The paper explained

PROBLEM:

Epithelial ovarian cancer exhibits considerable heterogeneity, which may lead to poor survival rates for patients treated with standard chemotherapeutic regimens. This has prompted the need for a robust classification scheme to unravel this heterogeneity and allow for the development of personalized treatment strategies.

RESULTS:

A large collection of gene expression data enabled the identification of five distinct subgroups of ovarian carcinoma. The existence of these five subgroups was validated in an independent collection. Genome-wide shRNA screening against a panel of ovarian carcinoma cell lines revealed two subtype-specific targets and the pathways that control cancer cell growth.

IMPACT:

We identified five distinct subgroups, allowing rational patient stratification. Subsequent assays uncovered genes and deregulated pathways, which will be instrumental in guiding future therapeutic strategies for ovarian cancer.

detected 60,002 and 65,533 shRNA hairpins in two independent screenings and 57,168 hairpins were intersected in both results. We compiled and subsequently standardized these two datasets by ComBat (Johnson et al, 2007). Binary comparisons were performed on the three subtypes (*e.g.* Epi-A subtype *versus* the others). We adopted the signal-to-ratio as the metric for ranking hairpins, 1000 as the number of permutations, and Kolmogorov–Smirnov in the RIGER settings. The false discovery rate was computed using the Benjamini and Hochberg procedure. Genes were considered significant at $q < 0.005$ in Fig 3B or $q < 0.03$ for the validation study. For heatmap presentation, we retained the hairpins with a hairpin score ≥ 0.2.

Validation of functional determinants in cell growth of Stem-A cell lines by siRNAs

We selected 135 genes as Stem-A-specific growth-promoting genes for further validation via siRNA transfection from the top hit gene list from RIGER analysis of shRNA lentivirus screens ($q < 0.03$). The validation experiments were performed via a process consisting of four steps (Fig 3C). Dharmacon SMART pool siGENOME siRNA (1st and 2nd steps) and Dharmacon SMART pool ON-TARGET*plus* siRNA (OTP; 3rd and 4th steps) formats (Thermo Fisher Scientific, Lafayette, CO) were used to validate the effect of gene knockdown on cell growth of ovarian cell lines (Fig 3C). PA-1 (1st, 2nd, and 3rd steps) and CH1, A2780 and OVCAR-3 (4th step) were used as representative cell line(s) for the Stem-A subtype. As reference(s) for the subtype, HeyA8 (1st step), HeyA8 and OVCA433 (2nd and 3rd steps), OVCA429, PEO1, ovary1847, SKOV-3 and HEY (4th step) were used (Fig 3C). Cells were reverse-transfected with each individual siRNA per well in a 96-well format in the following conditions: OVCA433, 2500 cells with 0.3 μl of DF1 (T-2001); HeyA8, 800 cells with 0.08 μl of DF4 (T-2004); PA-1, 1200 cells with 0.22 μl of DF2 (T-2002); OVCA429, 1500 cells with 0.22 μl of DF4 (T-2004); PEO1, 4000 cells with 0.24 μl of DF4 (T-2004); ovary1847, 2500 cells with 0.12 μl of DF2 (T-2002); SKOV-3, 2500 cells with 0.12 μl of DF2 (T-2002); HEY, 1000 cells with 0.08 μl of DF4 (T-2004); CH1, 1800 cells with 0.17 μl of DF4 (T-2004); A2780, 2000 cells with 0.16 μl of DF1 (T-2001); OVCAR-3, 4000 cells with 0.2 μl of DF3 (T-2003, Thermo Fisher Scientific). We used two negative controls for Dharmacon SMART pool siGENOME siRNA transfection (#D-001206-13-20 and #D-001206-14-20), and one negative control for Dharmacon SMART pool ON-TARGET*plus* siRNA transfection (#D-001810-10-20). Assays were performed in quadruplicate. After 96-h incubation, an MTS assay was used to measure cell growth using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay following the manufacturer's recommendations (#G5430, Promega, Madison, WI). Genes were considered as Stem-A-specific growth-promoting genes when their down-regulation caused $\geq$20% growth suppression on the Stem-A cell line ($p < 0.001$), and showed $\geq$20% more growth suppression on the Stem-A line than on the reference cell lines.

Cell line drug sensitivity *in vitro*

Eighteen ovarian cancer cell lines (12 non-Stem-A: OVCA433, OVCA429, OVCAR-8, PEO1, OVCA432, OVCA420, HeyA8, HEY, HeyC2, SKOV-3, ovary1847 and DOV 13; 6 Stem-A: PA-1, CH1, A2780, OVCAR-3, SKOV-4 and SKOV-6) were tested for their sensitivity to paclitaxel, vincristine and vinorelbine, as described previously (Bild et al, 2006). Paclitaxel (#T7402), vincristine (#V8879) and vinorelbine (#V2264) were purchased from Sigma–Aldrich. Cells were seeded in 96-well plates at an optimal density, which was determined for each cell line to ensure that it reached 80% confluency by the end of the assay. Following an overnight incubation, cells were treated with nine concentrations of each drug (twofold dilution series over a 128-fold concentration range) for 48 h. The percentage of the cell population responding to the drug relative to the negative controls was measured using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, following the manufacturer's recommendations (#G5430, Promega). Dose-response curves were plotted using GraphPad Prism, to derive a growth inhibitory concentration of 50% (GI50; drug concentration for 50% growth inhibitory effects on cells) for each cell line in at least three independent experiments. Mann–Whitney *U*-test of GraphPad Prism was used to statistically evaluate the averaged GI50s between non-Stem-A and Stem-A cell lines.

Western blotting analysis

Total cell lysates were prepared by direct lysis with RIPA buffer (#R0278, Sigma–Aldrich), supplemented with protease inhibitor cocktail (#539134, Calbiochem, Boston, MA). Protein concentrations were determined using BCA protein assay (#23225, Thermo Scientific,

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Rockford, IL). Electrophoresis of the cell lysates were carried out with a BioRad Mini Protean II apparatus and transferred onto PVDF membranes (#IPFL00010, Millipore, Billerica, MA) with a BioRad Mini Trans-Blot apparatus, following the manufacturer's recommendations. Membranes were immunoblotted with primary antibodies directed against PARP (#9542, Cell Signaling, Danvers, MA), Caspase-3 (#9662, Cell Signaling) or β-actin (#A1978, Sigma–Aldrich), followed by immunoblotting with secondary IRDye 800CW conjugated goat anti-rabbit (#926-32211) or IRDye 680 conjugated goat anti-mouse antibodies (#926-32220, LI-COR Biosciences, Lincoln, NE). The western blots were scanned using an Odyssey Infrared Imaging System from LI-COR Biosciences.

Author contributions

SM conceived the idea; SM, JPT, BCG and RYH devised the project and obtained funding; SM, TZT, QHM, JTC, JPT and RYH wrote the paper; SM, QHM, JY, JAL, LZW, NM and MKW performed the experiments; MCW, LHBAH and RS performed next-generation sequencing analysis; SM, TZT and JTC performed bioinformatics analyses; MC performed clinical parameter analyses; BD and JMN provided OSLO ovarian cancer samples; NM, MM, and IK provided ovarian cancer cell lines.

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Supporting Information is available at EMBO Molecular Medicine Online.

The authors declare that they have no conflict of interest.

For more information

National Library of Medicine (Ovarian Cancer):
http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001891/

RNAi Consortium:
http://www.broadinstitute.org/rnai/trc/lib

BinReg
http://dig.genome.duke.edu/software.html
http://www.stat.duke.edu/~mw/ABS04/Lecture_Slides/5.Stats_BinReg.pdf

ClaNC
http://www.stat.tamu.edu/~adabney/clanc/

RIGER
http://www.broadinstitute.org/cancer/software/GENE-E/

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Utilization of genomic signatures to identify high-efficacy candidate drugs for chemorefractory endometrial cancers

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Endometrial cancer, one of the most common gynecologic malignancies, is increasing in Japan, nearly doubling over the last decade. High-grade disease patients are often resistant to conventional chemotherapy with platinum agents; therefore, discovery of efficacious new drugs in this setting is required to benefit chemorefractory cases. The 50% growth-inhibitory (GI50) concentration of 27 clinically relevant drugs was measured in the NCI60 panel of cell lines. Gene expression data were analyzed using Bayesian binary regression, to first generate a response signature for each drug and then to calculate individual susceptibility scores using *in vivo* endometrial cancer data (GSE2109; http://www.ncbi.nlm.nih.gov/geo) and *in vitro* data (GSE25458), as well as to identify candidate drugs for chemorefractory cases. Using these candidates, cell proliferation, apoptosis and caspase assays were performed *in vitro*. The tumor growth-inhibitory effect of the candidate was also assessed *in vivo* using nude mice. Through microarray analysis, fludarabine and temsirolimus showed higher susceptibility scores in high-grade cases compared to cisplatin, doxorubicin and paclitaxel. Fludarabine significantly inhibited cell proliferation and increased apoptosis in the cisplatin-resistant endometrial cancer cell line, HEC1A, relative to HEC50B ($p < 0.001$). Fludarabine treatment also enhanced caspase-3/7 activity in HEC1A relative to HEC50B cells ($p < 0.001$), and inhibited the growth of HEC1A xenograft tumors relative to cisplatin ($p < 0.05$). These results support that identification and use of genomic signatures can lead to identification of new therapeutic candidates that may prove beneficial to chemoresistant cases. Fludarabine may be useful in targeting high-grade, chemorefractory endometrial cancer.

Endometrial cancer is the leading cause of gynecologic malignancy with 43,470 estimated cases diagnosed per year and 7,950 annual death in the United States, respectively, consisting of 6% of new cancer cases and 3% of all cancer deaths, and disease incidence has been steadily increasing.[1,2] The majority of endometrial cancers, more than 80%, are diagnosed at an early stage with the disease located within the uterus. When diagnosed at an early stage, primary surgery is frequently curative enough to be associated with a favorable prognosis. In contrast, extrauterine spread of cancer cells profoundly impacts patient prognosis as previous studies revealed high hazard ratios for Stage III and Stage IV compared to Stage I disease.[2] Clear cell and papillary serous carcinomas of the uterus are associated with aggressive behaviors, even at an early stage, with 5-year survival between 60 and 66%.[3] Besides staging and histology, several pathological factors, such as tumor grade, depth of invasion and lymph vascular invasion, are well known to determine the prognosis of each patient with an aggressively metastatic phenotype. Recently, adjuvant chemotherapy has been introduced after primary surgery as part of the first-line management for preventing recurrence of such high-risk disease.[4] First-line chemotherapy typically consists of a combination regimen followed by treatment with a single agent on disease progression. Throughout the phase II and III studies of the Gynecologic Oncology Group (GOG), platinum combined with doxorubicin and/or taxane has played an important role in the treatment of high-risk disease, but was also associated with infrequent complete response with recurrence in nearly half of these patients. Furthermore, there is no active second-line agent after failure of primary chemotherapies, as the response rate to paclitaxel was at most 25% for recurrent patients previously treated with doxorubicin and cisplatin.[5] With the objective of improving the prognosis of those with high-risk disease, it is essential to identify candidate cytotoxic agents that are effective against patients with resistance to conventional chemotherapies (chemorefractory tumors) or supportive agents that increase sensitivity to primary chemotherapies.

Key words: chemoresistant, fludarabine, chemodynamics, endometrial cancer
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What's new?
Patients with advanced endometrial cancer need something beyond conventional therapies. Genome wide microarray analysis of these difficult-to-treat cancers has shown that they have distinctive genetic signatures. In this paper, the authors profiled drug-resistant endometrial cancer cell lines to identify potentially effective chemical therapies. From their analysis, they determined that that five of seven cancer cell lines were likely susceptible to the chemotherapy agent fludarabine. They then demonstrated fludarabine's toxicity in vitro and in vivo, suggesting the drug may have promise for treating the cancer.

Identification of effective second-line agents for chemorefractory cancers has been a long-sought goal, and over the past few years various new cytotoxic agents have been synthesized for the treatment of malignancies. Clinical trials for endometrial cancer have also historically been conducted using drugs identified as effective for other solid malignancies, especially ovarian cancer. However, these trial-and-error approaches to drug mining are inefficient for this heterogeneous entity "endometrial cancer," and have mostly failed. To elevate the efficacy of second-line chemotherapy, individualized therapy will be necessary based on biological features of the patient and tumor, such as molecular mechanisms and clinical phenotypes.

Recent development of genome-wide analysis with microarray has revealed that chemorefractory cancers possess characteristic gene expression profiles, so called chemoresistant signatures.[6,7] A computational analysis using Bayesian binary regression methods enabled to project a phenotype signature extracted from one microarray onto another microarray to predict the phenotype probability of each sample in projected microarray. In our study, we utilized this bioinformatics approach to demarcate that fludarabine had potential efficacy in chemorefractory endometrial cancers. Furthermore, we performed several *in vitro* and *in vivo* approaches using endometrial cancer cell lines to demonstrate that fludarabine may be a potential alternative treatment for chemorefractory endometrial cancers.

Material and Methods

Patients

Clinicopathological information of 262 patients treated for endometrial cancer during 2004–2011 in Kyoto University Hospital was obtained with written consent from each patient and used under protocols approved by the Kyoto University Institutional Review Board. The prognostic risk of each case was determined as low, intermediate or high, as previously described.[8]

Cell lines and culture

Human endometrial cancer cell lines, AN3CA, HEC1A, HEC1B, KLE, RL95-2, TEN (ATCC, Rockville, MD), ACC230, ACC564 (DSMZ, Brunswick, Germany), HHUA, Ishikawa, JHUEM-1, JHUEM-2, JHUEM-3, JHUEM-7, JHUEM-14, Sawano (RIKKEN BRC, Tsukuba, Japan), HEC50B, HEC108, HEC265 and SNG-M (JCRB, Osaka, Japan), were maintained in RPMI1640 (Nikken, Kyoto, Japan) or DMEM/Ham's F12 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (v/v; Biowest, France) and penicillin–streptomycin (100 IU/ml penicillin and 100 μg/ml streptomycin; Nacalai Tesque, Kyoto, Japan). All cells were seeded into Cellstars® tissue culture plates (Greiner, Frickenhausen, Germany) and used for experiments after 18-hr incubation.

Chemicals

Following the manufacturer's instructions, a 5 mM stock solution of fludarabine (Alexis Biochemicals, San Diego, CA) was prepared in cold sterile water and stored at −20°C. Fresh thawed dilutions were used for each experiment. A 3.33 mM stock solution of cisplatin (Sigma Aldrich, St Louis, MO) was also prepared in sterile water following the manufacturer's instructions.

Bioinformatics analyses

Total RNA was extracted from cell lines using the RNeasy® Mini Kit (QIAGEN, Valencia, CA), and gene expression microarray data (Affymetrix U133 Plus 2.0) were generated in triplicate and robust multi-array average-normalized as described previously.[9] Expression microarray data of endometrial cancers (GSE2109) were also obtained from the Gene Expression Omnibus web site (http://www.ncbi.nlm.nih.gov/geo). Gene expression data for NCI60 cell lines were obtained from the National Cancer Institute along with 50% growth-inhibitory (GI50) values for 27 commercially available chemotherapeutic agents (http://www.dtp.nci.nih.gov/webdata.html). GI50 value data for 27 drugs from the NCI60 cell line data were normalized with median centering using Cluster 3.0 (http://rana.lbl. fgov/EisenSoftware.htm) and converted into a visual representation using JavaTreeView (http://jtreeview.sourceforge.net/). Heat maps were generated using Matlab (Mathworks, Natick, MA) or R with Bioconductor (http://www.r-project.org/) as described previously.[9–11] Genomic signatures of drug susceptibility were generated using Bayesian binary regression[10] from gene expression data of ten sensitive and ten resistant cell lines from the NCI60 drugs screening panel. Probabilities of chemosensitivity in response to NCI60 drugs were scored for each sample in dataset GSE 25458 as previously described.[9]

Cell proliferation assay

AN3CA, HEC1A and HEC50B cells were seeded into 96-well tissue culture plates at 2×10^3 cells per well. The cell culture medium was replaced with fresh medium containing 0, 10, 25, 50, 100, 250 or 500μM fludarabine and incubated for 24

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hr. The number of viable cells in each well was examined using the WST-1 assay kit (Premix WST-1®, Takara, Otsu, Japan) following the manufacturer's instructions.

Apoptosis detection

AN3CA, HEC1A and HEC50B cells were treated with medium containing 0, 25, 50 or 100 μM fludarabine for 24 hr. Cells were harvested by trypsinization for apoptosis detection by flow cytometry. After washing with phosphate-buffered saline, cells were resuspended in 200 μl of 1× annexin-V binding buffer (BD Pharmingen). Next, 5 μl of 7-amino-actinomycin D (7-AAD) (BD Pharmingen) and 5 μl of annexin-V were added to the tubes and incubated for 10 min at 4°C in the dark. Cells were washed with 500 μl of 1× annexin-V binding buffer and then resuspended in 200 μl of 1× annexin-V binding buffer followed by filtration through a 70-μm nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ). The samples were placed on ice and analyzed by FACSCalibur (Becton Dickinson). The data were analyzed using FlowJo v.7.6.3 (Tree Star, Ashland, OR).

Caspase-3/7 activity was measured using the Caspase-Glo® 3/7 Assay System (Promega, Madison, WI) following the manufacturer's instructions. After 2-hr incubation with the Caspase-Glo 3/7 Reagent, luminescence was measured using a Glo-Max® Luminometer (Promega) as directed by the manufacturer.

Western blot

AN3CA, HEC1A and HEC50B cells were treated with medium containing 0, 25, 50 or 100 μM fludarabine for 24 hr. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA) with a protease inhibitor cocktail (EMD, Madison, WI) and a phosphatase inhibitor cocktail (Nacalai Tesque). Protein was quantified using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). Twenty micrograms of sodium dodecyl sulfate (SDS)-treated protein was loaded onto a 10–20% Tris-Tricine Mini Protean® gel (Bio-Rad). Gels were electroblotted onto polyvinylidene fluoride membranes (Bio-Rad). Nonspecific binding of the antibody was blocked by 1-hr incubation at room temperature in Blocking One-P (Nacalai Tesque). The membranes were incubated overnight at 4°C with caspase-3 antibody (1:1,000, caspase-3 Rabbit mAb, Cell Signaling Technology, Danvers, MA) or cleaved caspase-3 (Asp175) antibody (1:1,000, cleaved caspase-3 rabbit polyclonal Ab, Cell Signaling Technology). After washing in Tris-buffered saline (TBS)-T, the blots were incubated with the appropriate peroxidase-coupled secondary antibody (1:6,000; anti-rabbit HRP, GE Healthcare Life Sciences, Uppsala, Sweden). β-Actin was used as an endogenous loading control and detected using an anti-human β-actin antibody (1:8,000; Rabbit mAb, Abcam, Cambidge, MA). Specific proteins were detected using ECL Plus Western Blotting Reagent (GE Healthcare Life Sciences). The bands were visualized using Molecular Imager® Gel Doc™ XR+ and ChemiDoc™ XRS+ Systems with Image Lab 2.0 software (Bio-Rad).

Real-time qPCR

Total RNA was extracted from cell lines using the RNeasy® Mini Kit (QIAGEN). To monitor gene expression, quantitative reverse transcriptase (RT)-PCR amplification of human caspase-3α, caspase-3β and *GAPDH* mRNAs was done by Light Cycler 480-II (Roche, Basel, Switzerland) using a Dual Color Hydrolysis Universal Probe System (Roche). The following primers that were obtained from the Universal Probe Library Assay Design Center (Roche) were used for analysis: caspase-3α, 5′-CTG GTT TTC GGT GGG TGT-3′ (forward), 5′-CCA CTG AGT TTT CAG TGT TCT CC-3′ (reverse); caspase-3β, 5′-TGG AAT TGA TGC GTG ATG TT-3′ (forward), 5′-TGG CTC AGA AGC ACA CAA AC-3′ (reverse); GAPDH, 5′-AGC CAC ATC GCT CAG ACA C-3′ (forward), 5′-GCC CAA TAC GAC CAA ATC C-3′ (reverse). Cycling parameters were 95°C for 10 sec followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec, followed by a dissociation cycle of 95°C for 15 sec, 60°C for 20 sec and 95°C for 15 sec. The expression of human caspase-3α and caspase-3β mRNAs was estimated by dividing the caspase-3α and caspase-3β threshold cycle (CT) values by the *GAPDH* CT values.

In vivo experiment

Female CD-1 Foxn/Nu mice were purchased from Oriental Bioservice (Kyoto, Japan). Animal care and experimental procedures under specific pathogen-free conditions were performed in accordance with the guidelines of the Institute of Laboratory Animals Graduate School of Medicine, Kyoto University. Subcutaneous xenografts were established in the flanks by inoculating 5×10^6 cells of the AN3CA, HEC1A or HEC50B cell lines. Three days after inoculation, each mouse was treated twice a day for 5 days with intraperitoneal administration of 125 mg/kg fludarabine, 1 mg/kg cisplatin or 50 μl sterile distilled water as a control treatment ($n = 4$ in each arm). Therapeutic effects were monitored by measuring tumor growth for 30 days after inoculation. The differences in tumor growth were analyzed statistically.

Statistical analysis

Group comparisons were done using Mann–Whitney *U*-tests. Prognostic analysis was done using log-rank test and Fisher's exact test. Statistical analyses were done using GraphPad Prism 5.5 software. Probability values below 0.05 were considered significant.

Results

Clinical significance of current chemotherapy in the treatment of endometrial cancer

Clinical features of the 262 patients studied here are listed in Table 1. Forty-three patients exhibited progression or recurrence of the disease (progressive disease, PD).

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Table 1. Patient characteristics. [Color table can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

		n	Recurrence/PD	p	Rate of 5 yr OS	p
Age	≦50	57	3		93.5	
	>50	205	40	0.0083	85.4	0.174
stage	I	174	10		98.5	
	II	19	2	0.3348	100	0.6355
	III	46	13	<0.0001	68.6	<0.0001
	IV	23	18	<0.0001	28.6	<0.0001
Myometrial invasion	≦1/2	145	6		97.0	
	>1/2	109	29	<0.0001	81.1	0.0004
LVSI	-	166	8		96.2	
	+	81	26	<0.0001	77.2	<0.0001
grade	Low	160	7		97.7	
	high	102	36	<0.0001	70.2	<0.0001
risk	Low	86	0		100	
	Intermediate	82	3	0.1141	98.4	0.3293
	high	94	40	<0.0001	64.9	<0.0001

Known risk factors are listed for the patients in our study. Recurrence/PD: the number of patients who showed recurrence or progressive disease during chemotherapy; LVSI means lymphovascular space invasion.

Clinicopathological analysis revealed that each known prognostic factor including advanced stage, outer-half myometrial invasion, lymphovascular space invasion and histological grade showed significant differences both in terms of the rate of PD and in the overall survival (OS) of patients. Based on the prognostic risk classification, 86 patients were categorized as "low-risk" without any PD. Three of 82 "intermediate-risk" patients recurred, but there was no significant difference in OS compared to "low-risk" patients, whereas "high-risk" patients exhibited higher PD and poor OS ($p < 0.0001$, Supporting Information Fig. 1). PD was significantly lower among patients who received chemotherapies with cisplatin + doxorubicin and paclitaxel + carboplatin in the "intermediate-risk" group ($p < 0.05$, Table 2), whereas not in the "high-risk" group ($p = 0.72$).

Drug sensitivity prediction from the NCI60 data

As a first step toward identifying compounds that exhibit efficacy toward chemoresistant endometrial cancer cells, we queried the NCI-60 database for the 50% growth-inhibitory doses (GI50) of conventional chemotherapeutic agents including cisplatin, paclitaxel and doxorubicin. GI50 values were normalized (see Material and Methods section) and visualized using a heatmap. Based on this representation, normalized GI50 values > 0.04 were colored in red or orange and 0.04 was thus determined as a threshold for sensitivity (Fig. 1*a*). By this criterion, 24 of 62 cell lines (39%) were resistant to cisplatin, doxorubicin and paclitaxel, and this rate, 39%, was very close to the rate of PD (44%) among the "high-risk" patients in our clinical data.

Second, the GI50 values were obtained from the NCI-60 database for another 24 commonly used chemotherapeutic agents for the 24 chemoresistant cell lines. An unsupervised hierarchical clustering analysis using the normalized GI50 values for the chemoresistant cells revealed that these 24 drugs were divided into two clusters: cluster 1 contains the anticancer drugs, whereas cluster 2 contains molecular targeting drugs (Fig. 1*b*). Some of the drugs exhibited efficacy against several cell lines, but fludarabine appeared to effectively target ten of these 24 chemoresistant cells.

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Table 2. Patient chemoresponsiveness profiles. [Color table can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

	Chemo	n	Recurrence/PD	p
Low	-	75	0	
	+	11	0	-
Intermediate	-	25	3	
	+	57	0	0.0260
High	-	8	4	
	+	86	36	0.7193

Effectiveness of chemotherapy was evaluated in each risk group using Fisher's exact test.

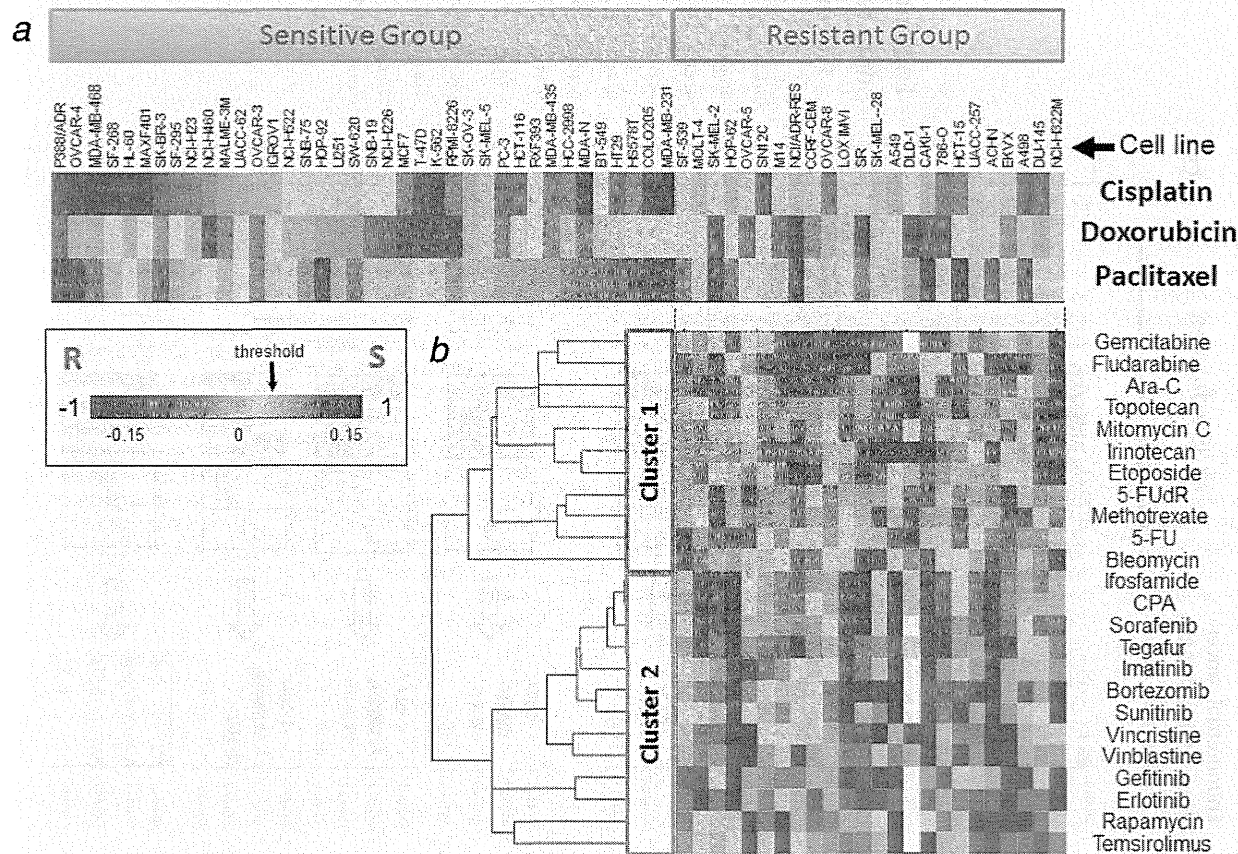

Figure 1. The 50% growth-inhibitory doses (GI50) of anticancer drugs in the NCI60 dataset in predicting chemoresponsiveness. (*a*) NCI60 cell lines were aligned according to their normalized GI50 values for cisplatin, doxorubicin and paclitaxel, shown in the top panel. Most (84.63%) of the normalized GI50 values ranged between 0.15 (red) and −0.15 (blue). Dark red and dark blue at the extreme ends of the color bar represent values >0.15 and <−0.15, respectively. [S: sensitive; R: resistant; threshold indicates the value (0.04) above which cells were considered as chemosensitive] (*b*) For the 24 chemoresistant cell lines in panel *a*, GI50 values were obtained for an additional 24 commonly used chemotherapeutic agents. Unsupervised hierarchical clustering produced two clusters: cluster 1 contains anticancer drugs; cluster 2 contains molecular targeting drugs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

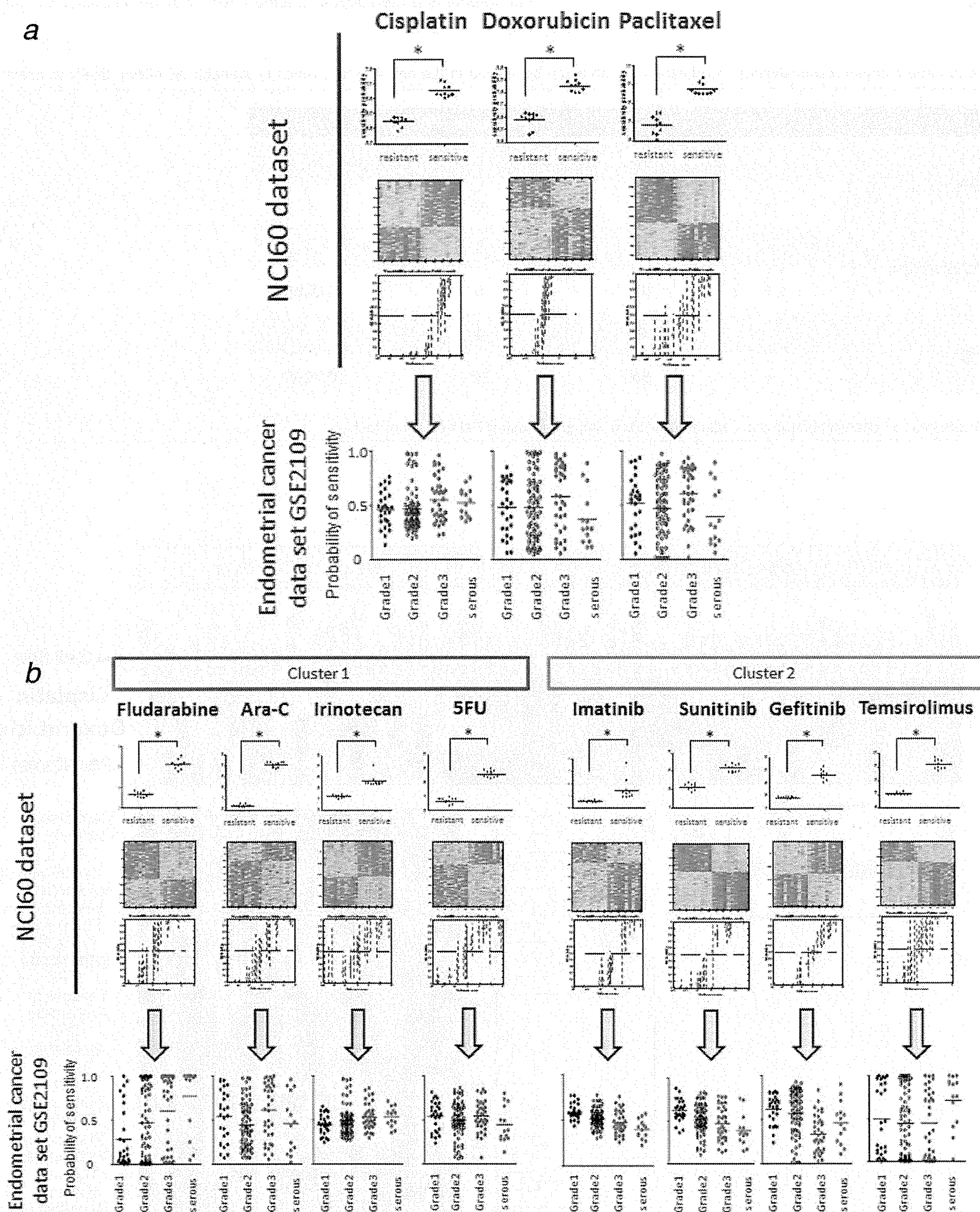

Figure 2. Predicted susceptibility of chemo agents in endometrial cancers *in vivo*. (*a*) Genomic signatures of susceptibility were generated using Bayesian binary regression from the NCI60 gene expression data and applied to microarray data from endometrial cancers in GSE2109 for predicting the probability of sensitivity to cisplatin, doxorubicin and paclitaxel. (*b*) Drug susceptibility signatures were also developed for fludarabine, Ara-C and irinotecan as representative anticancer drugs from cluster 1, and for temsirolimus, gefitinib and sunitinib as representative molecular targeting drugs from cluster 2. The probability of sensitivity (*y*-axis on a scale of 0–1, with 0 indicating high probability of resistance and 1 indicating high probability of sensitivity) to the three conventional chemotherapeutic agents was not superior in Grade 3 endometrioid adenocarcinoma and serous papillary adenocarcinoma to those in low-grade endometrioid adenocarcinoma. On the other hand, the probability of sensitivity to fludarabine was significantly higher in Grade 3 and serous and for temsirolimus was significantly higher in serous ($p < 0.001$ and $p < 0.05$, respectively). (*c*) Gene expression microarray analysis was performed in 20 endometrial cancer cell lines, and drug-susceptibility signatures for the conventional chemotherapeutic agents were applied to predict the probability of sensitivity of each cell line. Numbers in each box indicate the probability score with colors indicating probability of response. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

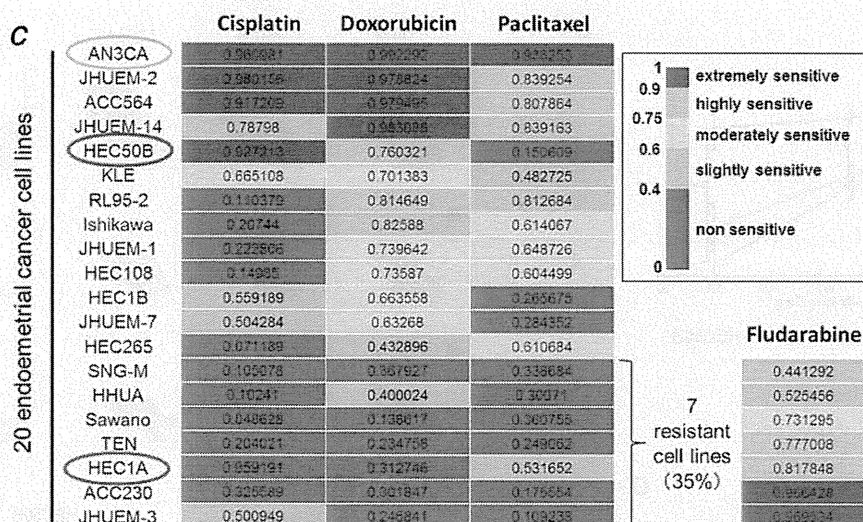

Figure 2. (Continued)

Predicted susceptibility of chemotherapeutic agents in endometrial cancers

The NCI60 drug screening panel also contains genetic information. By selecting ten sensitive and ten resistant cell lines, a genomic signature of drug susceptibility for each chemotherapeutic agent was derived using Bayesian binary regression[10] from the gene expression data. Such genomic signatures can be applied to an independent gene expression dataset to predict drug susceptibility for each sample in the dataset as previously described.[12] Drug-susceptibility signatures developed from the NCI60 dataset were applied to the microarray data of endometrial cancers in GSE2109 for predicting the probabilities of sensitivity to cisplatin, doxorubicin and paclitaxel. Meanwhile, drug-susceptibility signatures were also developed for fludarabine, Ara-C, irinotecan and 5-FU, as representative cluster 1 anticancer drugs, and for imatinib, gefitinib, sunitinib and temsirolimus, as representative molecular targeting drugs from cluster 2; these drugs were chosen as representative drugs for each subcluster based on chemosignatures for the NCI60 cell lines. The sensitivity probabilities of the three conventional chemotherapeutic agents in Grade 3 endometrioid adenocarcinoma and serous papillary adenocarcinoma were not superior to those in low-grade endometrioid adenocarcinoma (Fig. 2*a*). On the other hand, the probability of sensitivity to fludarabine was significantly higher in Grade 3 and serous, and the probability of sensitivity to temsirolimus was significantly higher in serous (Fig. 2*b*; $p < 0.001$ and $p < 0.05$, respectively).

Gene expression microarray analysis was performed in 20 endometrial cancer cell lines, and the drug-susceptibility signatures of conventional chemo agents were applied to predict the sensitivity of each cell line. There was a statistically significant correlation between cisplatin GI50 values of 37 ovarian cancer cell lines[10] and cisplatin sensitivity probability scores derived from the gene expression microarray data (GSE25428, $r = 0.3776$, $p = 0.02$, data not shown).

Seven cell lines exhibited low probabilities of sensitivity to cisplatin, doxorubicin and paclitaxel, whereas another 13 cell lines exhibited higher probabilities of sensitivity. Intriguingly, five out of these seven cells showed relatively high probabilities of sensitivity to fludarabine (Fig. 2*c*), and three had favorable probability scores to temsirolimus (Supporting Information Table 2). Further, we investigated HEC1A as a representative chemorefractory cell line with AN3CA (sensitive to all conventional chemo agents) and HEC50B (partly sensitive to conventional chemo agents) as counterpart controls for further experiments. Fludarabine was selected for further experiments as the most potent alternative agent for chemorefractory cases.

Cytotoxic activities of fludarabine in vitro

The cytotoxic effect of fludarabine was examined using proliferation assays. AN3CA, HEC1A and HEC50B were exposed to fludarabine (from 0 to 500 μM) for 24 hr. There were dose-dependent growth-inhibitory responses in the AN3CA and HEC1A cells ($p < 0.01$), and more than 90% growth abrogation was observed in HEC1A cells at 500 μM ($p < 0.001$). There was no significant difference in proliferation after fludarabine treatment for the HEC50B cells at any dose (Fig. 3*a*).

To investigate the growth-inhibitory mechanism of fludarabine in endometrial cancer cells, annexin-V/7-AAD apoptosis assays were performed using flow cytometry. After 24-hr exposure to fludarabine, apoptosis in AN3CA and HEC1A cells was increased in a dose-dependent manner, with 20 and 25% of the cells undergoing apoptosis after treatment with

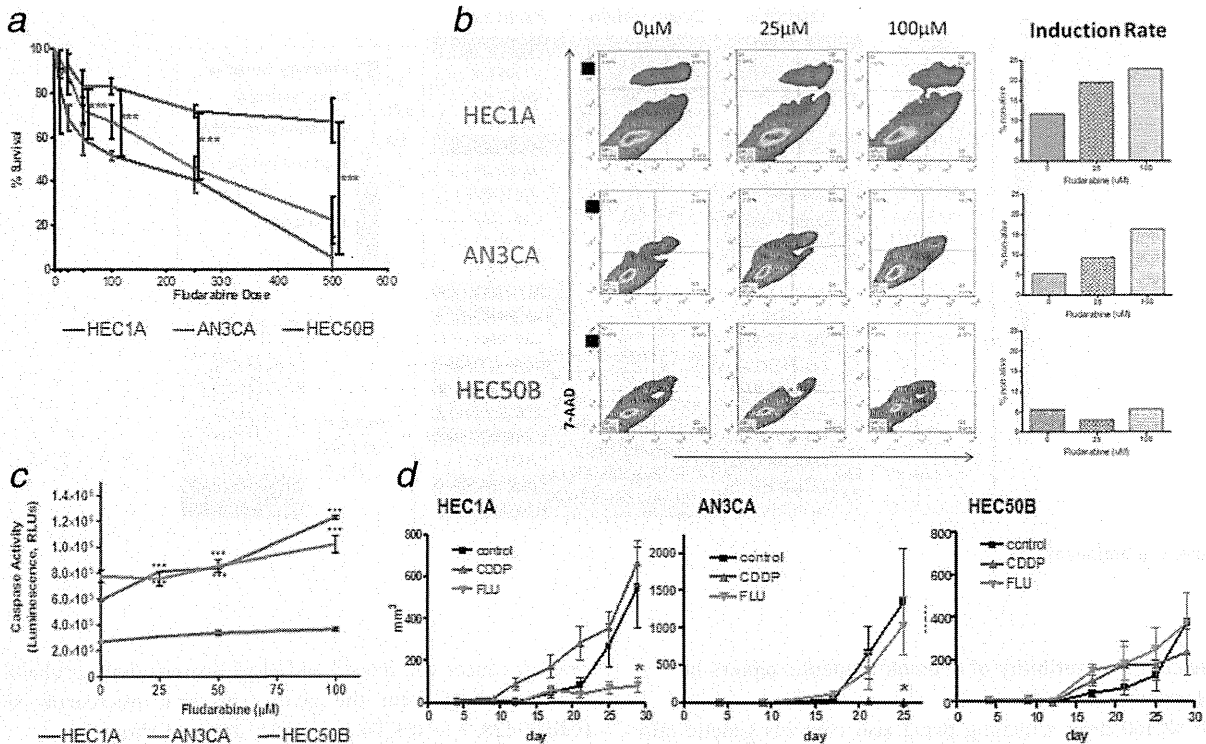

Figure 3. Cytotoxic activities of fludarabine. (*a*) The cytotoxicity of fludarabine was measured using WST-1 cell proliferation assays. There were growth-inhibitory responses in AN3CA (green) and HEC1A (blue) in a dose-dependent manner ($p < 0.01$), whereas this response was not significant in HEC50B (red). (*b*) Apoptosis induction after fludarabine treatment in HEC1A, AN3CA and HEC50B cells. The apoptotic effect of fludarabine was measured using the annexin-V/7-AAD apoptosis assay kit. (*c*) Caspase-3 protein induction by fludarabine. After 24-hr exposure to fludarabine, luminescence was gradually elevated in HEC1A cells in a dose-dependent manner, reflecting the activity of caspase-3/7, and this induction was almost sixfold higher than that in HEC50B cells ($p < 0.001$). (*d*) Fludarabine efficacy *in vivo*. Mice inoculated with 5×10^6 endometrial cancer cells subcutaneously were treated with fludarabine (125 mg/kg), sterile distilled water or cisplatin (1 mg/kg/day). Tumor growth was completely inhibited by cisplatin for mice inoculated with AN3CA ($p < 0.05$). Tumor growth in HEC1A-inoculated mice was significantly inhibited by fludarabine ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

100 μM fludarabine (Fig. 3*b*). Conversely, no induction of apoptosis was observed in the HEC50B cells.

Fludarabine-induced caspase-3 activity

Caspase-3 activation is involved in cell death signaling and induces chromatin condensation and DNA fragmentation, resulting in apoptosis. Caspase-3α and caspase-3β mRNA expression in HEC1A and AN3CA cells were upregulated by fludarabine in a dose-dependent manner ($p < 0.05$, Supporting Information Fig. 2A). HEC1A cells were still responsive to fludarabine at the lowest dose, 25 μM, whereas a similar low-dose response was not evident in AN3CA cells. There was no change in expression levels of these genes in the HEC50B cells, even at 100 μM. Similarly, caspase-3 protein expression increased in HEC1A cells after fludarabine treatment in a dose-dependent manner accompanied by a concomitant increase of cleaved-caspase-3 (Supporting Information Fig. 2B). Next, caspase-3/7 activity was assessed using a luminometer to investigate whether the upregulation of caspase-3 expression in fludarabine-treated cells was related to the activity of caspase-3 in facilitating and inducing apoptosis. After 24-hr exposure to fludarabine, the luminescence was gradually elevated in HEC1A cells in a dose-dependent manner to reflect the activity of caspase-3/7, and this induction was almost sixfold higher than that observed in HEC50B cells ($p < 0.001$, Fig. 3*c*). The AN3CA cells also showed increasing luminescence but the slope of the increase was lower than that in HEC1A cells.

Therapeutic effects of fludarabine in a mouse xenograft model of endometrial cancer

We investigated the *in vivo* therapeutic effects of fludarabine, cisplatin and sterile water on subcutaneously inoculated HEC1A, AN3CA and HEC50B xenografts in CD-1 Foxn/Nu mice. Tumor growth was completely inhibited by cisplatin in mice inoculated with AN3CA cells, consistent with the high probability of cisplatin sensitivity predicted from the microarray analysis ($p < 0.05$, Fig. 3*d*). Growth-inhibitory effects of

fludarabine were not evident in AN3CA-inoculated mice, and there was no therapeutic effect of cisplatin or fludarabine on tumor growth of HEC50B-inoculated mice. On the other hand and also consistent with the predicted sensitivity, tumor growth in HEC1A-inoculated mice was indeed significantly inhibited by fludarabine ($p < 0.05$, Fig. 3*d*).

Discussion

Endometrial carcinoma is frequently diagnosed at an early stage, at which point it is usually surgically curable. Surgical treatment includes hysterectomy, bilateral salpingo-oophorectomy and staging lymphadenectomy, which is a controversial but still common procedure in treating endometrial cancers. Adjuvant therapies to prevent relapse are mainly composed of platinum and doxorubicin or taxane, and are used for the patients bearing tumors with aggressive features such as pathologically high grade, invasion into the outer myometrium, lymphovascular space or cervix and extrauterine spread.[13,14] As shown in Table 2, the tumor recurrence rate in the group of intermediate-risk patients was significantly diminished by adjuvant chemotherapies, whereas recurrence was not improved in the high-risk group receiving similar therapies. Several randomized trials have reported effectiveness of adjuvant therapies. The carboplatin–paclitaxel regimen was previously reported as a well-tolerated and active regimen that provides a median time to progression of 13 months and a median overall survival of 47 months in high-risk patients.[15] The GOG 177 trial showed that addition of paclitaxel to the doxorubicin–cisplatin regimen significantly improved the rate of objective response and overall survival of high-risk or recurrent cases.[5] However, these results, such as the 57% of response rate and 17.3 months of overall survival, are not satisfactory in terms of therapeutic effect, and so the need for improved treatment strategies is urgently required to improve therapeutic benefit for patients with high-risk or recurrent disease.

The majority of high-risk or chemorefractory endometrial cancer cases are high grade, and the major challenge in developing more effective therapeutic strategies for these women involves confronting the heterogeneity of the disease and the distinct underlying mechanisms. The NCI60 database is comprised of more than 60 established cell lines from a diverse collection of malignant human tissues that have been tested for sensitivity to over 40,000 compounds to identify those with anticancer activity. Because of this diversity, the NCI60 is regarded as a reasonable representation of tumor heterogeneity and has been extensively used for discovering potent anticancer drugs.[11,16,17] Endometrial cancers are heterogeneous tumors that are classified into several types based on histological differentiation, and etiologic heterogeneity exists as well, especially within endometrioid adenocarcinoma. Thus, efforts have been made to establish molecular-based classifications, which may help in understanding the differences in biology and clinical outcome among subtypes.[18] In our study, 39% of the NCI60 cell lines were refractory to cisplatin, doxorubicin or paclitaxel, and intriguingly, this rate is quite similar to the 44% of high-risk patients who are chemorefractory from our data. With the aim of prompt clinical translation, the GI50 values from the NCI60 data were analyzed for 24 agents already in clinical use, and the results indicated that none was promising for the high-risk patient population. However, as the anticancer drugs and the molecular-targeting drugs exhibit distinct spectra, combined therapy using agents from each group may be a reasonable and more effective approach for use as second-line chemotherapy.

Gene expression profiling studies have been valuable tools for revealing the complex nature of cancer and for identifying new therapeutic strategies.[11] As the NCI60 dataset includes gene expression information, a pharmacogenomics approach allows the ability to define common genetic backgrounds as "signatures" of chemorefractory cell lines and to identify candidate drugs potentially effective against these cell lines. Using Bayesian binary regression methods, a sensitivity gene expression signature for each candidate drug, based on the results of the NCI60 analysis, can be projected onto independent microarray datasets including those from endometrial cancers to predict the probability of drug sensitivity for each sample. As expected, the sensitivity probabilities of three conventional chemotherapeutic agents were low in high-grade endometrial cancers (Fig. 2*a*). In contrast, temsirolimus was predicted as a potentially effective agent against serous adenocarcinoma. Temsirolimus is a molecular targeting agent that functions by inhibiting mTOR pathway signaling, a pathway impaired in endometrial cancers. This agent is now being studied under a GOG clinical trial to investigate efficacy for chemorefractory endometrial cancers. Considering our analysis, temsirolimus may be effective in a subset analysis for histology even when the results of primary endpoint analysis for the entire population might be negative. In endometrial cancers, loss of PTEN correlates with poor outcomes,[18] and cell lines with little or no PTEN are preferentially sensitive to temsirolimus as cell viability and Akt phosphorylation were regulated by temsirolimus in a dose-dependent manner.[19] There are no reports showing interaction between the mTOR pathway and p53 mutations in serous endometrial cancers, but upregulation of mTOR is observed in invasive bladder cancer accompanied by deletion or mutation of p53,[20] which suggests that temsirolimus, as an mTOR inhibitor, might be useful in serous endometrial cancers in which p53 is frequently mutated.

By analyzing the complexity of the genomic signatures from the cell lines, fludarabine was also predicted as a potential candidate for chemorefractory high-grade cases. Among seven endometrial cancer cell lines that were resistant to cisplatin, doxorubicin and paclitaxel, five exhibited favorable probability of sensitivity to fludarabine, whereas only three of those seven exhibited a favorable probability score of sensitivity to temsirolimus (Supporting Information Table 2). These results suggest that fludarabine is a promising alternative agent for chemorefractory endometrial cancers.

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The classes of antineoplastic drugs belonging to the group of purine nucleoside analogs play an important role and have had a substantial impact on the treatment of cancer.[21–23] Fludarabine is a purine analog that has demonstrated significant activity in B-cell malignancies, including CLL and indolent non-Hodgkin's lymphoma. Fludarabine is converted intracellularly into its active metabolite F-ara-ATP, which inhibits DNA as well as RNA synthesis, resulting in induction of growth arrest and apoptosis.[24] A single case report in the 1980s failed to show efficacy for recurrent endometrial cancers,[25] but as the number of cases was small and tumor histology was varied, it is hard to determine efficacy in high-grade tumors from this report. Our preliminary analysis for gene expression implies "purine metabolism" is augmented in G3 and serous endometrial cancers compared to G1 and G2 (data not shown), which is supportive of our prediction on fludarabine efficacy toward G3 and serous cases.

The exact mechanism of apoptosis induction by F-ara-A in proliferative and quiescent cells has not been completely clarified although purine nucleoside analogs are reported to activate d-ATP-dependent caspase pathways.[26] To investigate the cytotoxic effect of fludarabine, *in vitro* proliferation assays were done on three endometrial cancer cell lines, which were chosen as representative cell lines according to their probability of sensitivity to conventional chemo agents, as described in the Material and Methods section. As shown in Figure 3, fludarabine has a cytotoxic effect on endometrial cancer, inhibiting tumor growth and inducing apoptosis in a dose-dependent manner *in vitro,* suggesting that fludarabine may inhibit the proliferation of endometrial cancer cells through induction of apoptosis, consistent with reports using Jurkat cells.[24] Caspase-3 is activated *via* death receptor signaling to induce chromatin condensation and DNA fragmentation, resulting in apoptosis.[27,28] In both HEC1A cells and AN3CA cells, caspase-3 was activated *in vitro* by fludarabine more than fivefold higher than in the HEC50B cells that are resistant to fludarabine. On the other hand, fludarabine was not inhibitory to tumor growth in AN3CA-inoculated mice, but robustly inhibited HEC1A-derived tumors. The reason for this difference is not clear, but as growth inhibition and induction of apoptosis *in vitro* were higher in HEC1A cells at a lower dose, it may be that drug delivery to the tumor tissue *in vivo* was not as effective. Furthermore, *Caspase-3* expression was increased, suggesting the possibility that this augmentation may play a role in determining susceptibility to fludarabine despite the belief that constitutive expression of caspase-3 is not usually considered significant in apoptosis. These results may imply the existence of an unknown mechanism(s) enhancing the efficacy of fludarabine in each tumor.

In our study, we have used a pharmacogenomics approach with drug-specific signatures as a targeted method to identify new candidate drugs with potential efficacy against chemoresistant endometrial cancers. Through array-based analysis, fludarabine and temsirolimus were identified as candidate chemotherapeutic agents for chemorefractory endometrial cancers. This prediction was confirmed both *in vitro* and *in vivo.* Although further study is warranted, fludarabine may prove beneficial as an addition to the treatment strategy for managing high-risk endometrial cancers.

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「卵巣癌の腫瘍局所における包括的な免疫環境の解析と治療応用への基礎的研究」

(2010~2011年度 文部科学省科学研究費補助金採択研究)

がん研究に出会うまで

私は1999年に当教室に入局し，大学での2年間の研修後，関連病院で3年間臨床を学びました．初診から最後には死亡に至る婦人科癌を経験するたびに，「何かほかの治療は？ もっとほかにできることはないのだろうか？」と，学会や地方部会に出向いていました．そんなある日，藤井信吾教授(現名誉教授)から大学院での研究の誘いがあり迷うことなく受験をしました．入学後の最初のテーマは万代昌紀講師(現近畿大学教授)指導下での卵巣癌の新規治療開発でした．

卵巣癌とがん免疫？

進行・再発卵巣癌は，非常に予後不良であるため，新たな治療戦略が求められています．その候補の1つに以前から免疫療法があげられていますが，あまり注目はされていませんでした．そこでまず私たちは，卵巣癌における腫瘍局所の免疫状態を解明し，免疫治療への糸口を見つけようということになりました．そのきっかけは普段からみている病理組織と，本学免疫ゲノム医学研究室(本庶佑教授)との共同研究でした．術後病理所見で「炎症(免疫)細胞浸潤を伴う卵巣癌組織が…」とのコメントを見かけると，「腫瘍の中の免疫細胞はなぜ腫瘍細胞を排除しないのだろうか？」という疑問がでてきました．一般に，生体内では遺伝子変異などにより，1日に何千ものがん細胞が発生しても免疫系に排除される，いわゆる「免疫監視」が働いています．近年，ある種のがん細胞が，免疫抑制物質を発現したり放出することで，この免疫監視から攻撃を受けない「がん免疫逃避システム」を備えていることが明らかになり，このシステムを打破するような新しい免疫療法の開発が非常に注目されています．その1つが本学旧医化学研(現免疫ゲノム医学)教室で発見された免疫抑制性補助シグナル分子PD-1(programmed cell death-1)とそのリガンドPD-L1シグナルでした．私が大学院に入った2005年に，この研究室から当教室への共同研究の呼びかけから，卵巣癌でのPD-L1発現と腫瘍内に浸潤した免疫細胞の解析が始まりました．

卵巣癌におけるPD-L1発現と腫瘍内浸潤免疫細胞

まず当科で手術した卵巣癌患者の腫瘍組織を用いて，腫瘍局所における免疫状態の指標として大腸がんや肺がんでも報告のあるキラーT細胞の浸潤度と，病理学的因子や予後との関係を調べた結果，腫瘍内にキラー細胞の浸潤が多いほど患者予後が極めてよいことがわかりました．一方，卵巣癌におけるPD-L1高発現は，多変量解析にて独立予後不良因子であることがわかりました．また大変興味深いことに，卵巣癌のPD-L1発現は，キラーT細胞浸潤度とは逆相関したことから，少なくとも卵巣癌のPD-L1発現によって，腫瘍内にキラーT細胞が浸潤できない症例が数多くあることが示唆されました．この結果を2007年に米国科学アカデミー紀要(PNAS 2007；104：3360)に報告したのち，さらに本学トランスレーショナルリサーチ施設(探索医療センター)の支援を受け，卵巣癌に対

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するPD-1シグナル阻害剤を用いた臨床応用への橋渡しへと広がり，当科小西郁生教授の下，2011年より新規免疫療法として医師主導治験を開始しています．

卵巣癌における包括的な局所免疫の解析

腫瘍のPD-L1発現とキラー細胞浸潤度との逆相関が比較的緩やかであったことから，他の免疫抑制因子の存在が推察されました．そこで次に，キラーT細胞のほかに，初期免疫のナチュラルキラー細胞や樹状細胞，獲得免疫のヘルパーT細胞，制御性T細胞，PD-1陽性細胞の腫瘍内浸潤度を評価し，さらに腫瘍による免疫抑制因子としてPD-L1とそのアイソタイプPD-L2，シクロオキシゲナーゼCOX1とCOX2，形質転換増殖因子TGF-β1の発現を調べて，定量化したこれら11因子がどのように相互に作用し，どのような腫瘍局所の免疫環境をもたらしているのかを，網羅的遺伝子解析に用いる階層的クラスタリングという手法を行った結果，免疫細胞浸潤や免疫因子の発現のパターンが似かよった症例を4つの群（クラスター）に分類できることがわかりました．

この解析によると，各種免疫細胞が多く集まるが，腫瘍の免疫抑制因子発現が最も低いクラスター1と，免疫細胞浸潤が少なく，腫瘍における免疫抑制因子が高発現しているクラスター2，3，4に大きく分かれました．さらに，クラスター2は腫瘍のCOX1高発現，クラスター3では腫瘍のPD-L2高発現，クラスター4では腫瘍のPD-L1，TGFβ1，COX2が比較的高発現していることがわかりました．また4つのクラスターと進行期や組織型などの病理組織学的因子との間に相関性はないにもかかわらず，クラスター1の症例は，他のどのクラスターよりも予後がよいことがわかりました．

今回の免疫細胞浸潤や免疫抑制因子を用いた包括的解析により，卵巣癌患者の腫瘍局所における免疫状態は，腫瘍が発現する複数の免疫抑制因子により規定されている可能性が示唆されました（Clin Immunol 2011；141：338）．今後さらに，抗がん剤や免疫療法の開始前後の免疫状態を解析することによって，治療対象の個別化や効果予測への有用な指標になるのではないかと考えています．また，この結果は，前年に，マウス卵巣癌モデルにおいて卵巣癌局所に免疫細胞を誘導する特殊な実験系において，局所免疫が誘導されていると腫瘍抑制や生存期間延長につながることを報告（Stem Cells 2010；28：164）しており，実際のヒトの卵巣癌組織でこれを裏づける結果となりました．

この研究を通して

ここ数年，がん免疫療法，特に免疫逃避を標的にした治療については，世界的にその関心の高さや，期待の大きさがうかがえます（Pardoll, Nat Rev Cancer 2012；12；252）．これは卵巣癌についても例外でないのですが（Kandalaft et al：JCO 2011；29：925），わが国の婦人科腫瘍領域においてはまだ一般的な議論にはなっていません．本研究をはじめ，今後さらなる卵巣癌の免疫に関する病態解明により，がん免疫という視点から既存の治療に代わるあるいは補完するような新しい治療法の開発を求めて研究を続けていきたいと思っています．最後にこれらの研究を指導していただいている当教室の先生方や，腫瘍研究室の皆様に感謝申し上げます．

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編集主幹より

今回紹介した若手研究者はともに自身の研究成果が実際の臨床応用にまで到達している点ですばらしい．研究開始の動機も多忙な臨床実務の経験の中から自ら見出し，紆余曲折を経ながらも癌治療に役立たせることを目標としている点でも共通している．トランスレーショナルリサーチが提唱されて久しいが，臨床経験を有する若手研究者の研究成果が臨床研究のシーズとなるものは必ずしも多いとはいえない．しかし，わが国でも基礎から臨床への流れが着実に実践されつつあることを感じさせる．(青木大輔)

11日間マスター 輸液処方の実践に活かす 水・電解質・酸塩基平衡の基本
札幌北クリニック 大平整爾 日鋼記念病院腎センター 伊丹儀友 編集
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●研修医・若手医師が困る『体液異常への対応』と『輸液処方』に役立つ！
本書の特徴：①体液の基礎から，体液異常の鑑別診断，治療まで解説，②体液異常ごとのフローチャートで鑑別の手順がわかる，③学習内容ごとに症例解説・演習問題があり，理解を深められる．

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特集 がん免疫療法の最前線

7．再発・進行卵巣癌に対する抗PD-1抗体を用いた免疫療法

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要旨

腫瘍免疫学の進歩により，がん細胞が宿主免疫から逃避するメカニズム「がん免疫逃避機構」が提唱され，本機構を標的とする治療開発が世界中で注目されており，その代表がPD-1/PD-1リガンド経路である．本テーマでは，PD-1/PD-1リガンド経路について概説するとともに，これまで当科で行ってきた卵巣癌における本経路の基礎的検討から，抗PD-1抗体を用いた医師主導治験の実現までの道程，ならびに抗PD-1抗体治療の展望と課題について述べる．

KeyWords がん免疫逃避機構，PD-1，PD-L1

卵巣癌

卵巣癌は，婦人科悪性腫瘍の中で最も予後不良の疾患であり，その罹患率および死亡率ともに増加傾向にある．進行卵巣癌に対して，手術療法や化学療法含めた集学的治療を行っても，これらの半数以上は再発し，長期予後はいまだに改善されていない．そのため化学療法に代わるあるいは補完するような新しい治療戦略が求められており，その一つが免疫療法である．近年，腫瘍免疫学において「がん免疫逃避機構」の存在が示され，この機構を克服するような新たな治療開発が進められている．

がん免疫逃避機構

2002年Dunnらは，がん細胞が様々な方法で免疫細胞からの攻撃を逃れているという「がん免疫逃避機構」を提唱した[1]．その後，数多くの検証により，①腫瘍抗原・HLA発現の消失，②免疫抑制因子の発現や分泌（IL-6，IL-10，TGF-β，IDO，シクロオキシゲナーゼ［COX］，PD-1リガンド［後述］等）および，③免疫抑制性細胞の誘導（制御性T細胞や骨髄由来免疫抑制性細胞）などがかかわっていることが示され，本学説は広く支持されている[2]（**図1**）．近年，この中でも特に免疫抑制にかかわるPD-1（programmed cell death-1）/PD-1リガンド経路が注目されている．

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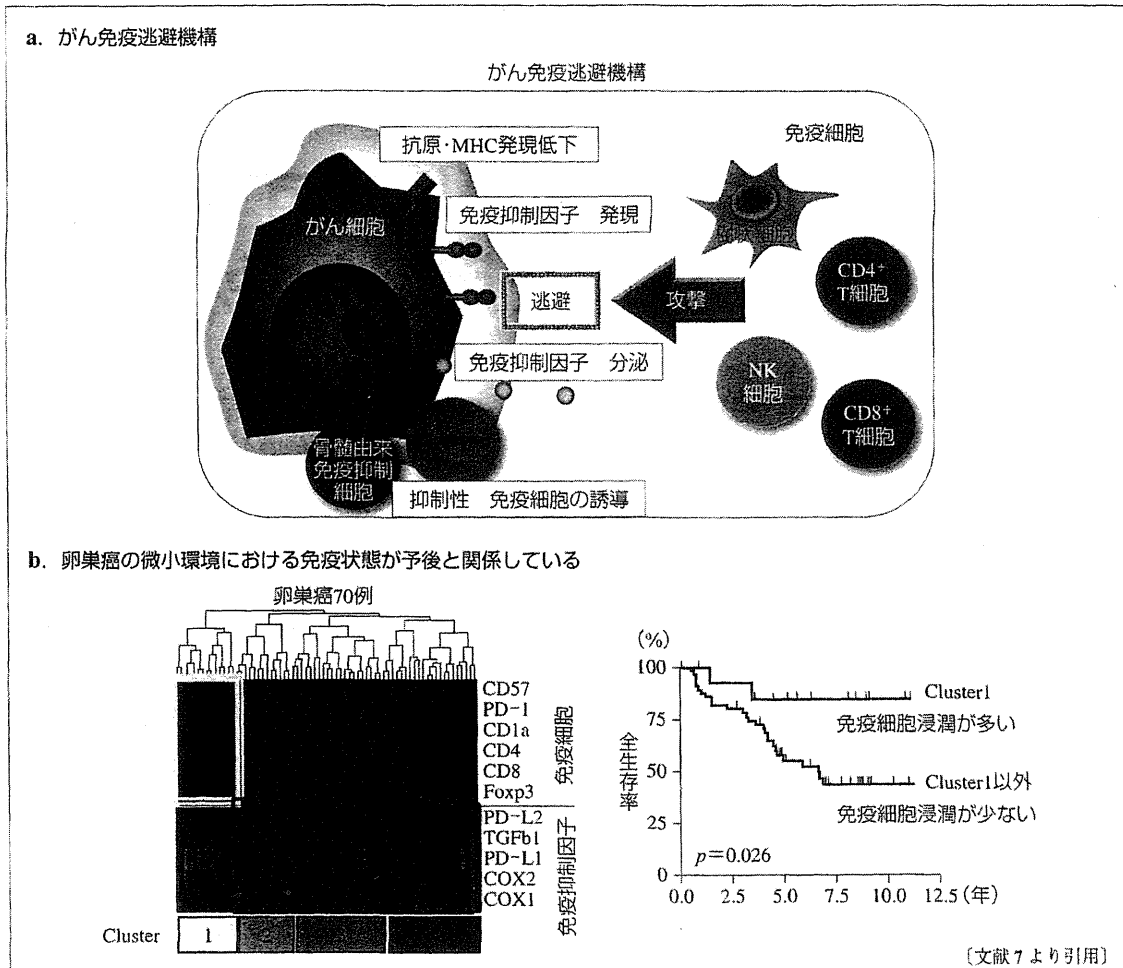

図1 卵巣癌におけるがん免疫逃避機構

PD-1/PD-1 リガンド経路

PD-1(CD279)は, CD28ファミリーに属する免疫抑制性副シグナル受容体であり, 1992年に京都大学旧医化学研(現 免疫ゲノム医学)の石田らによって分離同定された[3]. PD-1は, 活性化したT細胞, B細胞および骨髄系細胞に発現し, そのリガンドとの結合により抗原特異的にT細胞活性を抑制することから, 自己に対する末梢性免疫寛容を誘導する代表的な分子であるとともに, 免疫反応後の疲弊したT細胞のマーカー分子とされている.

PD-1のリガンドには, 免疫副シグナルB7ファミリーに属するPD-L1(CD274, B7-H1)とPD-L2(CD273, B7-H2)があり, PD-L2は樹状細胞にのみ発現しているが, PD-L1は樹状細胞ほか血管や心筋, 肺, 胎盤などに幅広く発現している. PD-1/PD-1リガンド経路は, 自己への不適切な免疫反応や過剰な炎症反応を制御する「免疫チェックポイント」ともよばれ, もう一つの免疫副シグナルCD28/CTLA4経路とともに免疫学的なホメオスターシスにかかわる重要な役割を果たしている.

一方, 腎癌, 悪性黒色腫, 非小細胞肺癌, 膵臓癌, 食道癌など数多くのがん細胞がPD-L1を高発現しており, 術後の予後不良とかかわっているもの多いことが報告されている(**表1**)[4].