency in the presence of serum in vitro. Therefore, the use of GTE-319 would be advantageous for clinical use in human cancer therapy.

To evaluate the anti-tumor effect of GTE-319 based on DC-6-14 in vitro, MTT assay was performed and the IC50 values of GCV were determined graphically. As shown in Table 2, the IC50 values of GCV for the lacZ gene transfection cells (therapeutic index) were 640-, 48-, 100-, 90-, 129- and 47-fold higher than those for the HSV-tk gene transfection cells of HRA, mEIIL, ES-2, KF, SKOV-3 and SW626, respectively. Our inference from this result is that HSV-tk/GCV suicidal gene therapy with GTE-319 liposomes was effective for treating cancer cells in vitro. Although transgene expression efficiency was generally low in vitro (1.9% to 18.1%, Table 1), malignant cells were killed by low concentrations of GCV. This phenomenon was probably due to the so-called bystander effect. Bystander effect describes a situation where neighboring cells suffer toxicity even though they are not genetically modification. The mechanism involved is poorly understood. It may require cell–cell contact via gap junction [31].

In this experiment, even though transfection efficiency was almost the same between cell lines (for example, HRA: 9.1%, KF: 9.4%), a difference was observed in therapeutic index (HRA: 640, KF: 90). This scenario may indicate differences in the level of gap junction expression by individual cell. Moreover, different sensitivities of cells to phosphated GCV mimic the clinical situation where different histological types of cancer have different sensitivities to anticancer chemotherapy. These possibilities will have to be explored.

Following confirmation of adequate therapeutic effect of gene therapy in six different cancer cell lines in vitro, we proceeded to evaluate gene therapy on HRA and mEIIL ascitic mice. We used survival time to evaluate in vivo anti-tumor effects. Although transfection efficiency was low in HRA cells (4.4%), we found that suicidal gene therapy using GTE-319

significantly prolonged survival of the two kinds of ascitic mice (Fig. 3). Part of the observed therapeutic effect may also be due to the phenomenon of bystander effect in vivo.

Although more researches still needed in this area, our findings are very encouraging and we feel that our approach has potential therapeutic application in the management of cancer.

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Case Report

Advanced malignant rhabdoid tumor of the ovary effectively responding to chemotherapy: A case report and review of the literature

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Abstract

Background. Malignant rhabdoid tumors (MRTs) are highly malignant neoplasms that consist of both renal and extrarenal subtypes. Primary ovarian cases are extremely rare. We herein describe the third known case of ovarian origin, which effectively responded to combination chemotherapy with ifosfamide, epirubicin, and cisplatin (IEP chemotherapy).

Case. A 19-year-old woman was diagnosed to have stage IIIc primary MRT of the ovary following the resection of tumors. Two months after surgery, an 8 cm-sized pelvic mass and enlarged retroperitoneal lymphnodes were detected. The patient received intravenous tri-weekly IEP chemotherapy. After the second course of chemotherapy, she demonstrated a complete clinical response.

Conclusion. Although this type of tumor is quite aggressive and chemotherapy is generally not considered to be effective, IEP chemotherapy may be useful in the treatment of MRT of the ovary.
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Keywords: Malignant rhabdoid tumor; IEP chemotherapy

Introduction

Malignant rhabdoid tumors (MRTs), consisting of renal and extrarenal rhabdoid tumors, are uncommon and have an extremely poor prognosis due to their rapid growth and tendency to metastasize early. Extrarenal rhabdoid tumor has been reported to occur in various sites throughout the body. The most common site of extrarenal rhabdoid tumors is the central nervous system, whereas ovarian cases are extremely rare with only two previous case report [1,2]. These tumors are aggressive and respond poorly to therapy. In general, chemotherapy does not appear to be effective and only a surgical extirpation is considered to offer a chance for controlling this disease. We herein describe the third patient with MRT of the ovary, who effectively responded to chemotherapy with ifosfamide, epirubicin, and cisplatin.

Case report

A 19-year-old gravida 0 para 0 woman presented with a 2-month history of abdominal distension. A pelvic examination revealed a large pelvic mass and an abdominal computed tomographic (CT) scan and magnetic resonance image showed a 14 × 12 × 10 cm sized lobulated mass in the pelvic cavity with massive ascites. Her laboratory studies including tumor markers, AFP, CEA, CA19-9, and SCC, were all within normal limits except elevated CA125 level (875 U/ml). During the operation, a solid right ovarian tumor, measuring 14 cm in diameter, and enlarged paraaortic lymphnodes were noted. The left ovary, uterus, omentum, and pelvic lymphnodes were unremarkable. The patient underwent an abdominal total hysterectomy,

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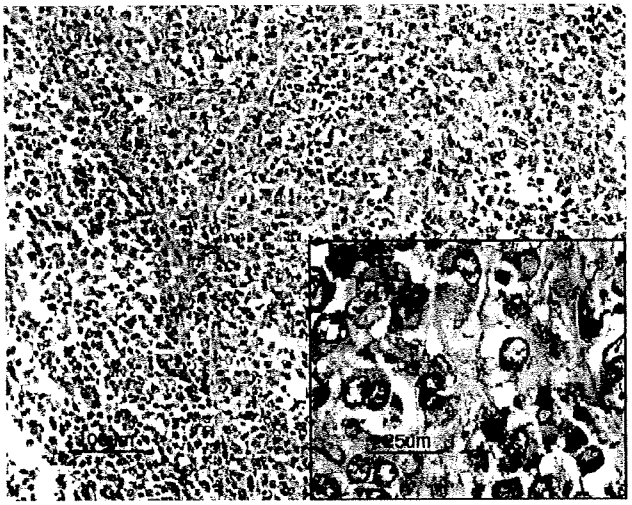

Fig. 1. Histopathology slide of the ovarian tumor showing diffusely infiltrating tumor cells in sheet-like alveolar structure (hematoxylin and eosin stain, original magnification ×100). The tumor is composed of round-shaped to pleomorphic atypical cells demonstrating large nuclei with prominent nucleoli (inset, original magnification ×400).

bilateral salpingo-oophorectomy, and pelvic and paraaortic lymphadenectomy. Intraoperative pelvic washing cytology was positive. Although the tumors were completely resected, metastasis to the paraaortic lymphnode was confirmed by postoperative histology and she was thus diagnosed to have stage IIIc ovarian carcinoma.

A histologic examination revealed a right ovarian tumor consistent with a malignant rhabdoid tumor (Fig. 1). The tumor is composed of round-shaped to pleomorphic atypical cells demonstrating large nuclei with prominent nucleoli, and abundant eosinophilic cytoplasm in which hyaline globular inclusions are conspicuously associated. Mitotic figures are frequently observed and lymphatic permeation is moderately observed. An immunohistochemical study revealed the tumor cells to be diffusely immunoreactive for vimentin, a common mesenchymal marker, and focally positive for cytokeratin markers, CAM5.2 (mixture of CK8, 18, and 19) and AE1/AE3 (mixture of CK1 to 8, 10, 14, 15, 16, and 19). No tumor cells were immunoreactive for striated muscular markers (HHF3.5, MyoD1, Myogenin, Myoglobin) or smooth muscular markers (HHF35, SMA, Desmin).

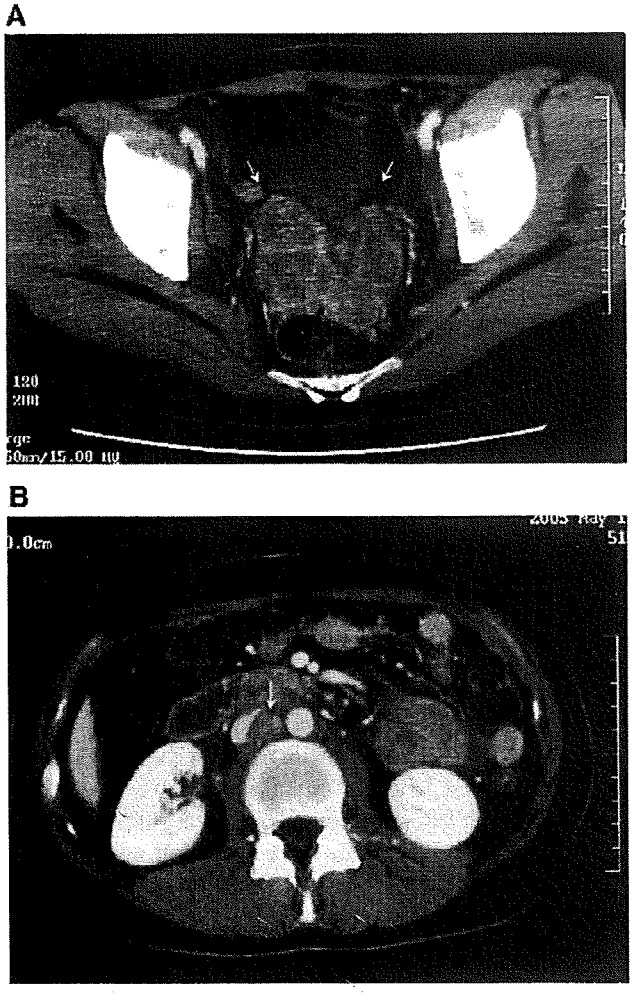

Fig. 2. CT scans showing a pelvic mass and paraaortic lymphnode swelling before IEP chemotherapy. (A) The 8 cm sized pelvic mass (white arrows) (B) and swelling of the paraaortic lymphnode (white arrow)

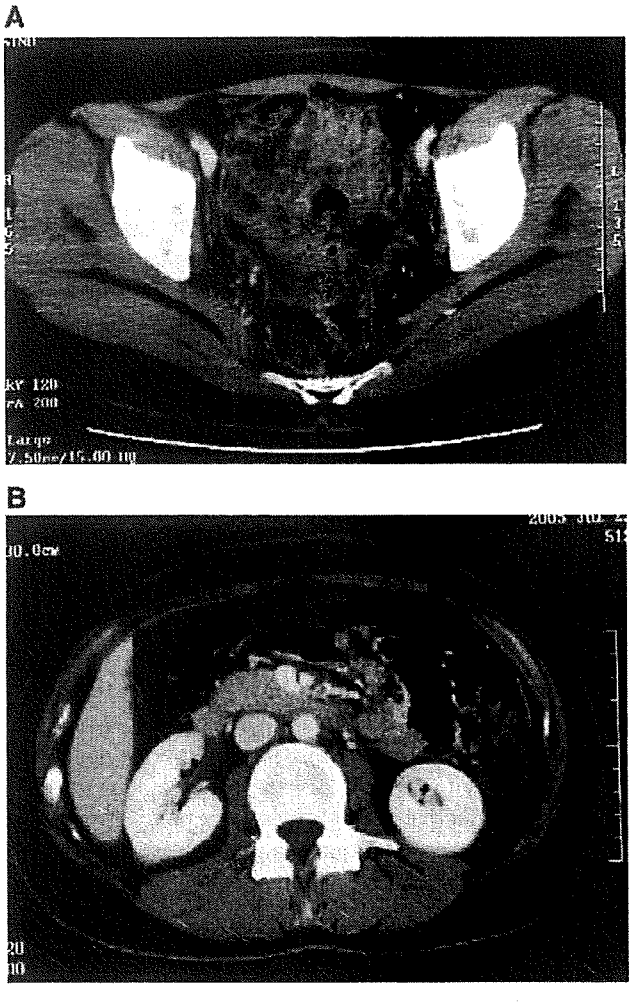

Fig. 3. CT scans after a second course of IEP chemotherapy. (A) The pelvic mass completely disappeared. (B) The swelling of paraaortic lymphnode completely resolved

Although no consistently effective regimen for MRT has yet been reported, we decided to initiate adjuvant chemotherapy because the case was advanced with extremely poor prognosis. Finally, the patient and her family decided not to have adjuvant chemotherapy and instead requested careful observation for any recurrence. Two months after surgery, an abdominal and pelvic CT scan showed an 8 cm-sized pelvic mass, enlarged paraaortic and right internal iliac lymphnodes, and small disseminated masses in the abdomen (Figs. 2A, B). The treatment was initiated with intravenous tri-weekly IEP chemotherapy (ifosfamide (1.5 g/body × 5 days), epirubicin (50 mg/m^2 × 1 day), and cisplatin (70 mg/m^2 × 1 day)). After the first course of IEP chemotherapy, the pelvic tumor decreased by −30%. After the second course, an abdominal and pelvic CT scan showed a complete resolution of the recurrent pelvic tumor, lymphnodes, and peritoneal dissemination (Figs. 3A, B). The CA125 level

Table 1
Malignant rhabdoid tumors of the female genital tract: summary of the reported cases

Case	Primary site	Age	Surgery	Stage	Site of metastasis at diagnosis	Recurrence (site (months after surgery))	Chemotherapy		Radiation	Prognosis	Reference
							Regimen	Response	Response		
1	Vulva	19	Radical resection, ILD	I	-	Vulva (26 m)	-	-	-	NED (38 m)	Perrone
2	Vulva	31	Wide local excision, ILD	I	-	Vulva, lung, liver, retroperitoneal node (35 m)	Adriamycin, cytoxan, vincristine, dacarbazine	PD	SD	DOD (11 years)	Perrone
3	Vulva	25	Excision	II	-	-	Regimen: NA	NE	-	NED (8 m)	Igarashi
4	Vulva	30	Wide local excision	II		Vulva, lung (2 m)	Adriamycin	PR		DOD (8 m)	Perrone
5	Vulva	39	Radical resection, ILD	II		Inguinal, pelvic, paraaortic lymphnode (3 m)	Cisplatin, etoposide	PD	PD	DOD (6 m)	Lupi
6	Vulva	40	Local excision	II		Vulva (1 m)	-	-	NE	NED (61 m)	Brand
7	Vulva	49	Local excision	II	-	Vulva, pubis (1 m)	Regimen: NA	NA	-	DOD (9 m)	Matias
8	Vulva	63	Radical resection, ILD	II	-	-	-	-	NE	NED (30 m)	Tzilinis
9	Vulva	44	Radical resection	IVb	Inguinal lymphnode, lung	Vulva, inguinal lymphnode (2 m)	Etoposide	PD	NE	DOD (7 m)	Sert
10	Uterine body	39	TAH, BSO, PLN, PAN	Ic	-	Lung, liver, abdomen (12 m)	-	-	NE	DOD (17 m)	Cattani
11	Uterine body	49	TAH, BSO	IIIb	Dougras pouch, vagina	Peritoneum, liver (3 m)	-	-	PD	DOD (4 m)	Gaetner
12	Uterine body	37	TAH, BSO, PLN, PAN	IIIc	Ovary, pelvic and paraaortic nodes	Abdomen, paraaortic lymphnode (2 m)	Cisplatin	PD	-	DOD (4 m)	Hseuh
13	Uterine body	46	TAH, BSO	IVb	Lung, liver	Vagina (1 m)	Ifosfamide, adriamycin, etoposide, vincristine, cyclophosfamide	NA	-	DOD (<12 m)	Cho
14	Uterine body	56	TAH, BSO, tumor debulking	IVb	Pelvic wall, sigmoid colon, omentum	-	Ifosfamide, etoposide	NA	-	Alive (7 m)	Niemann
15	Uterine body	72	SH, BSO, tumor debulking	IVb	Peritoneum	-	-	-	-	NED (6 m)	Levine
16	Ovary	18	RSO	I	-	Abdomen, retrosternal and retroperitoneal nodes (1 m)	Ifosfamide, etoposide	PD	-	DOD (2 m)	Leath
17	Ovary	36	TAH, BSO	IV	Lung	-	-	-	-	DOC (2 weeks)	Stastny
#	Ovary	19	TAH, BSO, PLN, PAN	IIIc	Paraaortic lymphnode, cul-de-sac	Abdomen, paraaortic lymphnode (2 m)	Ifosfamide, epirubicin, cisplatin	CR	SD	AWD (18 m)	

#: present case; BSO: bilateral salpingo-oophorectomy; TAH: total abdominal hysterectomy; SH: supracervical hysterectomy; PLN: pelvic lymphadenectomy; PAN: paraaortic lymphadenectomy; CR: complete response; DOD: died of disease; PR. partial response; DOC: died of complications; SD: stable disease; AWD: alive with disease; PD: progressive disease; NED: no evidence of disease; NA: not available; NE: not evaluable; ILD: inguinal lymphadenectomy.

returned to normal. She was treated with a further two courses of IEP chemotherapy. Her subsequent course was discontinued because she refused any further therapy due to severe nausea. She was clinically disease-free until 9 months following treatment, when enlarged paraaortic lymphnodes were detected by a CT scan. The patient underwent combination chemotherapy with only ifosfamide and cisplatin (she refused the inclusion of epirubicin because of anxiety for nausea). After the third course of the chemotherapy, sacral bone metastasis was found and a total 60 Gy of palliative irradiation was given. She is still alive with stable disease 18 months after the surgery.

Discussion

MRT was first described as a distinctive, highly malignant round cell neoplasm of the kidney in children. Those tumors are aggressive, are often widely metastatic at diagnosis, thus responding poorly to therapy, and they are uniformly fatal, except for localized disease. The diagnosis is based on light microscopic findings with supportive immunohistochemistry. The term "rhabdoid" refers to a histological resemblance to rhabdomyoblasts, although muscle markers are absent. Malignant extrarenal rhabdoid tumors are subsequently recognized and they have been reported to occur at various sites throughout body, such as the central nervous system, orbit, tongue, gum margin, esophagus, stomach, liver, colon, bladder, soft tissue, and extremities. The prognosis of extrarenal disease is also extremely poor similar to renal disease.

MRT in the female genital tract is rare, with only nine cases of the vulva [3–9], six cases of the uterine corpus [10–15], and only two cases of the ovary [1] being reported so far (Table 1). The initial treatment was surgery in all cases and 10 out of 14 completely resected cases the tumor recurred rapidly i.e., within 3 months, including present case. The most frequent recurrent pattern was local recurrence in cases of vulvar MRTs. The common recurrent sites were the pelvic and paraaortic lymphnodes and the lung. In 10 patients salvage chemotherapy was given for residual or recurrent tumors. The chemotherapy regimen tends to vary in each case including adriamycin, ifosfamide, cisplatin, etoposide, and others. All patients whose response was described in the report had progressive disease except for one partial response in a vulvar MRT patient. Seven patients received radiotherapy but it did not appear to be of any benefit. Eleven out of the 17 patients reported to date died with recurrence and systemic metastases (one case died of complications after surgery), and 9 of those died within 12 months of the diagnosis.

Primary ovarian cases are extremely rare, with only two cases previously reported in 18- and 36-year-old women [1,2]. The former case was stage I and the tumor was completely resected at the surgery. Shortly after the surgery, a recurrent tumor was detected in the pelvis, cul-de-sac, omentum, and retroperitoneal lymphnode. Combination chemotherapy with ifosfamide and etoposide for recurrent tumors was initiated; however, the disease progressed rapidly, and the patient died of disease 54 days after the initial presentation. The latter case was stage IV with lung metastasis and the patient died of complications 2 weeks after surgery.

Because of the small number of cases reported in the literature, no consensus exists regarding the standardized treatment or combination of treatments to effectively deal with the disease. In general, only a surgical extirpation is considered to provide the chance for controlling MRT because chemotherapy and radiotherapy have been minimally beneficial.

The surgical management of young patients with an ovarian mass can be complex because of concern regarding the preservation of fertility. In our case, the patient and her family desired standard management of her ovarian cancer; as such, she underwent an abdominal total hysterectomy, bilateral salpingo-oophorectomy, and pelvic and paraaortic lymphadenectomy. Given the progressive nature of the tumor of the patient described in this report, extensive surgical debulking should be performed especially in patients with advanced stages.

Various combination of chemotherapeutic agents, such as adriamycin, ifosfamide, etoposide, and cisplatin, have been tried in advanced or recurrent cases of MRT in the female genital tract with little benefit in one case with a brief response by adriamycin. For renal MRT, Roper et al. reported a patient who had a complete remission induced by cisplatin and adriamycin [16]. Our present case is the first patient to demonstrate a complete clinical response after salvage chemotherapy for MRT in the female genital tract. Unfortunately she experienced recurrence, but she has been able to survive for 18 months after surgery, which is a relatively better prognosis than that of the previously reported cases. Although the best treatment has yet to be determined, the prognosis might improve if the combination chemotherapy promptly initiated after surgery.

Based on the results for this single patient, IEP chemotherapy is thus considered to be potentially useful in the treatment of MRT of the ovary. Further experience is needed to confirm that this regimen may indeed result in a better prognosis.

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Enhancement of Gene Expression Efficiency Using Cationic Liposomes on Ovarian Cancer Cells

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We performed transfection using cationic liposome. According to the gene expression level, lined cells were divided into two groups, high and low. Introduced gene was monitored with a confocal laser-scanning microscope. The percentages of the cells introduced gene reached more than 90% in all line. Then, introduced gene was stable in high group, while in low group, it significantly decreased. With lysosomotrophic agents, gene expression efficiency was significantly reduced. With colchicine, gene expression efficiency did not change in high group, but was significantly elevated in low group. A method of liposomal transfection could be effective, particularly in low group.

Keywords Cationic Liposomes, Colchicine, Gene Delivery, Intracellular Stability, Ovarian Cancer Cells

Gene therapy is a method that utilizes genetic manipulation in the treatment of various diseases. The concept of gene therapy involves the transfer of genetic material into a cell, tissue, or whole organ, with the goal of curing a disease or at least improving the clinical status of a patient (Verma and Weitzman 2005).

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Various vectors for gene transfer have been developed and investigated both in vitro and in vivo. However, it is difficult to find a single method that meets all conditions for ideal gene transfer and vector expression. There are many concerns regarding the safety of vectors and the complicated pharmacodynamics involved that must be resolved. Various viral vectors, which have high transgene expression efficiency, are suitable for infection of mammalian host cells (Lundstrom 2003). However, they have serious drawbacks, which include risk of recombination and strong immunogenicity. Traditional nonviral chemical and physical methods such as calcium phosphate precipitation (Ginot et al. 1989), administration of diethylaminoethyl (DEAE) dextran (Ishikawa and Homcy 1992), and electroporation (Paquereau and Le Cam 1992) have relatively low efficiency.

Cationic liposomes are nonviral vectors and are superior to viral vectors in terms of reproducibility, simplicity, and safety of use. In addition, they do not invoke an immune response or protooncogene activation (Felgner and Ringold 1989; Zabner et al. 1995). However, they are inferior to viral vectors in transfection efficiency. We previously developed a series of new cationic liposomes containing *O,O'*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6-14), which have transgene activities in serum-containing media (Kikuchi et al. 1999a). Furthermore, we reported a correlation between gene expression efficiency and stability of the intracellular transferred plasmid using human cell hybrids derived from the fusion of the HeLa with a diploid of normal human fibroblast line (Serikawa et al. 2000). The stability of the transferred plasmid within the cells is crucial to the strength and duration of transgene expression and the difference in transfection efficiency

between high expression cells and low expression cells might be at least partly ascribed to the stability of the plasmid in the cells.

DNA-liposome complex (lipoplex) enters cells by endocytosis, and is delivered to endosomes and lysosomes. After avoiding lysosomal degradation, lipoplex is released into the cytosol and delivered to the nucleus (Wasungu and Hoekstra 2006). At first, we attempted to verify whether these phenomena would be applicable in ovarian cancer cells, and noticed the importance of stability in liposome-mediated transgene expression. We examined whether the control of intracellular stability of transferred plasmids would contribute to the enhancement of gene expression efficiency, especially in low expression cells, by the administration of endosomal or lysosomal inhibitor to increase the stability of intracellularly delivered plasmid.

MATERIALS AND METHODS

Cell Lines

Six human cancer cell lines were studied. The HRA cell line was derived from serous cystadenocarcinoma of the ovary (Kikuchi et al. 1987) and was kindly provided by Dr. Yoshinori Kikuchi (National Defense Medical College, Tokorozawa, Japan). The ES-2 cell line was derived from human ovarian carcinoma (Lau, Ross, and Sikic 1990) and was purchased from American Type Culture Collection. The Nakajima line was derived from human ovarian endometrioid cancer (Aoki et al. 1989) and was established in our laboratory. The OVHS-1 cell line was derived from human ovarian clear cell carcinoma (Ohta, Sakamoto, and Satoh 1998) and was kindly provided by Dr. Hideki Sakamoto (Nihon University, Tokyo, Japan). All six cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, Immuno-Biological Laboratories, Fujioka, Japan) supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA).

Plasmids

A humanized (Ser-65 to Thr converted) green fluorescent protein (GFP) plasmid, pGreen Lantern-1 (pGL-1), with cytomegalovirus promoter was purchased from Gibco-BRL. The plasmids grown in *Escherichia coli* were purified with a Qiagen kit (Qiagen, Hilden, Germany), diluted in distilled water at about 1–2 μg/mL, and stored at −20°C. A fluorescent probe, ethidium monoazide (EMA, Molecular Probes, Eugene, OR USA) can be photolyzed in the presence of nucleotides to yield fluorescently labeled nucleic acids. After its photocrosslinking to nucleic acids, EMA sends out red fluorescence (600 nm) at a similar excitation wavelength (464 nm) to GFP (Bolton and Kearns 1978). The EMA-labeled plasmid was prepared as previously described (Serikawa et al. 2000). The GFP protein stably emits green fluorescence at 511 nm when the excitation wavelength is 488 nm (Zolotukhin et al. 1996).

Cationic Liposome and Transfection

O,O'-Ditetradecanoyl-*N*-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6-14), a positively charged lipid, was purchased from Sogo Pharmaceutical (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE), a neutral helper lipid and essential to the formation of stable liposomes, was obtained from Nippon Oil and Fats (Tokyo, Japan). Cholesterol was purchased from Sigma (St. Louis, MO, USA). Cationic liposomes were prepared using freeze-dried empty liposomes (Kikuchi et al. 1999b). A molar ratio of 4:3:3 was used for DC-6-14/DOPE/cholesterol. Briefly, DC-6-14, DOPE, and cholesterol were dissolved in a chloroform–methanol mixture (4:1 v/v), and the solvent was removed in a rotary evaporator. After the lipids were mixed with 9% aqueous sucrose solution, they were hydrated at 60–70°C and extruded through a polyvinylidenedifluoride membrane filter with 0.22 μm pore size by using Liponizer LP-90 (Nomura Micro Science, Kanagawa, Japan). The dispersion was pipetted into glass vials (0.5 or 2 mL portion each), frozen at −50°C for 4 hr, and then lyophilized in a freeze drier (Virtis, New York, USA). The dried liposomes were then reconstituted with 0.5 or 2 mL of distilled water by gentle mixing prior to transfection.

To investigate the gene expression or transfer efficiency, the cells were seeded at a density of 1×10^5 on 6-well plates or 35-mm glass-base dishes with 2 mL of DMEM containing 10% FBS and incubated for 18–24 hr at 37°C in 5% CO_2. One hundred microliters of serum-free DMEM containing the appropriate amount of pGL-1 were mixed with 100 μL of serum-free DMEM containing the appropriate amount of cationic liposome with mild agitation in polystyrene tubes and incubated for 15 min at room temperature. Prior to transfection, cells were washed with serum-free DMEM and added to 800 μL of DMEM containing 12.5% FBS. Then 200 μl of complex solution (a final volume of one mL and final concentration of 10% FBS) was added. After 5 hr incubation at 37°C, the old medium was removed, the cells were washed twice with serum-free DMEM, 2 mL of DMEM containing 10% FBS was added, and the culture was continued for 19 hr.

Next, to investigate the role of the endosome or lysosome in the stabilization of the transfected plasmid in the cells, we used the endosomotrophic agent, chloroquine, and the lysosomotrophic agent, ammonium chloride. Furthermore, to investigate the role of intracellular transport, we used the microtubule-depolymerizing inhibitor, colchicine. When we transfected the plasmid, these reagents were added simultaneously to the medium for 5 hr.

FACS Analysis of GFP Expression Efficiency

To evaluate the gene expression efficiency, we used a fluorescein activated cell sorter (FACS, Becton-Dickinson, San Jose, California, USA) equipped for fluorescein isothiocyanate detection at an excitation wavelength of 488 nm (an argon laser). The fluorescence intensity of individual cells was measured as

relative fluorescence units. Transfected cells were harvested by incubation with 0.3 mL of 0.25% trypsin and 0.02% ethylenediaminotetraacetic acid (EDTA). After incubation, 1 mL of DMEM containing 10% FBS was added and cells were fixed with one mL of 4% paraformaldehyde (PFA, Wako, Osaka, Japan) for 30 min at 4°C. After centrifugation at 1,000 rpm for 5 min, the supernatant was discarded and the fixed cells were then rinsed with 2 mL of phospho-buffered solution (PBS) by centrifugation at 1000 rpm for 5 min. The final pellet was resuspended in 1 mL of PBS containing 0.05% sodium azide and 2 mM EDTA/2Na and delivered to 12 × 75-mm glass tubes. The fluorescence from approximately 10,000 individual cells collected by list-mode data, which consisted of forward and side scatter, was analyzed (Lysis software, Becton-Dickinson, San Jose, California, USA). The percentage of GFP positive cells was assessed by determining the percentage of highly fluorescent cells with fluorescence emission centered at 530 nm and subtracting the fluorescence of control cells that were exposed to the liposome only with a standard gating technique. No electric compensation was performed in these experiments.

Confocal Laser Scanning Microscopy of Gene Transfer Efficiency

To determine the percentages of cells that the fluorescently labeled plasmid had entered, we used a confocal laser-scanning microscope. The plasmid labeled with EMA was most efficiently excited using 488 nm illumination and was detected by an LP590 filter. Transfected cells were fixed immediately after transfection at 8 and 12 hr to retain fluorochrome within the cells. At 5 hr after transfection, the old medium was removed, the cells were washed twice with serum-free DMEM, 2 mL of DMEM containing 10% FBS was added, and the mixture was cultured further at 37°C. At 8 and 12 hr after transfection, the cells were thoroughly washed with PBS three times to remove extracellular or membrane-bound plasmid and fixed with 1 mL of 4% PFA for 30 min at 4°C. Then the cells were rinsed with PBS three times for 10 min at 4°C and enclosed with 1 mL of 80% glycerol. In each experiment, samples were prepared in triplicate for this determination.

FACS Analysis of the Amount of Intracellular Plasmid Transfected

The plasmid labeled with EMA was transfected into ovarian cancer cells. We measured the mean relative fluorescence intensity (RFI) of the cells containing the labeled plasmid and those without the plasmid. We calculated the arbitrary ratio of red fluorescent intensity to autofluorescence by FACS and considered the ratio to be the index of the amount of intracellular transfected plasmid.

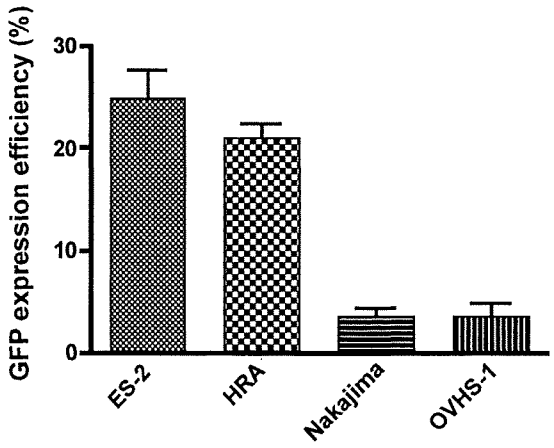

FIG. 1. GFP expression efficiency of four ovarian cancer cell lines. Gene expression efficiency was determined by FACS. Transfection was performed with 1μg of pGL-1 and 12.5 nmol of cationic liposome in the presence of 10% FBS. The data show that lined cells were divided into two groups, high and low. Data are shown as mean ± SD ($n = 3$).

RESULTS

Gene Expression Efficiencies of the GFP Gene

To examine the gene expression efficiency quantitatively on a cellular basis, we analyzed liposome-mediated gene transfer of the GFP plasmid into four kinds of human ovarian cancer cells in the presence of the 10% FBS. When this GFP expression was monitored by FACS, lined cells were divided into two groups, high (ES-2:24.9 ± 4.6%, HRA:20.9 ± 2.3%) and low (Nakajima:3.6 ± 1.3, OVHS-1:3.5 ± 2.1%) (Figure 1). High-expression group cells started to express GFP at 4 hr after gene transfer and low-expression group cells at 8 hr after gene transfer (data not shown).

Detection of a Fluorescent-Labeled Plasmid by Confocal Laser Microscopy

EMA, a fluorescent intercalating probe, was used to label the GFP plasmid DNA covalently by photoactivation. This EMA-labeled plasmid was detected within cells by confocal laser scanning microscopy under the conditions in which liposome-mediated GFP expression occurred, but not under the uninduced condition. This detection also eliminated the EMA-labeled plasmid adsorbed to the cell surface.

When this lipofection-dependent delivery of the EMA-labeled plasmid into cells was examined quantitatively at 8 h, the percentages of the cells containing labeled plasmid reached more than 90% in all four cell lines (HRA: 95.6 ± 1.2%, ES-2: 91.6 ± 5.1%, Nakajima: 90.3 ± 0.3%, OVHS-1: 92.8 ± 1.8%) (Figure 2). At 12 h, intracellular plasmid was kept stable in HRA (89.5 ± 1.8%) and ES-2 (88.8 ± 2.7%) cells, while the dose of plasmid detected in Nakajima (79.6 ± 6.5%) and OVHS-1 (81.8 ± 4.6%) cells decreased significantly (Figure 2).

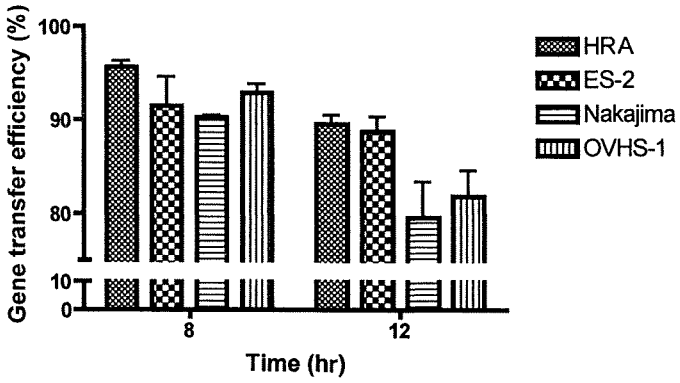

FIG. 2. Changes in gene transfer efficiency of four cell lines. Cells were transfected with EMA-labeled plasmid plus cationic liposome' and the level of intracellular plasmid DNA was determined at the indicated times after transfection and was expressed as a percentage of the total cells: 200–300 cells. Data are shown as mean ± SD ($n = 3$).

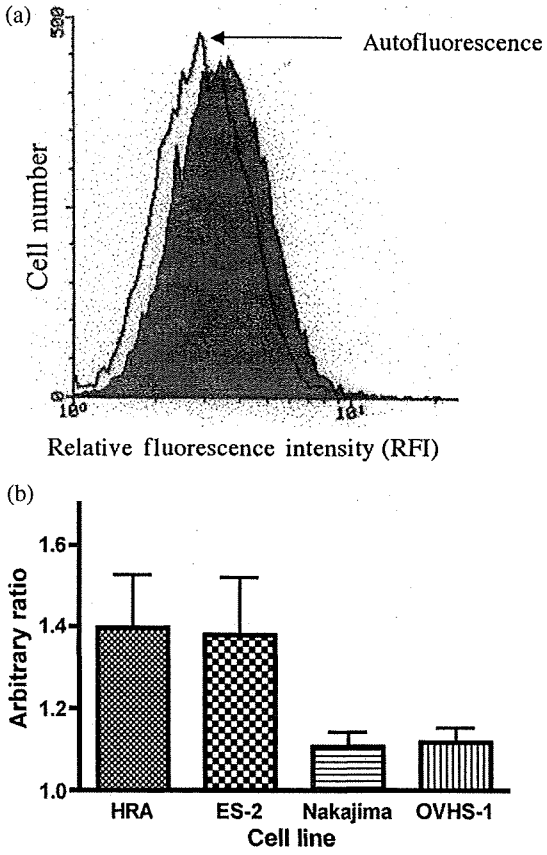

FIG. 3. Arbitrary ratio of the fluorescence intensity of the cells containing EMA to autofluorescence. Transfection was performed with 1 μg of EMA-labeled pGL-1 and 12.5 nmol of cationic liposome in the presence of serum. (a) Mean RFI of the cells was determined by FACS. We regard this ratio as an index of the amount of intracellular plasmid. (b) The ratio of the high-expression group (HRA and ES-2) was consistently larger than that of the low-expression group (Nakajima and OVHS-1). Data are shown as mean ± SD ($n = 3$).

The Ratio of Mean Relative Fluorescence Intensity as the Index of Intracellular Plasmid

At 8 hr after transfection of the EMA-labeled plasmid into the cells, we measured the arbitrary ratio of the red fluorescent intensity to autofluorescence by FACS (Figure 3a). The ratio of the high-expression group was consistently larger (HRA: 1.40 ± 0.22, ES-2: 1.38 ± 0.24) than that of the low-expression group (Nakajima: 1.11 ± 0.06, OVHS-1: 1.12 ± 0.06) (Figure 3b).

The Effect of Endosomotrophic and Lysosomotrophic Agents

As gene transfer efficiency in the low gene expression efficiency group significantly decreased at 12 hr after transfection, we supposed that intracellular stabilization of transduced plasmid might have an important role in cationic liposome-mediated transfection. We therefore tried an endosomotrophic agent, chloroquine. Gene expression efficiency in all cell lines decreased significantly (Figure 4). When we experimented using the lysosomotrophic agent ammonium chloride, similar results were obtained (Figure 5).

The Effect of Microtubule-Depolymerizing Agent

When the microtubule-depolymerizing and intracellular transport inhibitor agent, colchicine, was added to the cell at the time of transfection, the gene expression efficiency did not changed in the high-expression group (ES-2 and HRA). However, the efficiency was significantly elevated in the low-expression group (Nakajima and OVHS-1) (Figure 6).

DISCUSSION

We previously developed a series of novel cationic liposomes based on DC-6-14 that have high transgene activities in serum-containing media for gene therapy (Serikawa et al. 2000). DC-6-14 has unique properties for gene delivery into human cancer cells in serum-containing media. We reported that it was effective to use these cationic liposomes for peritoneally disseminated tumors (Serikawa et al. 2006) and cisplatin-resistant cells (Sato et al. 2005). However, there were differences in gene expression efficiency among cell lines, and we showed a correlation between gene expression efficiency and the stability of DNA introduced into the cells (Serikawa et al. 2000). These results suggested that intracellular stabilization of an introduced plasmid may be important. A major limitation with cationic liposomes is their low efficiency in producing transgene expression. We need many improvements in cationic liposome-mediated transfection.

In our study, we used four ovarian cancer cell lines. According to the levels of GFP expression, lined cells were divided into two groups, high (ES-2: 24.9 ± 4.6%, HRA: 20.9 ± 2.3%) and low (Nakajima: 3.6 ± 1.3%, OVHS-1: 3.5 ± 2.1%) (Figure 1). To examine the difference in transfection efficiency of cationic liposomes among these two groups, the plasmid, photolabeled with EMA and transferred along with the liposome into the cells, was quantitatively analyzed by confocal laser microscopy. The

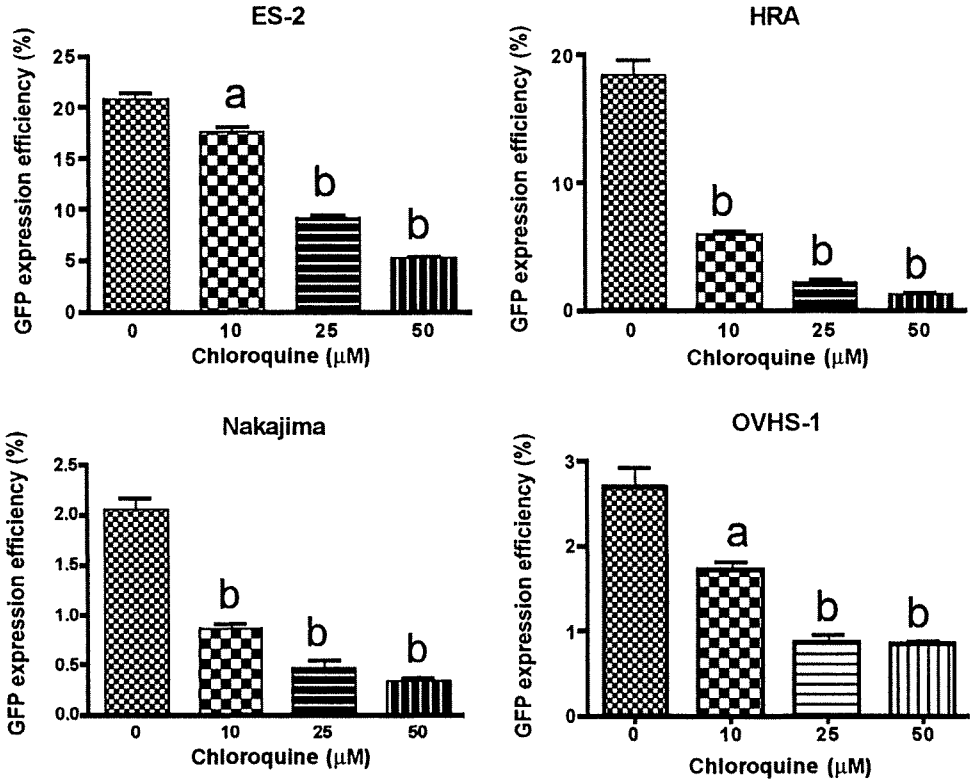

FIG. 4. Effect of chloroquine on the transfection. Endosomotrophic agent chloroquine was added to the cells at the transfection. Gene expression efficiency in all cell lines significantly decreased. Data are shown as mean ± SD ($n = 3$). Letters (a and b) denote $p < 0.01$ and 0.001, respectively, significant differences were found with respect to the control.

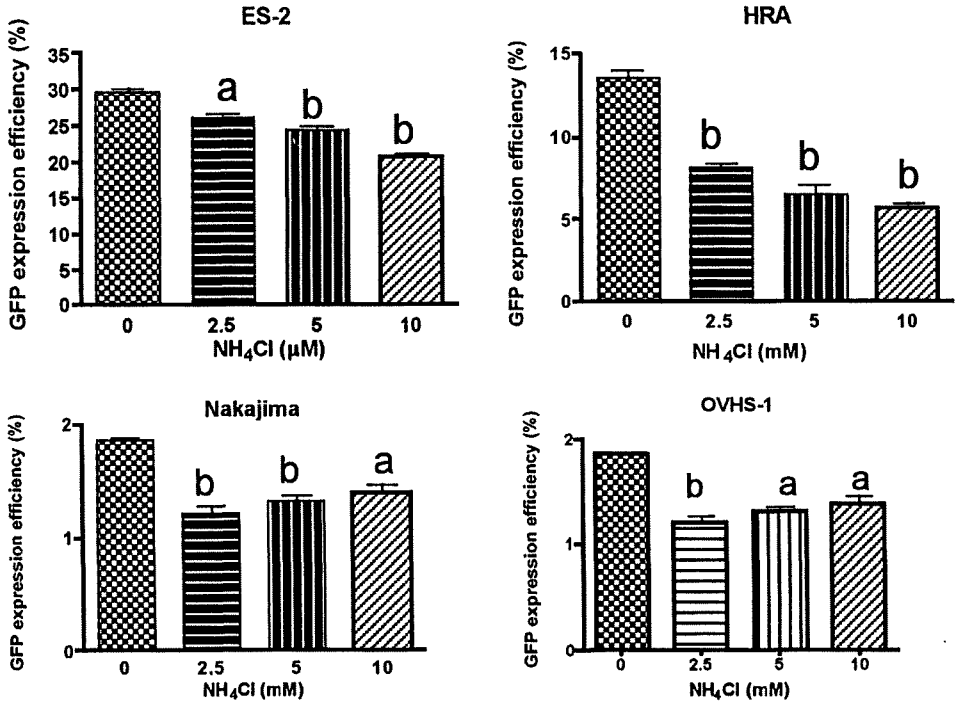

FIG. 5. Effect of ammonium chloride on the transfection. Lysosomotrophic agent ammonium chloride was added to the cells at the transfection. Gene expression efficiency in all cell lines significantly decreased. Data are shown as mean ± SD ($n = 3$). Letters (a and b) denote $p < 0.01$ and 0.001, respectively, significant differences were found with respect to the control.

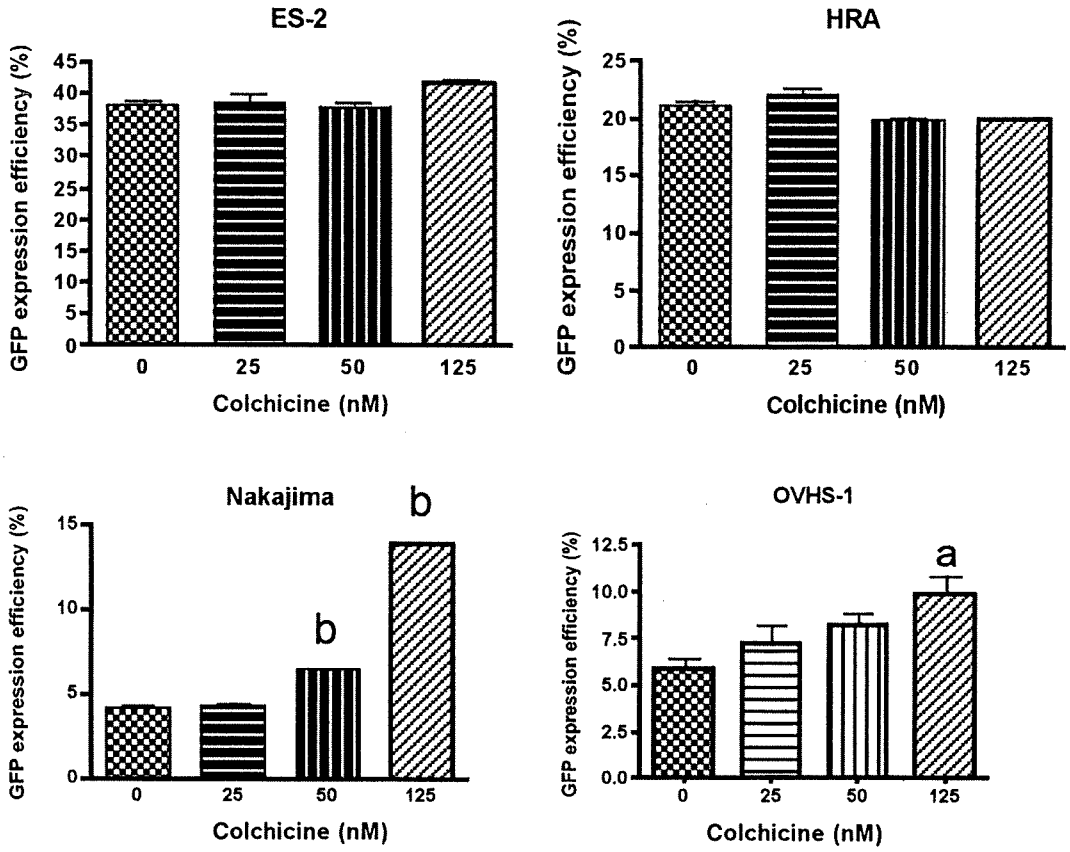

FIG. 6. Effect of colchicine on the transfection. Microtubule-depolymerizing agent chloroquine was added to the cells at the transfection. Gene expression efficiency in high-expression group cells did not change but significantly elevated the levels of efficiency in the low-expression group. Data are shown as mean ± SD ($n = 3$). Letters (a and b) denote $p < 0.05$ and 0.001, respectively, significant differences were found with respect to the control.

percentage of cells containing labeled plasmid was more than 90% in all cell lines at 8 hr after transfection. Therefore, the transfer of the labeled plasmid into the cells was similarly enhanced by cationic liposomes. The labeled plasmid was maintained in about 90% of cells after 12 hr in ES-2 and HRA cells, whereas in Nakajima and OVHS-1 cells, which showed transgene expression as low as 5%, the percentage of EMA-positive cells decreased significantly and was about 80% at 12 hr after transfection (Figure 2). These results suggest that intracellular plasmid in the high transgene expression group was more stable when compared to the low group and the difference in transfection efficiency between the high- and low-expression groups with cationic liposomes may be at least partly ascribed to the stability of the plasmid in the cells.

To evaluate the amount of transferred plasmid per cell, we determined the arbitrary ratio of fluorescent intensity to autofluorescence by FACS in both the high and low GFP expression groups (Figure 3A). The ratio of the high-expression group was consistently larger than that of the low-expression group (Figure 3B). These results suggested that gene expression efficiency might be dependent on the stability of the transferred plasmid and the total amount of intracellular plasmid transfected.

As the importance of the stability in liposome-mediated transgene expression has also been documented (Mahato et al. 1998), we attempted to examine the role of the endosome–lysosome in order to enhance the intracellular stabilization of transferred plasmid.

In general, liposome–DNA complexes are endocytosed into the cells and degraded in the endosome or lysosome (Felgner and Ringold 1989). After degradation, DNA is released in the cytoplasm and delivered to the nucleus (Wong, Scales, and Reilly 2007). We added endosomal or lysosomal inhibition agent, chloroquine and ammonium chloride, to the medium to evaluate the effect of the escape from endosomal or lysosomal degradation for the cationic liposomes. Both chloroquine and ammonium chloride significantly reduced gene expression efficiency in all four cell lines (Figures 4 and 5). These results suggest that it is necessary for our cationic liposome to be degraded in the endosome or lysosome and then be released to the cytoplasm.

Microtubules transport the liposome from the endosome to the lysosome. The microtubule-depolymerizing agents were found to dramatically increase the transfection of cationic phospholipid–DNA complexes on cultured vascular smooth muscle cells (Wang and MacDonald 2004). We hypothesized

that avoidance of microtubular transportation with colchicine inhibited lysosomal degradation after endosomal degradation and increased gene expression efficiency by more plasmids being released into the cytoplasm. Gene expression efficiency did not change in the high-expression group, but significantly increased in the low-expression group using colchicine (Figure 6). The diversity of gene expression efficiency according to cell type might be due to this difference of microtubular activity and sensitivity.

The present results suggest that microtubule-depolymerizing agent is useful for low-expression group cells to increase the gene expression efficiency. Microtubule-depolymerizing agents such as Vincristine and Vinblastine and microtubule-polymerizing agents such as Paclitaxel and Docetaxel are already used for clinical ovarian cancer. Although more research is still needed in this area, our findings are very encouraging and we feel that combination therapy with our cationic liposome and anticancer drug has potential therapeutic applications for ovarian cancer.

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Genome-Wide Expression of Azoospermia Testes Demonstrates a Specific Profile and Implicates *ART3* in Genetic Susceptibility

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Infertility affects about one in six couples attempting pregnancy, with the man responsible in approximately half of the cases. Because the pathophysiology underlying azoospermia is not elucidated, most male infertility is diagnosed as idiopathic. Genome-wide gene expression analyses with microarray on testis specimens from 47 non-obstructive azoospermia (NOA) and 11 obstructive azoospermia (OA) patients were performed, and 2,611 transcripts that preferentially included genes relevant to gametogenesis and reproduction according to Gene Ontology classification were found to be differentially expressed. Using a set of 945 of the 2,611 transcripts without missing data, NOA was further categorized into three classes using the non-negative matrix factorization method. Two of the three subclasses were different from the OA group in Johnsen's score, FSH level, and/or LH level, while there were no significant differences between the other subclass and the OA group. In addition, the 52 genes showing high statistical difference between NOA subclasses ($p < 0.01$ with Tukey's *post hoc* test) were subjected to allelic association analyses to identify genetic susceptibilities. After two rounds of screening, SNPs of the ADP-ribosyltransferase 3 gene (*ART3*) were associated with NOA with highest significance with ART3-SNP25 (rs6836703; $p = 0.0025$) in 442 NOA patients and 475 fertile men. Haplotypes with five SNPs were constructed, and the most common haplotype was found to be under-represented in patients (NOA 26.6% versus control 35.3%, $p = 0.000073$). Individuals having the most common haplotype showed an elevated level of testosterone, suggesting a protective effect of the haplotype on spermatogenesis. Thus, genome-wide gene expression analyses were used to identify genes involved in the pathogenesis of NOA, and *ART3* was subsequently identified as a susceptibility gene for NOA. These findings clarify the molecular pathophysiology of NOA and suggest a novel therapeutic target in the treatment of NOA.

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Introduction

Spermatogenesis, a major function of mammalian testes, is complex and strictly regulated. While spermatogenesis is a maturation of germ cells, other cells including Sertoli, Leydig, and peritubular myoid cells also play important roles, and defects at any differentiation stage might result in infertility. Male infertility is estimated to affect about 5% of adult human males, but 75% of the cases are diagnosed as idiopathic because the molecular mechanisms underlying the defects have not been elucidated. In consequence, an estimated one in six couples experiences difficulty in conceiving a child despite advances in assisted reproductive technologies. Male-factor infertility constitutes about half of the cases, and a significant proportion of male infertility is accompanied by idiopathic azoospermia or severe oligozoospermia, which may well have potential genetic components. It is well-recognized that men with very low sperm counts (<1 million/ml), identified through an infertility clinic, have a higher incidence of Y-chromosome microdeletion (up to 17%) [1,2]. However, the genetic causalities of most cases of azoospermia are not known.

Global gene-expression profiling with microarray technologies has been applied with great promise to monitor biological phenomena and answer biological questions. Indeed, microarray technologies have been successfully used to identify biomarkers, disease subtypes, and mechanisms of toxicity. We applied microarray analysis to testis specimens from infertile individuals including patients with obstructive azoospermia (OA) and non-obstructive azoospermia (NOA [OMIM %606766]) to characterize NOA and to identify the specific pathophysiology and molecular pathways of the disease. In addition, we attempted to identify genetic susceptibility to NOA from genes differentially expressed in NOA testes.

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Abbreviations: ART3, ADP-ribosyltransferase 3; AZF, azoospermia factor; coph, cophenetic correlation coefficient; Cy3, cyanine 3-CTP; Cy5, cyanine 5-CTP; EM, expectation-maximization; FDR, false discovery rate; FSH, follicle-stimulating hormone; GO, Gene Ontology; HC, hierarchical clustering; LD, linkage disequilibrium; LH, leutenizing hormone; MAF, minor allele frequency; MESA, microsurgical epididymal sperm aspiration; NMF, non-negative matrix factorization; NOA, non-obstructive azoospermia; OA, obstructive azoospermia; R^2, square of correlation coefficient; SNP, single nucleotide polymorphism; TESE, testicular sperm extraction

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☯ These authors contributed equally to this work.

Author Summary

Worldwide, approximately 15% of couples attempting pregnancy meet with failure. Male factors are thought to be responsible in 20%–50% of all infertility cases. Azoospermia, the absence of sperm in the ejaculate due to defects in its production or delivery is common in male infertility. In this study, we focused on non-obstructive azoospermia (NOA) because the etiologies of obstructive azoospermia are well studied and distinct from those of NOA. Microdeletions of the Y chromosome are thus far the only genetic defects known to affect human spermatogenesis, but most cases of NOA are unsolved. Because NOA results from a variety of defects in the developmental stages of spermatogenesis, the stage-specific expressions of genes in the testes must be investigated. Thus, genome-wide gene expression analyses of testes of NOA can provide insight into the several etiologies and genetic susceptibilities of NOA. In the present study, we analyzed several differentially expressed genes in NOA subclasses and identified ART3 as a susceptibility gene for NOA.

Genes related to spermatogenesis and candidate genes for azoospermia have been surveyed in humans and mice, especially since gene targeting technology accelerated the identification of genes that play crucial roles in spermatogenesis [3]. Because spermatogenesis is a complex process including meiosis, a germ cell–specific event, gene expression profiles specific to the differentiation stage, clinically classified by the Johnsen's score, were examined to provide insight into the pathogenesis of azoospermia [4]. In the current study, we performed microarray analyses on biopsied testes obtained from 47 NOA patients at diverse clinical stages without prior selection and 11 OA patients. The 47 NOA samples showed a wide range of heterogeneity, including a series of impairments at the differentiation stage of spermatogenesis that so far have been evaluated mainly by pathological findings. Thus, classification of NOA at the transcriptome level is a necessary first step in elucidation of the molecular pathogenesis of NOA. To do this, we adopted the non-negative matrix factorization (NMF) method, an unsupervised classification algorithm developed for decomposing images that has been applied in various fields of science including bioinformatics because of its potential for providing insight into complex relationships in large data sets [5–7]. 47 of the NOA-samples were divided into three subclasses by the NMF method, and each class was associated with clinical features. 149 transcripts were identified as differentially expressed genes among the NOA subclasses according to a statistical criterion, and the features involved in spermatogenesis based on Gene Ontology classification were demonstrated.

The genetic causality of NOA most likely involves the expression level of a susceptibility gene, which might be detected by genome-wide gene expression analysis. While it is daunting to identify genetic susceptibility from 100-1000 differentially expressed genes, genetic susceptibility might more readily be identified from random genes differentially expressed with high significance rather than by investigating only genes in a specific biological pathway. Based on a well-defined statistical procedure, 52 candidate genes for NOA were catalogued by gene expression profile and screened for allelic association study in a total of 442 NOA patients and 475 fertile male controls. After gene-centric selection of SNPs, 191 SNPs of 42 candidate genes were initially evaluated for allelic association with NOA. After two rounds of screening, SNPs of the ADP-ribosyltransferase 3 (*ART3*) gene were found to be significantly associated with NOA, and five of these SNPs were selected for haplotype construction. The most common haplotype was significantly under-represented in the patients and may be protective. The functional impact of this haplotype was further investigated.

Results

Extraction of NOA-Related Gene Expression Profile

As shown in Figure 1A, the most notable difference in histological findings between NOA and OA testes was that the NOA patients exhibited, at varying degrees, incomplete sets of spermatogenic germ cells (spermatogonia, spermatocytes, spermatids, and spermatozoa) in the seminiferous tubules. In severe NOA patients, we could not even detect Sertoli cells, the major somatic cells of the seminiferous tubules, on histological examination (figure not shown), indicating clinical heterogeneity of NOA testes. In order to elucidate the molecular systems underlying NOA at the transcriptome level, it is important to extract genes reflecting the diversity of NOA phenotypes. For this purpose, we first compared global gene expression profiles in NOA testes to those of OA testes using the Agilent Human 1A(v2) Oligo Microarray system. We chose the 'standard reference design' in two-color microarray experiments as an experimental design for the expression analysis [8], where a single microarray was used to compare either NOA or OA to the testicular reference RNA as described in Materials and Methods (Figure 1B).

Of the 18,716 transcripts screened with the microarray, we obtained transcripts that showed a 2-fold mean expression difference between NOA and the reference, the NOA group; the OA group comprised transcripts showing less than 2-fold mean expression difference between OA and the reference (Figure 1B). Of the transcripts overlapping the two groups, 2,611 transcripts were found to be differentially expressed between NOA and OA testes after statistical filtering (based on lowess-normalized natural log[Cy5/Cy3], Bonferroni's corrected $p < 0.05$). This gene list, termed NOA-related target genes, comprised 902 elevated and 1,709 decreased transcripts in NOA testes. To characterize the gene list from the biological aspect, the 2,611 transcripts were subjected to functional clustering according to Gene Ontology (GO) classification for biological processes with GeneSpring software. We identified a total of 190 GO categories that were significantly ($p < 0.05$ without multiple testing correction) over-represented among the 2,611 transcripts. Table 1 shows the ten top-ranked GO categories in descending order of significance based on p-values with Fisher's exact test. It is notable that the GO categories involved in gametogenesis (GO:48232; 7283; 7276), reproduction (GO:19953; 3), and the cell cycle (GO:279; 51301; 7049; 7067) are significantly associated with the gene list. We further analyzed two separate subsets comprising 1,709 decreased (Figure 2, upper) and 902 elevated (Figure 2, lower) transcripts, based on their GO annotations. The top-ranked GO categories for NOA-related target genes are more similar to those of the 1,709 decreased transcripts than to those of the 902 elevated ones (Figure 2; Table 1). Thus, the predominant features may reflect spermatogenic defects common to NOA testes. On the

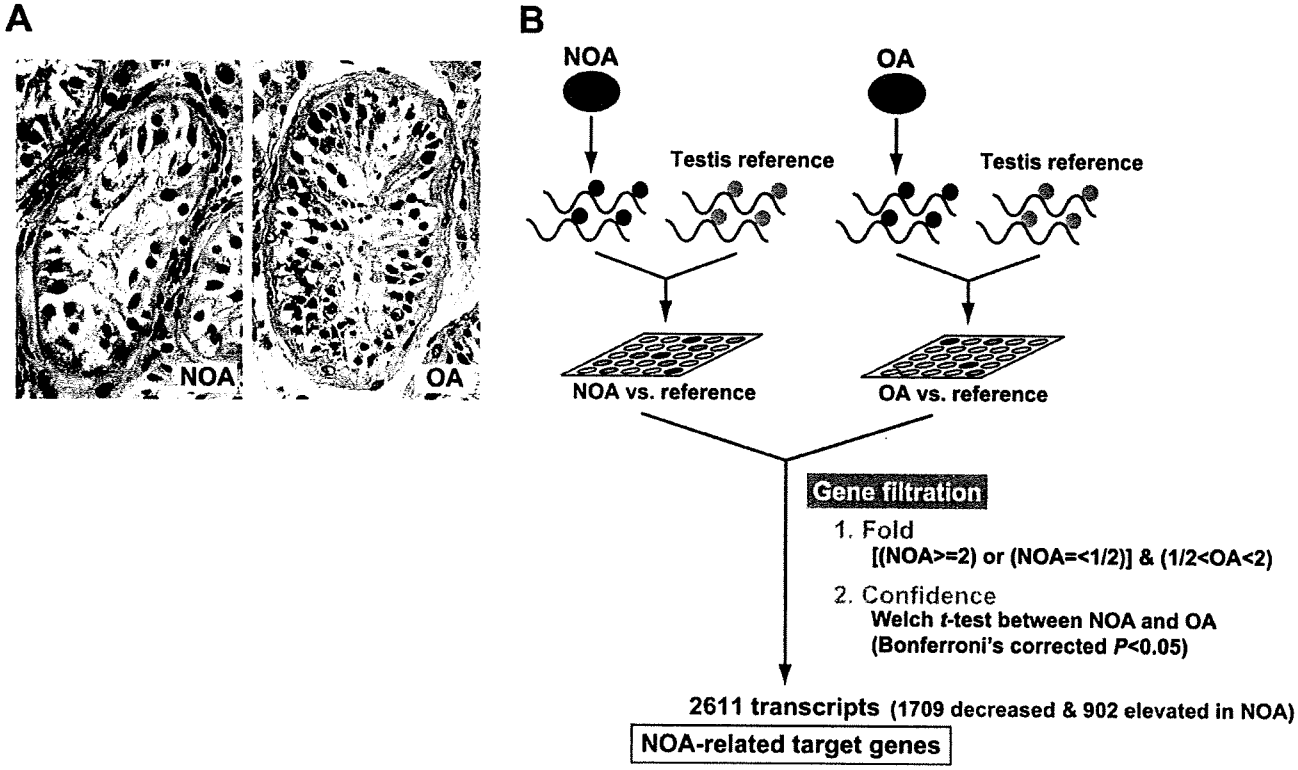

Figure 1. Experimental Design to Extract NOA-related Target Genes

(A) Representative testicular biopsies from NOA (left panel) and OA (right panel) patients. The seminiferous tubules in NOA testis show Sertoli cells only; testicular histology of the OA patient indicates the presence of germ cells at all stages of spermatogenesis.

(B) Strategy for discovering the NOA-specific expression profile, i.e., the NOA-related target genes. Compared with the expression level of reference RNA, the NOA group, with expression undergoing 2-fold or more mean change, was extracted; the OA group comprised transcripts with less than 2-fold mean expression change. Of the overlapping transcripts, only those with statistically significant difference between NOA and OA ($p < 0.05$ with Bonferroni's correction) were identified as differentially expressed.

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Table 1. Top-Ranked Ten Categories of Gene Ontology Significantly Overrepresented among 2,611 Transcripts

GO Category[a]	Genes within GO Category		−logP[c]
	Number	Percent[b]	
Male gamete generation (GO:48232)	55	3.1	12.0
Spermatogenesis (GO:7283)	55	3.1	12.0
Sexual reproduction (GO:19953)	70	3.9	11.2
Gametogenesis (GO:7276)	59	3.3	10.5
Reproduction (GO:3)	75	4.2	8.3
M phase (GO:279)	57	3.2	6.7
Microtubule-based process (GO:7017)	42	2.4	6.5
Cell division (GO:51301)	48	2.7	6.5
Cell cycle (GO:7049)	144	8.1	6.4
Mitosis (GO:7067)	45	2.5	5.6

[a]All GO categories are from the subontology *biological process*.

[b]Percent denotes the percentage of coverage of NOA-related target genes. Of the 2,611 transcripts in the gene list, 1,784 are used for calculating the percentage of genes with a given GO annotation because the GO annotations regarding *biological process* for the others are not available.

[c]*p*-Value was determined by Fisher's exact test, comparing the observed percentage of NOA-related target genes with a given GO annotation to that of genes on the Agilent Human 1A(v2) microarray with the same GO annotation.

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other hand, 902 transcripts elevated in NOA testes exhibited a distinct GO profile that included several GO categories involved in biosynthesis and metabolism in cytoplasm (Figure 2), implying an increase in cytoplasmic turnover rates such as steroid turnover in NOA testes.

Discovery of Three Molecular Subclasses of NOA Testes

To clarify heterogeneity of NOA testes at the transcriptome level, we further examined NOA-related target genes to identify NOA subclasses without prior classification with pathological features. We adopted the NMF algorithm coupled with a model selection method [6] to a complete dataset of 945 out of the 2,611 transcripts without missing values of signal intensities for a total of 47 NOA samples. Figure 3A shows reordered consensus matrices averaging 50 connective matrices generated for subclasses $K = 2$, 3, 4, and 5. Distinct patterns of block partitioning were observed at models with 2 and 3 subclasses ($K = 2$ and 3), whereas higher ranks ($K = 4$ and 5) made block partitioning indistinct. Thus, the NMF method predicts the existence of reproducible and robust subclasses of NOA samples for $K = 2$ and 3. This prediction was quantitatively supported by higher values of cophenetic correlation coefficients (coph) for NMF-clustered matrices. The NMF class assignment for $K = 3$ showed the highest coph value (coph = 0.993), indicating that three molecular subclasses, termed NOA1, NOA2, and NOA3, are