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General Summary

The main research topics of our department are the development of molecularly targeted agents for gynecologic tumors, including ovarian cancer; clarification of the mechanisms of successful pregnancy; and the development of assisted reproductive techniques. These topics were investigated both experimentally and clinically.

Research Activities

Gynecologic oncology

1. High-risk ovarian cancer based on a 126-gene expression signature is uniquely characterized by downregulation of the antigen-presentation pathway

High-grade serous ovarian cancers are heterogeneous both in terms of clinical outcomes and at the molecular level. Our aim was to establish a novel risk-classification system based on gene expression signatures for predicting overall survival which we hope will lead to novel therapeutic strategies for high-risk patients. In this large-scale cross-platform study of 6 microarray data sets from 1,054 patients with ovarian cancer, we developed a gene expression signature for predicting overall survival by applying elastic net analysis and 10-fold cross-validation to Japanese data set A (n=260) and evaluated signatures in 5 other data sets. Subsequently, we investigated differences in biological characteristics between patients with high-risk and low-risk ovarian cancers. Elastic net analysis of Japanese data set A identified a 126-gene expression signature for predicting overall survival in patients with ovarian cancer (multivariate analysis, $P=4\times10(-20)$). We validated its predictive ability through multivariate analysis with 5 other data sets (Tothill's data set, $P=1\times10(-5)$; Bonome's data set, P=0.0033; Dressman's data set, P=0.0016; The Cancer Genome Atlas data set, P=0.0027; Japanese data set B, P=0.021). Through gene ontology and pathway analyses, we identified a significant reduction in expression of immune-response–related genes, especially on the antigen-presentation pathway, in patients with high-risk ovarian cancer. This risk classification based on a 126-gene expression signature is an accurate predictor of clinical outcome in patients with advanced high-grade serous ovarian cancer and might lead to new therapeutic strategies for patients with high-grade serous ovarian cancer.

2. Short-term serum deprivation confers sensitivity to taxanes in platinum-resistant

human ovarian cancer cells

On the basis of evidence that serum deprivation provokes apoptosis in variety of cells, the effect of serum deprivation on drug sensitivity was examined. Serum deprivation resulted in significant increases in paclitaxel sensitivity by factors of 148 and 10 in platinum-resistant C13 and CP70 cells, respectively. Similarly, serum deprivation induced significant docetaxel sensitivity in these cell lines. However, serum deprivation did not enhance docetaxel sensitivity in platinum-sensitive cells. Furthermore, serum deprivation did not have any effect on the sensitivities to cisplatin, vincristine, or doxorubicin in any of these cells. Increases in apoptotic cells of more than 700% were observed in C13 and CP70 cells when they were subjected to serum deprivation followed by exposure to paclitaxel. Serum deprivation decreased mitochondrial membrane potential ($\Delta\Psi m$) in C13 and CP70 cells. This finding indicates that serum deprivation induces depolarization specifically in platinum-resistant cells. These results indicate that serum deprivation confers hypersensitivity to taxane specifically in platinum-resistant cells by improving their impaired mitochondrial functions. This finding might be clinically beneficial for the development of new chemotherapeutic technology, particularly for patients with platinum-resistant ovarian cancer.

3. Cytokine gene expression signature in ovarian clear cell carcinoma

Cytokine expression in a tumor microenvironment can affect both host defense against the tumor and tumor cell survival. In this study, we sought to clarify whether the cytokine gene expression profile has clinical associations with ovarian cancer. We analyzed the expression of genes for 16 cytokines (interleukin [IL]-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, interferon γ, tumor necrosis factor α, IL-6, HLA-DRA, HLA-DPA1, and colony-stimulating factor 1) in 50 ovarian carcinomas. Hierarchical clustering analysis of these tumors was performed with Cluster software, and differentially expressed genes were examined between clear cell carcinoma (CCC) and other subtypes. Following this examination we evaluated the biological significance of IL-6 knockdown in CCC. Unsupervised hierarchical clustering analysis of cytokine gene expression revealed 2 distinct clusters. The relationship between the 2 clusters and clinical variables showed statistically significant differences in CCC compared with other histologic types. The CCC showed a dominant Th-2 cytokine expression pattern driven largely by IL-6 expression. Inhibition of IL-6 in CCC cells suppressed signal transducer and activator of transcription 3 (Stat3) signaling and rendered cells sensitive to cytotoxic agents. The unique cytokine expression pattern found in CCC may be involved in the pathogenesis of this subtype. In particular, high IL-6 expression appears likely to be driven by the tumor cells, fueling an autocrine pathway involving IL-6 expression and Stat3 activation, and may affect survival when the tumor cells are exposed to cytotoxic chemotherapy. Modulation of IL-6 expression or its related signaling pathway is a promising treatment strategy for CCC.

4. Hypoxia promotes glycogen synthesis and accumulation in human ovarian CCC

Ovarian CCC has several significant characteristics based on molecular features that are distinct from those of ovarian high-grade serous carcinoma. Cellular glycogen accumulation is the most conspicuous feature of ovarian CCC, and in the present study its metabolic mechanism was investigated. The amount of glycogen in cells cultured under

hypoxic conditions increased significantly and approximately doubled after 48 hours ($P<0.01$) as compared with cells cultured under normoxic conditions. Periodic acid-Schiff staining also demonstrated intracellular glycogen storage. Western blot analysis revealed that hypoxia-inducible factor 1α, which was overexpressed and stabilized under hypoxic conditions, led to an increase in the level of cellular glycogen synthase 1, muscle type (GYS1), and, conversely, to a decrease in inactive phosphorylated GYS1 at serine (Ser) 641. Additional increases were observed in both protein phosphatase 1, which dephosphorylates and thereby induces GYS1 enzyme activity, and glycogen synthase kinase 3 beta (GSK3β) phosphorylated at Ser9, which does not act on phosphorylated GYS1 and subsequently induces its enzyme activity. In contrast, levels of phosphorylase, glycogen, muscle (PYGM)-b decreased. These results indicate that the glycogen accumulation in a hypoxic environment promotes glycogen synthesis but does not inhibit glycogen degradation or consumption. Under hypoxic conditions, HAC2 cells showed activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway caused by a mutation in exon 20 of *PIK3CA*, encoding the catalytic subunit p110α of PI3K. The resulting activation of AKT (phosphoSer473) also plays a role as a central enhancer in glycogen synthesis through suppression of GSK3β via phosphorylation at Ser9. Hypoxia decreased the cytocidal activities of cisplatin and doxorubicin to various degrees. In summary, hypoxic conditions, together with the expression and stabilization of hypoxia-inducible factor 1, increased the intracellular glycogen contents and the resistance to anticancer drugs.

5. Identification of a novel long noncoding RNA involved in the tumorigenicity of ovarian CCC

Ovarian CCC has a poor prognosis because of its chemoresistance. Therefore, identifying novel therapeutic targets is important. Recently, several long noncoding RNAs (lncRNAs), such as HOTAIR, have been reported to be aberrantly expressed in human cancers. However, lncRNAs involved in the tumorigenicity of ovarian CCC have not been reported. In this study, we attempted to identify novel lncRNAs that are required for the tumorigenicity of CCC. Apoptotic cell death was detected with the MEBCYTO Apoptosis Kit (MBL International, Woburn, MA, USA). Cell growth was measured with the methylthiotetrazol assay. For subcutaneous xenograft experiments, ovarian CCC JHOC5 cells infected with a lentivirus expressing a short hairpin RNA targeting lncRNA were injected stereotactically into 6-week-old nude mice. We found that knockdown of a novel lncRNA, termed ASBEL (antisense noncoding RNA in the BTG3 locus), resulted in a marked increase in the apoptotic death of JHOC5 cells. Moreover, knockdown of ASBEL significantly repressed the tumorigenicity of JHOC5 cells. We have identified a novel lncRNA, ASBEL, which is required for the tumorigenicity of CCC and, thus, is a promising target for the diagnosis and therapy of CCC.

6. Development of second-generation photodynamic therapy for cervical cancer aiming at reducing photosensitivity and shortening length of stay

Uterine cervix conization has become a standard method of uterus-preserving therapy for early stage cervical cancer. However, because a 2006 article in *The Lancet* reported that conization increases the risks of premature birth, low birth weight, and cesarean delivery, the 2011 guidelines for the treatment of cervical cancer of the Japan Society of Gyneco-

logic Oncology states that informed consent concerning these risks is necessary before conization. On the other hand, photophrin photodynamic therapy (PDT) for cervical cancer shows a high complete response rate (97%) and low obstetrical risk, as mentioned above, but PDT is not recommended as the standard treatment for cervical cancer because it has the side effect of photosensitivity and requires a hospital stay as long as 3 weeks. Therefore, to develop second-generation PDT for cervical cancer with the aim of reducing photosensitivity and shortening hospital stays, we tested a semiconductor laser and its adaptation to the existing probe for uterine cervix irradiation in collaboration with Professor Kunio Awazu (Department of Engineering, Osaka University). First, we produced an FC adapter to connect a direct irradiation probe for lung cancer to an existing probe for uterine cervix irradiation. Then, we connected the direct irradiation probe for lung cancer to the main apparatus of the PD laser, and through the FC adapter connected an existing probe for uterine cervix irradiation to a probe for lung cancer. We performed laser irradiation under different experimental conditions. Next year, we plan to perform a phase I clinical trial of second-generation PDT using the photosensitizer talaporfin sodium (Laserphyrin, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) with less photosensitivity.

Perinatology

1. Antiphospholipid antibodies and dysregulated clotting factors differentially affect the villous trophoblast in fetal growth restriction

Antiphospholipid antibodies (aPLs) and dysregulated clotting factors are associated with pregnancy pathologies, including preeclampsia and fetal growth restriction. Here we investigated the effect of aPLs and dysregulated clotting factors on the biology of the villous trophoblast. Placental samples were collected from healthy control subjects (n=8, 25-38 weeks) and patients (all with a small for gestational age infant) with aPLs/antiphospholipid syndrome (APS; n=10, 25-36 weeks) or dysregulated clotting factors (n=9, 27-35 weeks). Placentas were stained for Ki-67 and cytokeratin 7 to identify proliferating villous cytotrophoblast. Images were systematically and randomly selected, and Ki-67-positive cytotrophoblast and fibrin deposits were counted. There were no significant differences in gestational age at delivery, maternal age, or body-mass index. Fetal weight differed significantly between control subjects (2,291±833 g) and patients with APS (1,160±413 g) and between control subjects and patients with dysregulated clotting factors (1,182±516 g). In patients with APS, placental weight tended to be lower and the P/F ratio tended to be higher than in patients with dysregulated clotting factors. The percentage of Ki-67-positive cytotrophoblast in control subjects gradually decreased with gestational age, whereas this decrease was not apparent in patients with APS or dysregulated clotting factors. The relative number of Ki-67-positive cells was significantly lower in patients with APS (12.1%±7.1%) and dysregulated clotting factors (12.7%±5.8%) than in control subjects (22.6%±6.4%). The total number of cytotrophoblasts was lower in patients with APS (578±209) than in patients with dysregulated clotting factors (830±258) or in control subjects (780±149). There was no significant differences in total fibrin deposition or intravillous fibrin deposition. However, perivillous fibrin deposition was significantly greater in patients with dysregulated clotting factors

(3.7%±1.5%) than in patients with APS (1.9%±1.1%). We suggest that aPLs downregulate cytotrophoblast proliferation throughout pregnancy, whereas in patients with dysregulated clotting factors, an increasing amount of fibrin deposition finally leads to a decrease in trophoblast proliferation but does not affect the total cell count.

2. Pathological changes in aborted tissue from patients who have had recurrent spontaneous abortions with aPLs induced by paternal lymphocyte immunization

Immunization with paternal lymphocytes for patients with recurrent spontaneous abortion (RSA) reportedly stimulates maternal immune response and transmits blocking antibodies and, therefore, contributes to the maintenance of pregnancy. However, this treatment may induce autoantibodies, including aPLs, that may be unfavorable for pregnancy. Seventy-one patients with RSA of unknown cause but without autoantibodies, including aPLs, underwent immunization with paternal lymphocytes at The Jikei University Hospital from April 2003 through December 2011. The presence of aPLs was assessed after treatment. Therapeutic outcomes were compared between patients in whom aPLs were induced and patients in whom they were not. We investigated pathological changes in aborted tissues from patients who miscarried after treatment. Of the 71 patients, 15 patients (21.1%) showed induced aPLs and 56 patients (78.9%) did not. Of the patients with induced aPLs, 14 had anticardiolipin immunoglobulin G antibodies. The pregnancy success rate was 63.6% (7 of 11 pregnancies) in patients with induced aPLs and 67.5% (27 of 40 pregnancies) in patients without induced aPLs. In patients with induced aPLs, anticoagulant therapy was performed for 7 patients, 6 of whom (85.7%) successfully maintained pregnancy; in contrast, of 4 patients who did not receive anticoagulant therapy, only 1 successfully maintained pregnancy (25%). Investigation of pathological changes in aborted tissues with a normal karyotype revealed characteristic changes in 4 of 7 cases in patients with induced aPLs, whereas no changes were observed in patients without induced aPLs (0 of 6 cases). Changes included abortive vessels; pink, amorphous deposits; and lymphocyte infiltration. After immunization with paternal lymphocytes the possible induction of aPLs should be assessed. Subsequent anticoagulant therapy may prevent abortion even under these adverse conditions.

Reproductive endocrinology

We investigated potential clinical indicators of the outcome of in vitro fertilization (IVF) treatment in women 40 years or older undergoing infertility treatment. We retrospectively examined the results of IVF treatment in a total of 111 women 40 years or older. We found that in addition to patient age and the number of treatment cycles, cancellation of a treatment cycle could be a useful indicator of pregnancy outcome. Moreover, we investigated whether anti-Mullerian hormone would be a useful indicator for infertile women 40 years or older to consider ending infertility treatment. Our study indicated that anti-Mullerian hormone is not a definitive indicator to consider ending infertility treatment for infertile women 40 years or older.

In our next study, we investigated the relationship among outcomes of infertility treatment, ovarian reserve, and endometriosis in infertile women 40 years or older to establish an indicator for ending infertility treatment. Our results suggest that endometriosis decreases oocyte quality and is a useful predictor of treatment outcome in infertile women

40 years or older.

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