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Interleukin-34 expression in ovarian cancer: A possible correlation with disease progression

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Ovarian cancer is the second lethal gynecological malignancy and the seventh cause of cancer-related death in women around the world. Most of the ovarian cancer patients are diagnosed at advanced-stages and suffer from recurrence after primary cytoreductive surgery and standard first-line chemotherapy. Thus, the successful management of ovarian cancer patients requires the identification of factors that contribute to progression and relapse. Interleukin-34 (IL-34) is a novel cytokine that acts as a tissue-specific ligand of colony stimulating factor-1 receptor. In cancer, IL-34 exerts pro-tumorigenic functions that promote tumor growth, metastasis, angiogenesis, immune suppression, and therapeutic resistance. In this study, we evaluate the impact of IL-34 on progression and survival of ovarian cancer patients. First, IL-34 was found to be expressed in several human ovarian cancer cell lines and cancer tissues from patients. The expression of IL-34 was enhanced by cytotoxic chemotherapy in ovarian cancer cell lines and cancer tissues from chemotherapy-treated ovarian cancer patients. Importantly, high IL-34 expression correlated with worse progression-free survival (PFS) and overall survival in different cohorts. The assessment of PFS based on a combination between *IL34* expression and other related genes such as *CSF1R* and *CD163* helped further to reach more statistical significance compared with *IL34* alone. Furthermore, in murine ovarian cancer cell HM-1 *in vivo* model, it was suggested that IL-34-derived tumor cells was correlated with tumor progression and survival by modulating immune environment. Collectively, these findings indicate a possible correlation between IL-34 expression and disease progression in ovarian cancer patients and mouse model.

Keywords

Interleukin-34, ovarian cancer, disease stage, progression-free survival, overall survival

Introduction

Epithelial ovarian cancer is one of the most frequent female gynecological cancers, characterized by high mortality and poor prognosis (1-3). In the USA, 22500 women develop new cases of epithelial ovarian cancers, of whom 14000 (>60%) die every year (1-3). Most patients have advanced disease on the first diagnosis, due to the metastatic characteristic of ovarian cancers, which results in high mortality rates. Despite recent advances in the management of ovarian cancers that helped to approach a complete response in a significant proportion of patients, most of those suffer a recurrence within 18 months (1-3). The management of epithelial ovarian cancers combines surgery and platinum-based chemotherapy to achieve optimum clinical outcomes (4, 5). Treatment options include neoadjuvant chemotherapy followed by surgical debulking of tumors and adjuvant chemotherapy, or primary debulking surgery followed by adjuvant chemotherapy (4, 5). In most cases, tumors remain sensitive to periodic retreatment with cytotoxic chemotherapy, until the development of chemoresistance that restricts further treatment options (4). Thus, identifying factors that correlate with disease progression becomes critical for treatment strategies and survival improvement.

In this context, interleukin-34 (IL-34) has been identified as an important factor that exerts pro-tumorigenic roles at the tumor microenvironment (TME) (6). IL-34 was reported in 2008 as a cytokine that binds colony-stimulating factor-1 receptor (CSF-1R), in addition to colony-stimulating factor-1 (CSF-1) (7). Under physiological conditions, IL-34 contributes to the development and maintenance of Langerhans cells and microglia (6, 8, 9). However, IL-34 can be induced under pathological conditions and importantly contributes to the etiology of various diseases including autoimmune diseases, inflammatory disorders, infections and cancer (6, 8, 9). In cancer, IL-34 plays essential roles in tumor growth, metastasis, angiogenesis,

immune suppression and therapeutic resistance (10-20). Importantly, the expression of IL-34 in cancer is correlated with enhanced chemoresistance, such as in malignant pleural mesotheliomas, lung cancers and colon cancer, and correlates with poor survival when highly expressed (11 ,15 ,17 ,19). In this regard, the impact of IL-34 expression on disease progression and patients' survival in ovarian cancers remains unknown. In this study, we examine the expression of IL-34 in human ovarian cancers and evaluate the correlation between IL-34 expression and disease progression in retrospective cohort studies. Furthermore, we demonstrate whether or not IL-34 expression in ovarian cancer cell correlates prognosis and survival in mouse model.

Methods

Cell lines

Human ovarian adenocarcinoma (KF28, OVISE and OVTOKO) and lung adenocarcinoma (A549) cell lines were obtained and utilized in this study as follows. KF28 cell line was provided kindly by Prof. Yoshihiro Kikuchi (National Defense Medical College, Saitama, Japan). OVISE and OVTOKO cell lines were obtained from the Japanese Collection of Research Bioresources, Osaka, Japan (JCRB). A549 cell line was obtained from the American Type Culture Collection. Murine ovarian cancer cell line HM-1 was purchased from JCRB. The Lenti-X 293T cell line was purchased from TaKaRa. All cell lines were cultured at 37℃ with 5% CO_2 in an appropriate culture medium.

Generation of chemo-resistant cancer cell lines

Chemo-resistant cell lines were established as we previously described (15). Ovarian cancer or lung cancer cells were exposed to stepwise increasing concentrations of standard chemotherapies, including doxorubicin (0.01–1 μM) or cisplatin (0.01–0.1 μM). After reaching maximal concentrations, chemo-resistant cells were exposed to maximal toxic concentrations at regular intervals to maintain their drug resistance. In experiments that use supernatants of cell culture, chemo-resistant cells were washed five times with sterilized phosphate-buffered saline (PBS) and cultured in media without these drugs.

Generation of IL-34 knockout HM-1 cell line

Firefly luciferase (Luc) lentiviral particles were generated by transfecting Lenti-X 293T cells with psPAX2 (Addgene), pMD2.5 (Addgene) and pLenti-PGK-V5-Luc Neo (W632-2) using TransITX2 transfection reagent (Mirus). Supernatants containing

lentiviral particles were collected and used to infect HM-1 cells, which were then continuously selected by G418 (500 mg/ml).

Then, *Il34*^{KO} HM-1 cell line was generated by using IL-34 CRISPR/Cas9 KO Plasmid (m) (Santa Cruz Biotechnology). The plasmids were transfected by using *Trans*IT-X2 (Mirus). Cells were selected by GFP expression 48 h after transfection.

Cell viability assay

To assess cell viability, MTT assay was performed using MTT Cell count kit (Nacalai Tesque). Absorbance at a test wavelength of 570 nm and a reference wavelength of 650 nm was measured by using a Multiskan FC (Thermo Fisher Scientific). Cell proliferation was followed up to 3 days.

Enzyme-linked immunosorbent assay (*ELISA)*

The production of IL-34 in cell lines was measured with ELISA. Culture supernatants were collected at 48 h after seeding the cells at a density of 1×10^6 in 6-well plate. The IL-34 contents were measured with LEGEND MAX Human IL-34 ELISA kit with Pre-Coated Plates (Biolegend) or LEGEND MAX Mouse IL-34 ELISA kit with Pre-Coated Plates (Biolegend).

Clinical samples

Ovarian cancer patients were diagnosed at Hokkaido University Hospital (Sapporo, Japan), St. Marianna University School of Medicine (Kanagawa, Japan) or Kanagawa Cancer Center (Yokohama, Japan) between June 2006 and January 2016. Patients were staged according to International Federation of Gynecology and Obstetrics (FIGO) criteria and graded by gynecologic pathologists based on the World Health Organization guidelines. Clinical samples were selected if sufficient

formalin-fixed paraffin-embedded (FFPE) tissues were available for immunohistochemistry (IHC) staining. Cancer tissues were obtained either from primary cytoreductive surgery or interval surgery after neoadjuvant chemotherapy. Patients who underwent primary cytoreductive surgery received platinum-based chemotherapeutic regimen combined with paclitaxel. In the case of neoadjuvant chemotherapy, patients received platinum-based chemotherapeutic regimen. The follow-up period was calculated from the date of initial treatment either surgery or neoadjuvant chemotherapy and was last updated in July 2017. Informed consent was obtained from all patients, and all experiments were approved and performed in accordance with the relevant guidelines and regulations indicated by the institutional ethics committees of Hokkaido University Hospital (Approval no. 17-0001), Hokkaido University Institute for Genetic Medicine (17-0001), Kanagawa Cancer Center and St. Marianna University School of Medicine (3520). All clinical samples were collected with written informed consent from all patients.

Chemotherapy stimulation

KF28, OVISE and OVTOKO human ovarian cancer cell lines were seeded in 96-well culture plate and stimulated with increased concentrations of two chemotherapeutic agents: cisplatin or doxorubicin (0.001–10 μM). Cell viability was evaluated after 48 h by MTT assay (Cell Proliferation Kit I, Roche). In other experiments, cells were treated with cisplatin or doxorubicin at concentrations at which 60-80% cells are viable (1 μM). Following treatment for 48 h, total RNAs were collected for qRT-PCR analysis as described in the following section.

Quantitative reverse transcription PCR analysis

For experiments described in Figs. 1A and 4, total RNAs were extracted using TriPure Isogen Reagent (Roche Molecular Biosciences), and 1 μg of total RNAs was used for first strand cDNA synthesis using ReverTraAce (TOYOBO). qRT-PCR was performed on cDNA products using Fast SYBR green PCR Master Mix (Applied Bioscience), and samples were run on Applied Step One real-time PCR system (Applied Biosystems). For experiments described in Fig. 1B, total RNAs were isolated from cultured cells and ovarian cancer or normal tissues using Maxwell 16 LEV simply RNA Tissue Kit (Promega). Complementary DNA was synthesized using ReverTraAce qPCR RT Kit (TOYOBO) and quantified by real-time PCR using TaqMan Universal Master Mix II and TaqMan assays on a StepOne Plus thermocycler (Applied Biosystems) according to the manufacturer's instructions. Primers for *β-ACTIN* (*ACTB*: Hs01060665_g1) and IL-34 (*IL34*: Hs01050926_m1) were purchased from Applied Biosystems. Primers used in Figs. 1 and 4 were as follows: *β-ACTIN* (forward: 5'-TCACCCACACTGTGCCCATCTACG-3' and reverse: 5'-CAGCGGAACCGCTCATTGCCAATG-3'), *IL34* (forward: 5'-GTGCCTTACGAGGGGGTGTTC-3' and reverse: 5'-CACCTTGGGGCTGACCTCCAC-3'), *CSF1* (forward: 5'-CCTGAAGAGCTGCTTCACCAA-3' and reverse: 5'-CATTCTTGACCTTCTCCAGCAA-3'), *CSF1R* (forward: 5'-TGCCTTACAACGAGAAGTGGGAG-3' and reverse: 5'-ATCTTCACAGCCACCTTCAGGAC-3'). All experiments were performed in triplicate for each sample. Each target gene expression was normalized to *β-ACTIN* expression. Relative quantitation was calculated using the $2^{\Delta Ct}$ method. Fold induction was calculated based on the gene expression of control group (vehicle group or normal ovarian epithelium sample) in each experiment.

Immunohistochemistry

To investigate the expression level of IL-34 in clinical samples from ovarian cancer patients, FFPE tissue sections were prepared from surgical specimens and stained with a rabbit anti-IL-34 antibody (1D11 clone, Abcam 101443 or Millipore-QVP 1311236) after protein blocking. The sections were immersed in PBS with 0.3% H_2O_2 + 40% MeOH at 4℃ overnight for blocking endogenous peroxidase. Then, the sections were incubated with HRP-labeled anti-rabbit IgG as the secondary antibody (DakoCytomation). Substrate-chromogen were added, and the sections were counterstained with hematoxylin. IL-34 showed homogeneous staining in tumor tissues. The intensity of IL-34 staining was evaluated semiquantitatively as absent, weak or strong without prior knowledge of clinicopathological data. For IHC scoring, tumor areas were objectively judged by two independent researchers at 200× magnification for each section, and quantification of IL-34 immunoreactivity on the randomly-selected tumor areas in each section was carried out on 20× images using Image J software (National Institutes of Health). For calculating average and errors of quantify, 100 images captured randomly were used. Based on color deconvolution, the following three images were produced: DAB image, hematoxylin image, and a complementary image. DAB images were binarized and assigned a score. In digital image analysis, the pixel intensity values for any color range from 0 to 255, where 0 represents the darkest shade of color, while 255 represents the highest shade of the color as the standard.

Ion AmpliSeq targeted sequencing analysis

Tumor tissue specimens were collected upon operative surgery from 39 patients with a preoperative diagnosis of ovarian cancer. Tissue specimens were rapidly (within 10 min after tumor resection) collected in vials containing RNA later (QIAGEN) and

stored at −80℃ until use. Total RNAs were extracted from tumor tissues using RNeasy Mini (QIAGEN) and quantified using Qubit 3.0 Fluorimeter (Thermo Fisher Scientific). Ten nanograms of total RNAs were used to prepare libraries for transcriptome using Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific). Prepared libraries were purified using AMPure XP (Beckman Coulter), quantified using Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific), diluted to 50 pM, and pooled equally with eight samples per pool. Emulsion PCR was performed on Ion Chef™ System. The template libraries were then sequenced on Ion Proton™ system using Ion P1 Hi-Q Chef Kit and Ion P1 Chip Kit v3 (Thermo Fisher Scientific). Based on this method, the medians of *IL34*, *CSF1*, *CSF1R*, and *CD163* expressions were calculated from AmpliSeq values (RPM: Reads Per Million), and the cohort was divided accordingly into patients with high or low expression of *IL34* (≧0.40 or <0.40), *CSF1* (≧1.62 or <1.62), *CSF1R* (≧1.79 or <1.79), *CD163* (≧1.78 or <1.78), and a Kaplan Meier curve was generated to evaluate the correlation between gene expression and survival.

Mouse experimental model

Six- to eight-week-old female B6C3F1 mice (Japan SLC, Inc.) were inoculated with 1 × 10^6 Luc^+ HM-1 cells into ovary or subcutaneously. For *in vivo* bioluminescence imaging, mice were intravenously injected with A-Luciferin (150 mg/kg dissolved in PBS; Avidin Ltd) and imaging was started within 30 s from the injection by using IVIS Spectrum Imaging Systems (Spectrum-FL-TKHD; Caliper Life Sciences Ltd). Exposure time in *in vivo* experiment was 1 min and in *in vitro* experiment was 40 s.

Isolation of tumor-infiltrating immune cells from solid tumor

Isolation of tumor-infiltrating immune cells from solid tumors was performed by using BD Horizon™ Dri Tumor & Tissue kit, according instructions of manufacture (Becton, Dickinson and Company). The recovered tumor-infiltrating cells were used for flow cytometry.

Flow cytometry

Cells were washed and blocked with FcR Blocking Reagent (TONBO) and stained with 4',6-diamidino-2-phenylindole (DAPI, Cayman Chemical Company) and the antibodies against following molecules; CD45, CD3, CD4, CD8, F4/80 and CD11b (BioLegend). Data were acquired using BD FACSCelesta flow cytometer and analyzed using FlowJo software.

Statistics

All statistical analyses were performed using StatView software. Overall survival (OS) was defined as the period from the date of surgery or first chemotherapeutic treatment until cancer-related death or last follow-up. Progression-free survival (PFS) was defined as the period from the date of first chemotherapeutic treatment until recurrence. Kaplan Meier curves were generated for each relevant variable for IL-34, CSF-1, CSF-1R or CD163 expression, and differences in survival among patient subgroups were analyzed using the log-rank test. *P*-value of <0.05 was considered significant, and all tests were performed two-sided.

Results

IL-34 expression in ovarian cancer patients correlates with disease progression

Previous studies have reported the expression of IL-34 in various cancers including colon cancers, cholangiocarcinoma, giant cell tumors, glioblastoma, hepatocellular carcinoma, lung cancers, malignant pleural mesothelioma, melanoma, and osteosarcoma (10-20). In this study, we first examined whether IL-34 expression can be detected in ovarian cancer cell lines and clinical samples from ovarian cancer patients. In our previous report, *IL34* mRNA and IL-34 protein expression levels were high in human lung cancer cell line A549 acquired resistance to doxorubicin (A549-DR) compared with doxorubicin-sensitive A549 (A549-DS) (15). We prepared A549-DS as a negative control to calculate IL-34 expression level in ovarian cancer cell lines. Furthermore, we established the cisplatin resistant KF28 (KF28-CR) to evaluate the difference IL-34 expression level compared with cisplatin sensitive KF28 (KF28-CS). qRT-PCR analysis showed that *IL34* mRNA is expressed at different levels in human ovarian and lung cancer cell lines including A549-DS, KF28-CS, OVTOKO, OVISE and chemo-resistant cell line. IL-34 level in the culture supernatant of each cell line was measured by ELISA (Fig. 1A).

Additionally, IL-34 expression was evaluated in clinical samples from a cohort of ovarian cancer patients. This cohort comprises ovarian cancer patients (n = 113) diagnosed at Kanagawa Cancer Center with serous adenocarcinoma (n = 43, 38.1%), clear cell carcinoma (n = 35, 31.0%), endometrioid tumors (n = 16, 14.2%) in addition to mucinous adenocarcinoma (n = 10, 8.8%) and others including teratoma, york sac tumor, dysgerminoma and adenocarcinofibroma (n = 9, 8.0%). Tumors were surgically removed from these patients without any prior neoadjuvant chemotherapy. qRT-PCR analysis showed different levels of *IL34* mRNA in primary ovarian cancer

297 tissues when compared with control (normal ovary epithelium or normal fallopian
298 tissue) (Fig. 1B). Using statistical analysis, *IL34* mRNA expression level in tumor
299 tissue was higher than normal fallopian tissue and normal ovarian epithelium
300 (P=0.0017, Student's t-test). Furthermore, IHC staining of clinical samples unveiled
301 the expression of IL-34 at the protein level in ovarian cancer tissues but not in normal
302 tissues (Fig. 1C). By calculating positivity rates in each group classified according to
303 FIGO stages, IL-34 showed positive staining in 37.3% of patients at stage I (n=19/51),
304 43.8% at stage II (n=7/16), 62.5% at stage III (n=20/32) and 85.6% at stage IV
305 (n=12/14) (Fig. 1D). Strong staining of IL-34 showed high frequencies in patients at
306 stages III and IV (21.9% and 42.8%, respectively) compared with stages I and II
307 (15.7% and 12.5%, respectively) (Supplementary Table 1, P = 0.002, Fisher's exact
308 test). However, it should be noted here that this analysis was collectively performed
309 on all patients in this cohort. When divided into subgroups according to disease
310 stages, Kaplan Meier analysis showed that the expression of IL-34 has no impact on
311 the OS of ovarian cancer patients at the same disease stage (Supplementary Figure
312 1). This indicates that there is a possible confounding relationship between IL-34 and
313 disease stage. Furthermore, when performing FIGO classification for each
314 histological type of tumors, IL-34 showed a tendency to correlate better with serous
315 cystadenocarcinoma than other tumors (Supplementary Table 1). Therefore, there is
316 a possibility that the results could be affected by various factors associated with IL-34
317 expression. Thus, these findings should be confirmed in larger cohorts in future
318 studies. Together, these results indicate that IL-34 is a factor which is upregulated in
319 an advanced stage of ovarian cancer and has a possibility for affecting patient
320 prognosis depending on the histological type.
321
322 **IL-34 expression correlates with poor PFS in ovarian cancer patients**

RNA-Seq has emerged as a powerful technology that can help to improve the predictive performance of the survival analysis method (21, 22). As described above, IL-34 is suggested to correlate with disease stage and poor survival in ovarian cancer patients. To strengthen this conclusion, we next examined the correlation between *IL34* expression and survival in a cohort of ovarian cancer patients based on RNA-Seq analysis. In this cohort, a total of 39 cancer patients were pathologically diagnosed at the Department of Obstetrics and Gynecology in Hokkaido University Hospital with various histological types of ovarian cancers. The clinicopathological characteristics of the cohort are summarized in Supplementary Table 2. Upon operative surgery, total RNAs were extracted from tumor tissues and subjected to Ion AmpliSeq Targeted Sequencing analysis as described in Materials and methods. The median of AmpliSeq values of *IL34* expression was calculated and utilized to divide the cohort into patients with high or low *IL34* expression. Based on this classification, a Kaplan Meier curve was generated to evaluate the correlation between *IL34* expression and patients' survival. Since only 8 out of 39 patients reached the clinical end-point (death) during the follow-up period in this study, we chose to examine the relation between *IL34* expression and PFS, which in this case refers to recurrence upon initial treatment. Interestingly, the Kaplan Meier curve revealed a correlation between high expression of *IL34* and poor PFS in ovarian cancer patients (P = 0.0421) (Fig. 2A). In addition to *IL34*, a Kaplan Meier curve was generated to evaluate the correlation between *CSF1*, *CSF1R* and *CD163* (M2-macrophage marker) expressions and PFS. While there was no association between *CSF1* expression and PFS in this cohort (Fig. 2B, P = 0.4870), high expression of *CSF1R* (Fig. 2C, P = 0.0226) or *CD163* (Fig. 2D, P = 0.0174) was associated with worse PFS, similar to *IL34*. Furthermore, we performed Pearson correlation analysis to investigate correlation between expression of *IL34* with *CSF1*, *CSF1R* or *CD163*. As

results, among these factors, only *CSF1R* expression showed correlation with *IL34* expression (R^2=0.2294, *P*=0.0018) (Fig. 2E-G). Although we previously reported that IL-34 expression correlates with CSF-1R and CD163 and consequently with poor survival in lung cancer patients (19), this was not fully recapitulated in ovarian cancer. However, above findings prompted us to evaluate *IL34* correlation with PFS in combination with *CSF1R* and *CD163*.

_IL34_ combined with _CSF1R_ and _CD163_ associates with worse PFS in ovarian cancers

Thus, we next examined the impact of *IL34* expression on PFS when combined with *CSF1R* and *CD163* in our cohort of ovarian cancer patients described above. Based on the method described above, the cohort was re-divided into groups of patients with (i) high expression of both *IL34* and *CSF1R*, (ii) high expression of both *IL34* and *CD163*, (iii) high expression of *IL34*, *CSF1R* and *CD163* against other groups. Kaplan Meier analysis of PFS in ovarian cancer patients showed that the combination between high expression of *IL34* with *CSF1R* (*IL34*^{high} *CSF1R*^{high}: *n*=15, others: *n*=31) (Fig. 3A, *P* = 0.011) or *CD163* (*IL34*^{high} *CD163*^{high}: *n*=13, others: *n*=33) (Fig. 3B, *P* = 0.004) significantly correlated with worse PFS. The combination of *IL34*, *CSF1R* and *CD163* showed the most statistical significance (*IL34*^{high} *CSF1R*^{high} *CD163*^{high}: *n*=9, others: *n*=37) (Fig. 3C, *P* = 0.001) compared with other groups. Together, the evaluation of the *IL34* expression in combination with other related genes such as *CSF1R* and *CD163* may help to further improve the predictive performance of survival assessment in ovarian cancer patients.

Enhanced IL-34 expression in chemotherapy-treated ovarian cancer cell lines

Chemotherapy is an essential component in the regimens of ovarian cancer treatment. Previous reports suggest that chemotherapeutic agents can induce the cellular expression of IL-34, such as in cisplatin- or doxorubicin-treated lung cancer cells (15). Thus, we next asked whether chemotherapy could similarly enhance IL-34 expression in ovarian cancer cells. To answer this question, we stimulated various human ovarian cancer cells lines with two chemotherapeutic agents cisplatin (Fig. 4A) and doxorubicin (Fig. 4B) at concentrations at which 60-80% cells are viable (1 μM). In KF28, OVISE and OVTOKO cell lines, the treatment with cisplatin (Fig. 4C) or doxorubicin (Fig. 4D) was effective to induce IL-34 expression compared with vehicle control. Furthermore, the treatment of KF28 with increasing concentrations of cisplatin could induce the expression of IL-34 in a dose-dependent manner (Supplementary Figure 2). Together, these results indicate that chemotherapy treatment may induce IL-34 expression in ovarian cancer cells.

Enhanced IL-34 expression in chemotherapy-treated ovarian cancer patients

We asked whether chemotherapy treatment could result in an enhancement of IL-34 expression in patients with ovarian cancers. To answer this question, we compared the expression levels of IL-34 in clinical samples from chemotherapy-treated ovarian cancer patients. IHC staining of ovarian cancer tissues unveiled an enhanced expression of IL-34 in recurrent cancer tissues upon chemotherapy treatment compared to primary cancer tissues (Fig. 5). Thus, IL-34 expression can be enhanced by chemotherapy treatment in ovarian cancer patients.

IL-34 derived from cancer cell is correlated with cancer progression in murine ovarian cancer model

Finally, we carried out *in vivo* experiments using murine ovarian cancer cell line HM-1

which expresses IL-34 at high level. First, we established *Il34* knockout (*Il34*^{KO}) HM-1 and mock-transfected (Mock) HM-1 by CRISPR-Cas9 system. The expression of IL-34 of individual cell lines were measured by ELISA (Fig. 6A). These cell lines express CSF-1 equally at the protein level (data not shown). Both cell lines showed equivalent growth rate *in vitro* (Supplementary Figure 3A, B). In addition, luciferase-expression vector was transfected into both HM-1 cell lines by using lentivirus. We confirmed that the luciferase expression levels were nearly equal in both cell lines (Supplementary Figure 3C, D). To represent naturally occurring ovarian cancer, we performed orthotopic inoculation of HM-1 cells. Luc-Mock HM-1 and Luc-*Il34*^{KO} HM-1 resuspended with Matrigel were inoculated into ovary directly, and we observed the spread of bioluminescence signal of tumor cells. As results, Mock HM-1 signal spread more widely compared with *Il34*^{KO} HM-1 (Fig. 6B). Moreover, the survival rate of mice inoculated Mock HM-1 was lower than inoculated *Il34*^{KO} HM-1 (Fig. 6C). Similarly, when the cells were subcutaneously inoculated, rate of tumor growth of *Il34*^{KO} HM-1 was lower than that of Mock HM-1 (Supplementary Figure 4). These results suggested that IL-34-derived tumor cells promoted ovarian cancer progression in mouse model. Then, to evaluate the effect of IL-34 for TME in ovarian tumor, we investigated the population of infiltrating immune cells into primary tumor site by flow cytometry. Interestingly, we found that the population of CD11b^{+}F4/80^{+} macrophage was decreased in *Il34*^{KO} HM-1 inoculated group compared with Mock HM-1 inoculated group (P=0.048) (Fig. 6D) while the population of CD3^{+} T cell within the tumors showed increasing trend in *Il34*^{KO} HM-1 inoculated group (P=0.062) (Fig. 6D). These results suggest that IL-34 derived from ovarian cancer cells can modulate the population of immune cells in tumor sites and contribute to form pro-tumorigenic environment.

Discussion

Chemotherapy is still an essential component of ovarian cancer treatment, along with surgery. Unfortunately, cancer cells acquire resistance to chemotherapy during the treatment course, resulting in tumor recurrence, metastasis, and, ultimately, patient death (23). In this study, we evaluated for the first time the expression of IL-34 and its correlation with disease progression in ovarian cancer patients and mouse model. Notably, IL-34 expression was observed in chemo-resistant ovarian cancer cells and prone to be expressed in advanced stage tumor.

Growing evidence from clinical studies and experimental animal models has suggested an association between IL-34 expression and tumor progression, metastasis, angiogenesis, and acquired resistance to cancer therapy (10-20). Consistent with these roles, IL-34 expression correlates with poor survival, such as in blood, brain, colorectal and lung cancers (14). Similarly, we found in this study that IL-34 expression can be detected in ovarian cancers. The expression of IL-34 was suggested to possibly correlate with disease progression and poor survival in ovarian cancer patients.

By evaluating the expression of IL-34 in ovarian cancer tissues using Ion AmpliSeq sequencing technology, we found that high expression of IL-34 correlates with worse PFS in our cohort of ovarian cancer patients, and with poor survival in another independent cohort registered at the cBioPortal database that utilizes RNA-Seq technology. These technologies have recently emerged as powerful tools that hold new promise for their diagnostic, prognostic and therapeutic applicability in various diseases (21,22). Based on the results above, we suggest that evaluating the expression of IL-34 by these technologies may help to improve the predictive performance of the RNA-Seq-based survival analysis method in ovarian cancer patients.

452 In contrast to *IL34*, *CSF1* expression showed no statistical significance on PFS or
453 OS in the cohorts of ovarian cancer patients described in this study. These findings
454 are inconsistent with a previous study that suggested the diagnostic usefulness of
455 CSF-1 in epithelial ovarian cancers (24). This might be explained by the biological
456 differences between CSF-1 and IL-34. First, IL-34 is suggested to have high affinity
457 to CSF-1R than CSF-1 and can induce strong activation of signaling molecules
458 downstream of CSF-1R (7,25). Thus, an elevated level of IL-34 at the TME is
459 expected to strongly activate various signaling pathways in CSF-1R-expressing cells
460 (tumor or non-tumor) and consequently enhances tumor progression. Second, while
461 CSF-1 activities are restricted to CSF-1R, IL-34 may interact with other molecules
462 such as PTPRZ1 and syndecan-1 in addition to CSF-1R (26, 27). Thus, IL-34 is
463 expected to have broad effects on various cells and may show more impact on the
464 TME than CSF-1. This also indicates the importance of direct targeting of IL-34 rather
465 than CSF-1R alone, due to the existence of various receptors and regulators for IL-
466 34, which should be considered in the treatment of patients with IL-34-producing
467 cancers.
468 We also performed *in vivo* experiments using murine ovarian cancer cell HM-1
469 expressing IL-34 to further evaluate the relationship between IL-34 and ovarian
470 cancer prognosis. From our results that *Il34*^{KO} HM-1 tumor showed mild spread and
471 better prognosis of tumor-bearing mice, it was suggested that cancer cell-derived IL-
472 34 correlates with tumor progression and poor survival of mouse model. Additionally,
473 we performed flow cytometry analysis to evaluate how IL-34 effects the TME in
474 ovarian tumor site. Then, in the immune microenvironment of primary tumor formed
475 by *Il34*^{KO} HM-1, the population of CD11b^{+}F4/80^{+} macrophage was decreased and
476 CD3^{+}CD8^{+} T cell tended to increase compared with Mock HM-1 group. It has been
477 reported that infiltrating M2 TAMs into ovarian tumor site correlates with tumor

progression and poor survival in patients (28). Moreover, in a previous report published by Zhang *et al*., the number of $CD3^+$ infiltrating T cells in tumor sites correlated with significantly increase long term survival in patients with advanced ovarian cancer (29). According to our *in vivo* experiments data and these reports, it is suggested that IL-34 derived from ovarian tumor cells contribute to creating pro-tumor environment by altering the population of macrophage and T cell that results in promoting tumor progression and poor survival rate in mouse model.

In our recent report, we have identified IL-34 as an important factor that correlates with disease stage and poor survival in lung cancer patients (19). In this study, we extend these findings to ovarian cancers, showing a strong correlation between IL-34 expression and disease progression of ovarian cancer patients and mouse model. On the basis of current evidence, our data suggest that IL-34 would be a new target of therapies to ovarian cancer patients including someone has resistance to chemotherapy.

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Conflict of interest statement: the authors declared no conflicts of interest.

Author contribution

Endo H, Baghdadi M, and Seino KI designed research and experiments. Endo H, Hama N, Baghdadi M, Ishikawa K, Asano H, Endo D, Konno Y, Kato T, Tozawa A, Takano A, and Kato H performed experiments. All authors analyzed data and discussed the results. Endo H, Hama N, Baghdadi M, Otsuka R and Seino KI wrote the manuscript, and all authors approved the final manuscript.

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592 associated macrophages is associated with extended survival in ovarian cancer
593 patients. *J. Ovarian Res*. 7:19.
594

Figure legends

Fig. 1. IL-34 expression in ovarian cancer. (A) qRT-PCR and ELISA analysis of *IL34* or IL-34 expression in human ovarian cancer cell lines. The expression level of *IL34* was normalized to housekeeping gene, *β-ACTIN*. Fold induction was calculated based on A549-DS not expressing *IL34* cell line in qRT-PCR. One representative of three independent experiments is shown. (B) Representative data of *IL34* qRT-PCR analysis in primary ovarian cancer tissues from patients diagnosed with clear cell or serous carcinoma. Fold induction was calculated based on the normal ovarian epithelium sample 51N. Boxplots show deference of the *IL34* expression level between normal tissues and ovarian cancer. The expression level in tumor tissues is significantly higher compared with normal tissues. (P=0.0017; Student's t-test). (C) Representative data of immunohistochemical staining of IL-34 in ovarian cancer tissues or normal fallopian tubal tissues. (D) Bar graph analysis of IL-34 positivity rates in ovarian cancers according to each stage of FIGO classification. (E) Kaplan Meier analysis of OS in a cohort of ovarian cancer patients was performed based on IL-34 expression. Time line refers to days after surgery.

Fig. 2. A correlation between *IL34* expression and worse PFS in ovarian cancers. Kaplan Meier analysis of PFS in a cohort of ovarian cancer patients was performed based on the expression of *IL34* (A), *CSF1* (B), *CSF1R* (C) or *CD163* (D) expression in tumor tissues. Statistical significance was compared using the log-rank test. (E-G), Correlation between expression of *IL34*, *CSF1*, *CSF1R* and *CD163* according to RNA-Seq, assessed by Spearman's correlation coefficient.

Fig. 3. The impact of *IL34*/*CSF1R*/*CD163* on PFS in ovarian cancers. Kaplan Meier analysis of PFS in a cohort of ovarian cancer patients, as compared between patients that show high expression of both *IL34* and *CSF1R* (*IL34*^{high} *CSF1R*^{high}: *n*=15, others: *n*=31) (A) *IL34* and *CD163* (*IL34*^{high} *CD163*^{high}: *n*=13, others: *n*=33) (B) or all high (*IL34*^{high} *CSF1R*^{high} *CD163*^{high}: *n*=9, others: *n*=37) (C) against other groups. Statistical significance was compared using the log-rank test.

Fig. 4. IL-34 induction by chemotherapy in human ovarian cancer cell lines. A dose-response curve of cisplatin (A) or doxorubicin (B) in KF28, OVISE and OVTOKO human ovarian cell lines were generated based on cell viability 48 h after stimulation. In next experiments, cells were treated with cisplatin (C) or doxorubicin (D) at concentrations at which 60-80% cells are viable (1 μM), and qRT-PCR analysis was performed to evaluate *IL34* expression 48 h upon stimulation. The expression levels of *IL34* mRNA were normalized to housekeeping gene, *β-ACTIN*. Fold induction was calculated based on the vehicle control group (added NaCl or DMSO instead of chemotherapeutic agent). One representative of three independent experiments is shown. Data are shown as mean ± SEM.

Fig. 5. IL-34 expression in chemotherapy-treated ovarian cancer patients. IHC staining of IL-34 in primary (P) or recurrent (R) cancer tissues from ovarian cancer patients. One handred images in each sample were captured and used for calculating average and errors of quantity of IL-34 immunoreactivity. The bar graph on the right shows fold induction of IL-34 staining in tumor areas when compared between primary and recurrent tumors (primary tumors =1). IL-34 staining in normal skin is shown as a positive control for the specificity of the anti-IL-34 antibody.

644 Statistical significance was compared using the log-rank test. Data are shown as
645 mean ± SEM. *P<0.05.
646
647 **Fig. 6.** IL-34 expression derived from cancer cells correlates tumor progression and
648 poor survival in mouse model. (A) IL-34 concentration in supernatants of HM-1 cell
649 lines. ELISA was performed in triplicate for each sample. One representative of three
650 independent experiments is shown. (B) B6C3F1 mice that received inoculation of 1 ×
651 10^6 Luc$^+$ Mock HM-1 or *Il34*^{KO} HM-1 cells were monitored using the IVIS imaging
652 system at 3 weeks following the inoculation. Mice were i.v. injected with A-Luciferin
653 and the imaging was started within 30 s from the injection. The exposure time was 1
654 min. (C) Kaplan Meier analysis of survival rate in two groups; inoculated Mock HM-1
655 or *Il34*^{KO} HM-1 (n=9-11/group). (D) Bar graphs represent the frequency of each
656 subset CD3$^+$, CD3$^+$CD8$^+$, CD3$^+$CD4$^+$ T cells and CD11b$^+$F4/80$^+$ macrophage within
657 the tumor-infiltrating CD45$^+$ cells on day 10. (n=4/group). Control means ovaries
658 collected from healthy mice (n=3). Data represent mean ± SEM. *P<0.05,
659 ***P<0.001; the log-rank test (C) and Student's t-test (D).
660
661 **A list of all supporting information**
662 **Supplementary Figures**
663 Figure 1: The correlation between IL34 expression and OS in ovarian cancer patients
664 in each disease stage
665 Figure 2: Induction of IL34 expression by cytotoxic chemotherapy
666 Figure 3: Characterization of Luc-transduced Mock and *Il34*^{KO} HM-1 cells
667 Figure 4: Tumor growth of subcutaneously inoculated Mock and *Il34*^{KO} HM-1 cells
668
669 **Supplementary Tables**

Table 1: Association of *IL34* expression in ovarian cancers with patients' characteristics in cohort 1

Table 2: Association of *IL34* expression in ovarian cancers with patients' characteristics in cohort 2

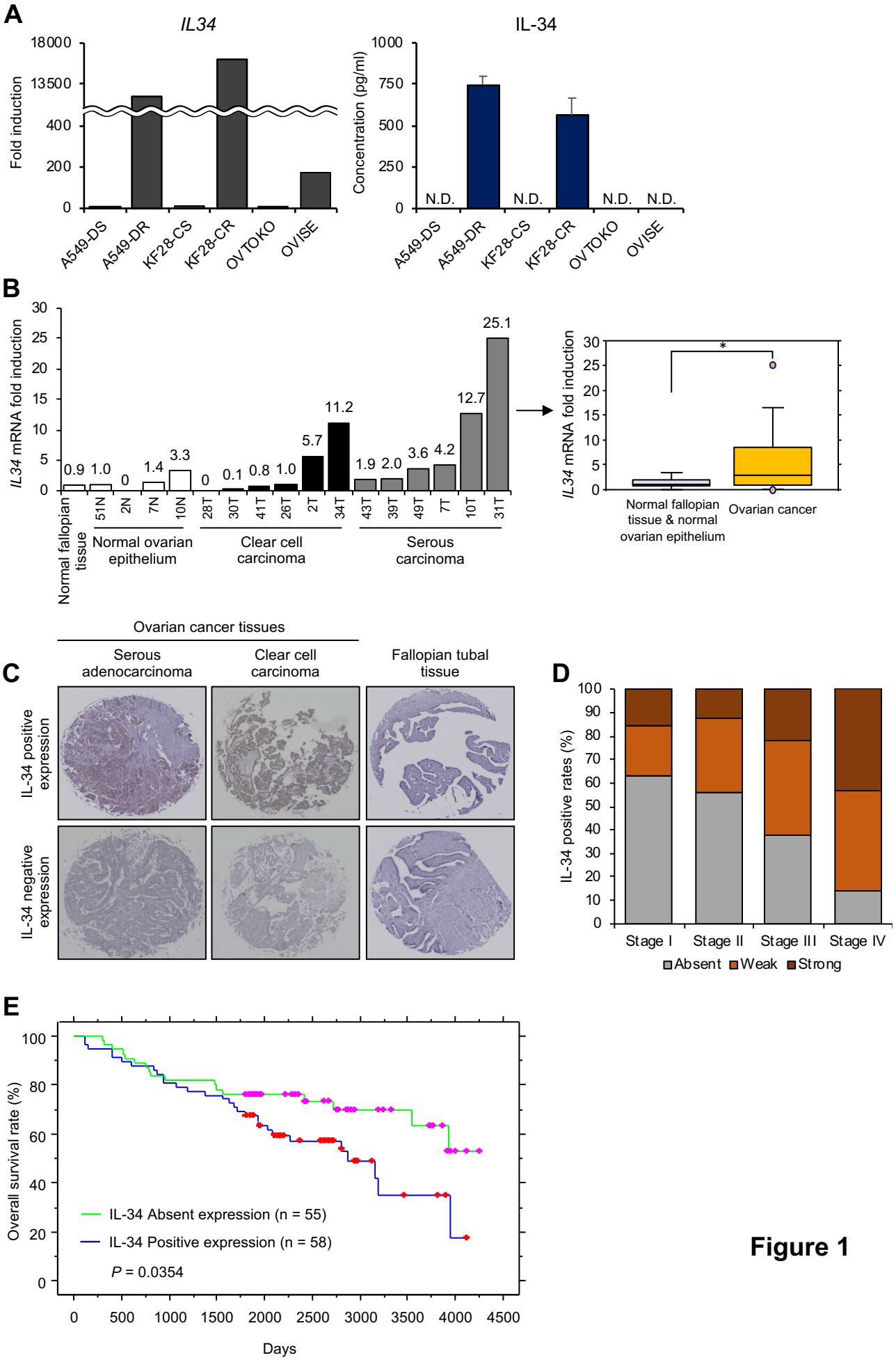
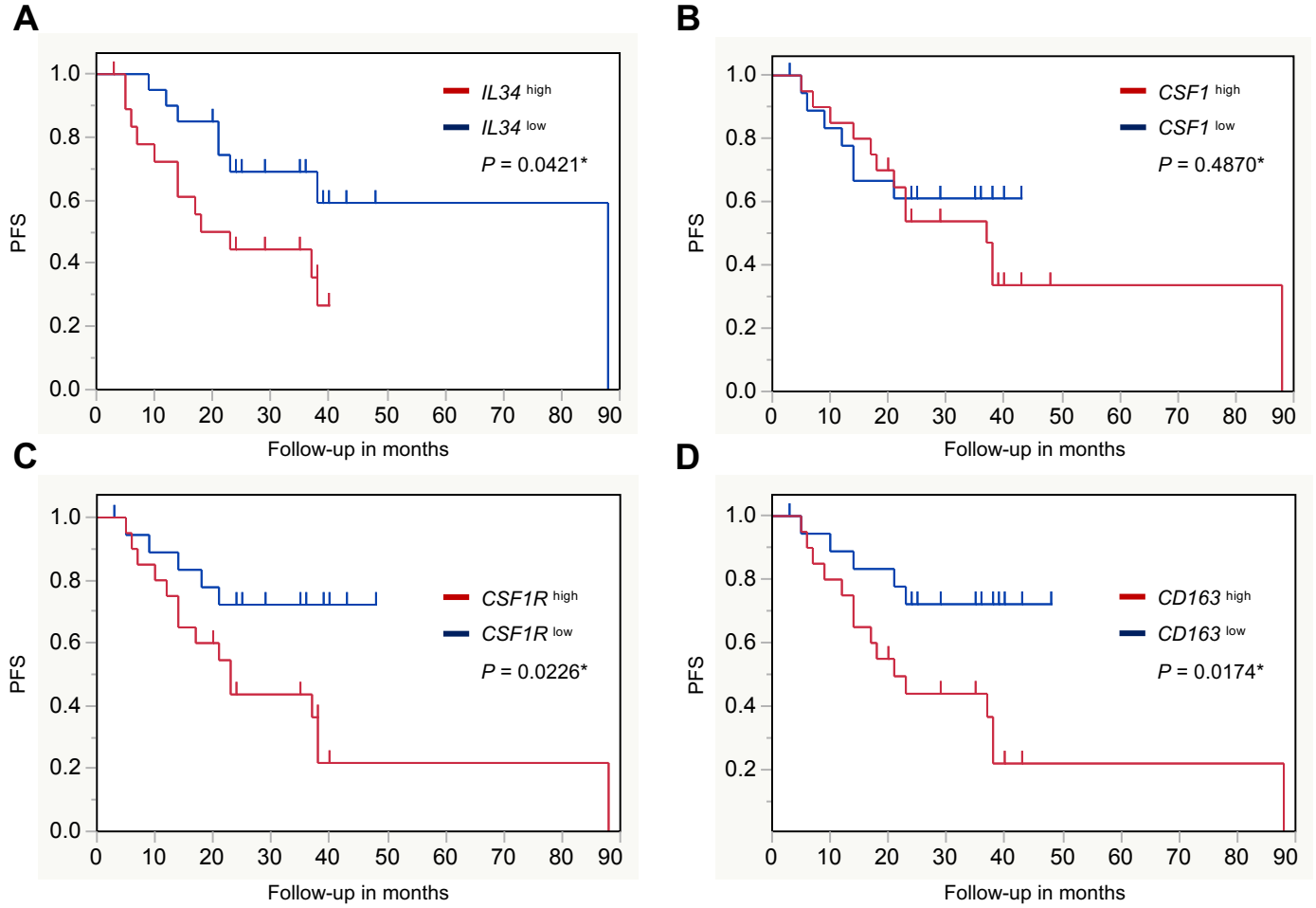

A
IL34
Fold induction
18000
13500
400
200
0
A549-DS
A549-DR
KF28-CS
KF28-CR
OVTOKO
OVISE
IL-34
Concentration (pg/ml)
1000
750
500
250
0
N.D.
N.D.
N.D.
N.D.
B
IL34 mRNA fold induction
0.9 1.0 0 1.4 3.3 0 0.1 0.8 1.0 5.7 11.2 1.9 2.0 3.6 4.2 12.7 25.1
51N 2N 7N 10N 28T 30T 41T 26T 2T 34T 43T 39T 49T 7T 10T 31T
Normal fallopian tissue
Normal ovarian epithelium
Clear cell carcinoma
Serous carcinoma
Normal fallopian tissue & normal ovarian epithelium
Ovarian cancer
*
C
Ovarian cancer tissues
Serous adenocarcinoma
Clear cell carcinoma
Fallopian tubal tissue
IL-34 positive expression
IL-34 negative expression
D
IL-34 positive rates (%)
Stage I
Stage II
Stage III
Stage IV
Absent
Weak
Strong
E
Overall survival rate (%)
IL-34 Absent expression (n = 55)
IL-34 Positive expression (n = 58)
P = 0.0354
Days

Figure 1


A
PFS
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90
Follow-up in months
IL34 high
IL34 low
P = 0.0421*
B
PFS
Follow-up in months
CSF1 high
CSF1 low
P = 0.4870*
C
PFS
Follow-up in months
CSF1R high
CSF1R low
P = 0.0226*
D
PFS
Follow-up in months
CD163 high
CD163 low
P = 0.0174*

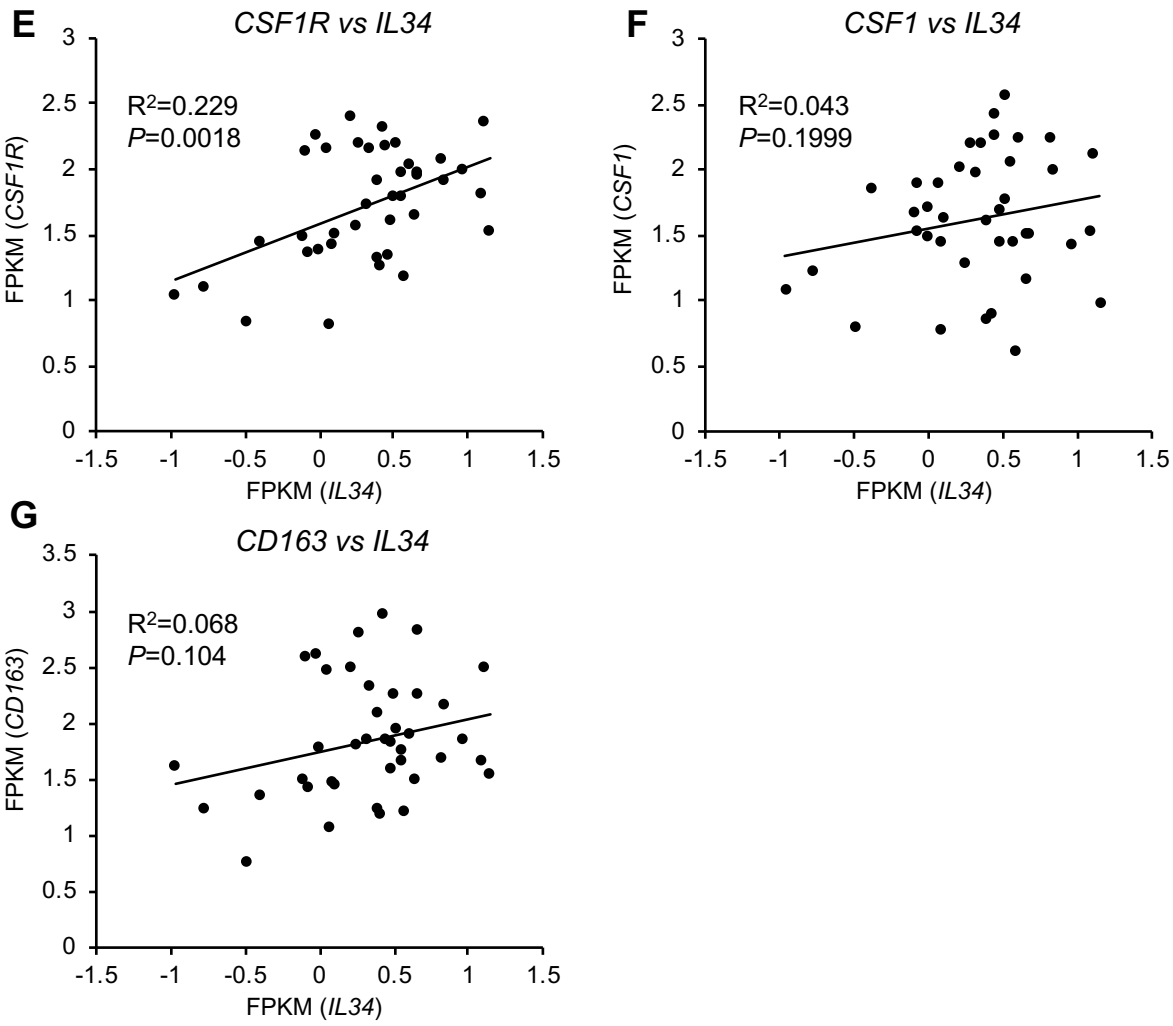
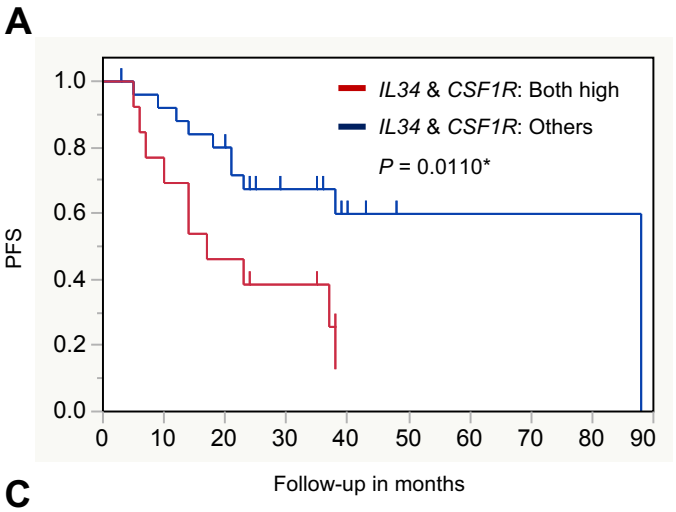
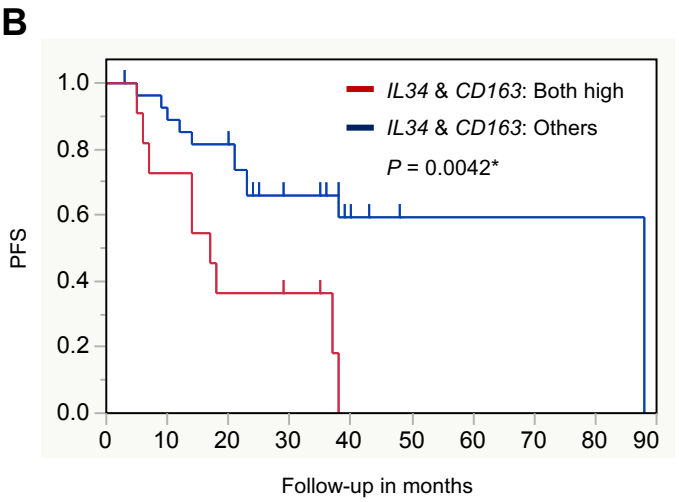

E
CSF1R vs IL34
R²=0.229
P=0.0018
FPKM (CSF1R)
FPKM (IL34)
F
CSF1 vs IL34
R²=0.043
P=0.1999
FPKM (CSF1)
FPKM (IL34)
G
CD163 vs IL34
R²=0.068
P=0.104
FPKM (CD163)
FPKM (IL34)

Figure 2


A
1.0
0.8
0.6
0.4
0.2
0.0
PFS
0
10
20
30
40
50
60
70
80
90
Follow-up in months
IL34 & CSF1R: Both high
IL34 & CSF1R: Others
P = 0.0110*


B
1.0
0.8
0.6
0.4
0.2
0.0
PFS
0
10
20
30
40
50
60
70
80
90
Follow-up in months
IL34 & CD163: Both high
IL34 & CD163: Others
P = 0.0042*

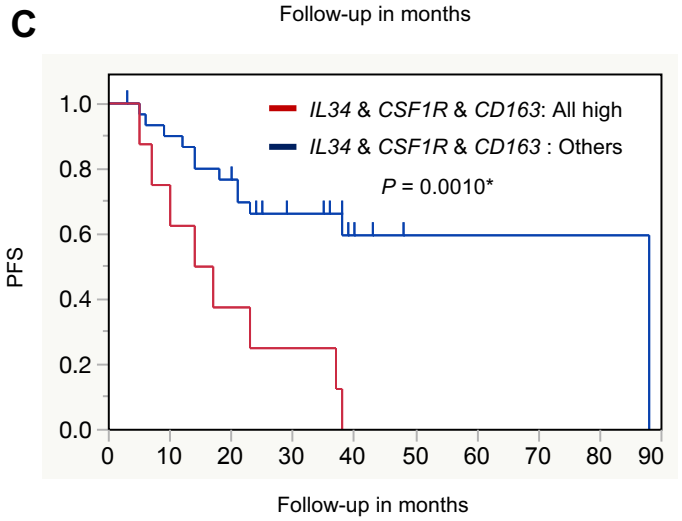

C
1.0
0.8
0.6
0.4
0.2
0.0
PFS
0
10
20
30
40
50
60
70
80
90
Follow-up in months
IL34 & CSF1R & CD163: All high
IL34 & CSF1R & CD163 : Others
P = 0.0010*

Figure 3

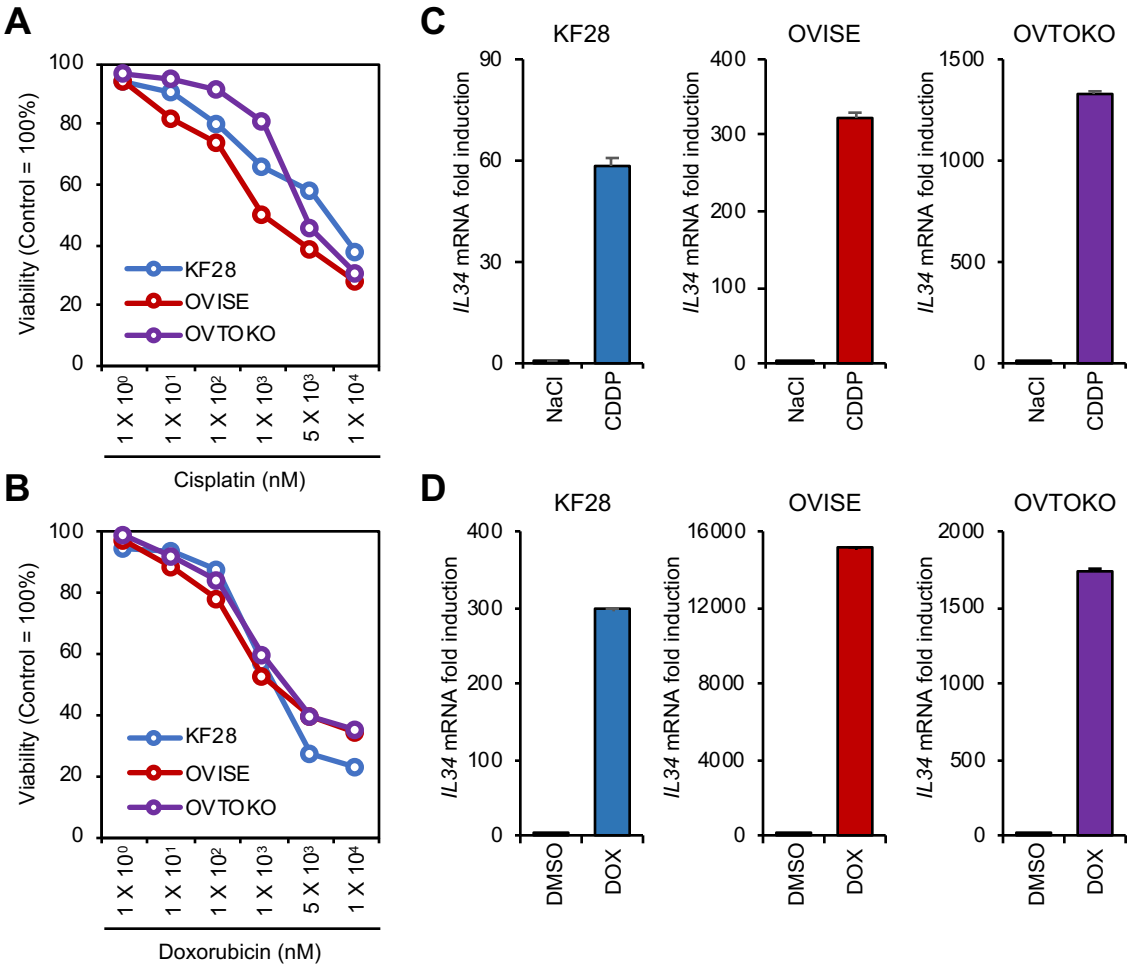
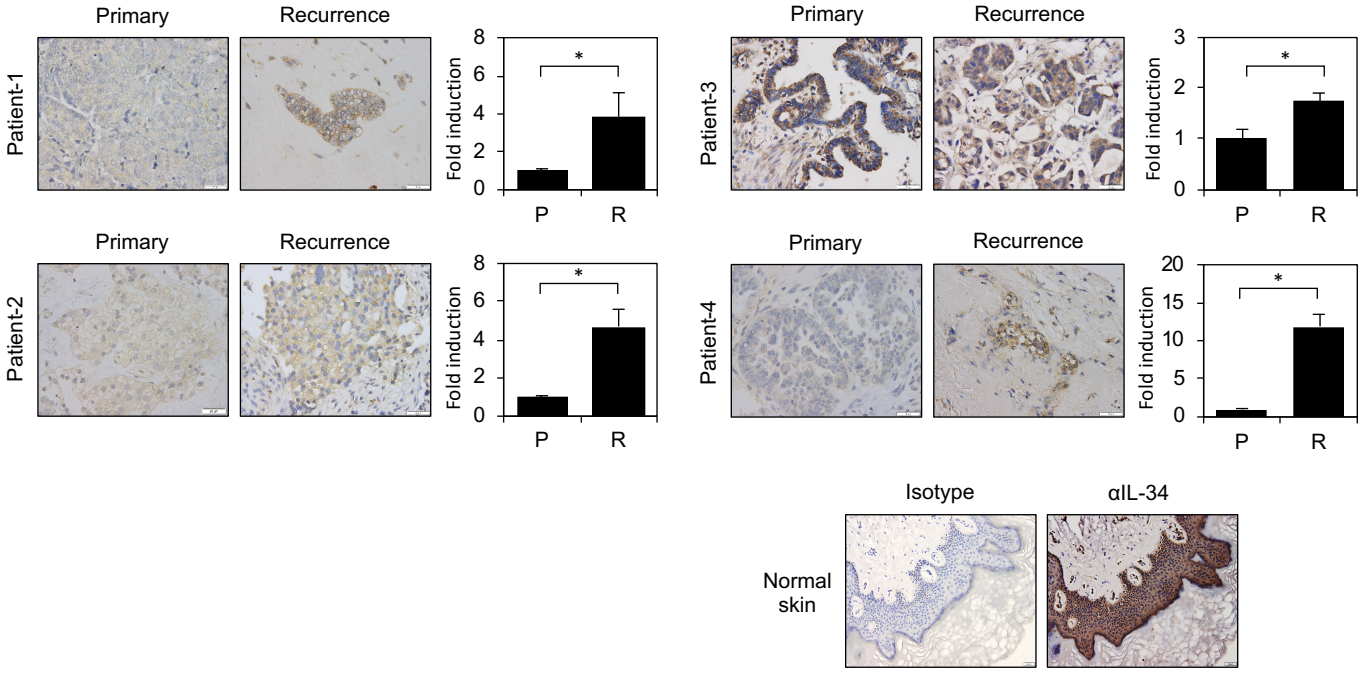

A
Viability (Control = 100%)
KF28
OVISE
OVTOKO
1 X 10⁰
1 X 10¹
1 X 10²
1 X 10³
5 X 10³
1 X 10⁴
Cisplatin (nM)
B
Doxorubicin (nM)
C
KF28
OVISE
OVTOKO
IL34 mRNA fold induction
NaCl
CDDP
D
DMSO
DOX

Figure 4


Primary
Recurrence
Patient-1
Fold induction
8
6
4
2
0
*
P
R
Primary
Recurrence
Patient-3
Fold induction
3
2
1
0
*
P
R
Primary
Recurrence
Patient-2
Fold induction
8
6
4
2
0
*
P
R
Primary
Recurrence
Patient-4
Fold induction
20
15
10
5
0
*
P
R

Isotype
αIL-34
Normal skin

Figure 5

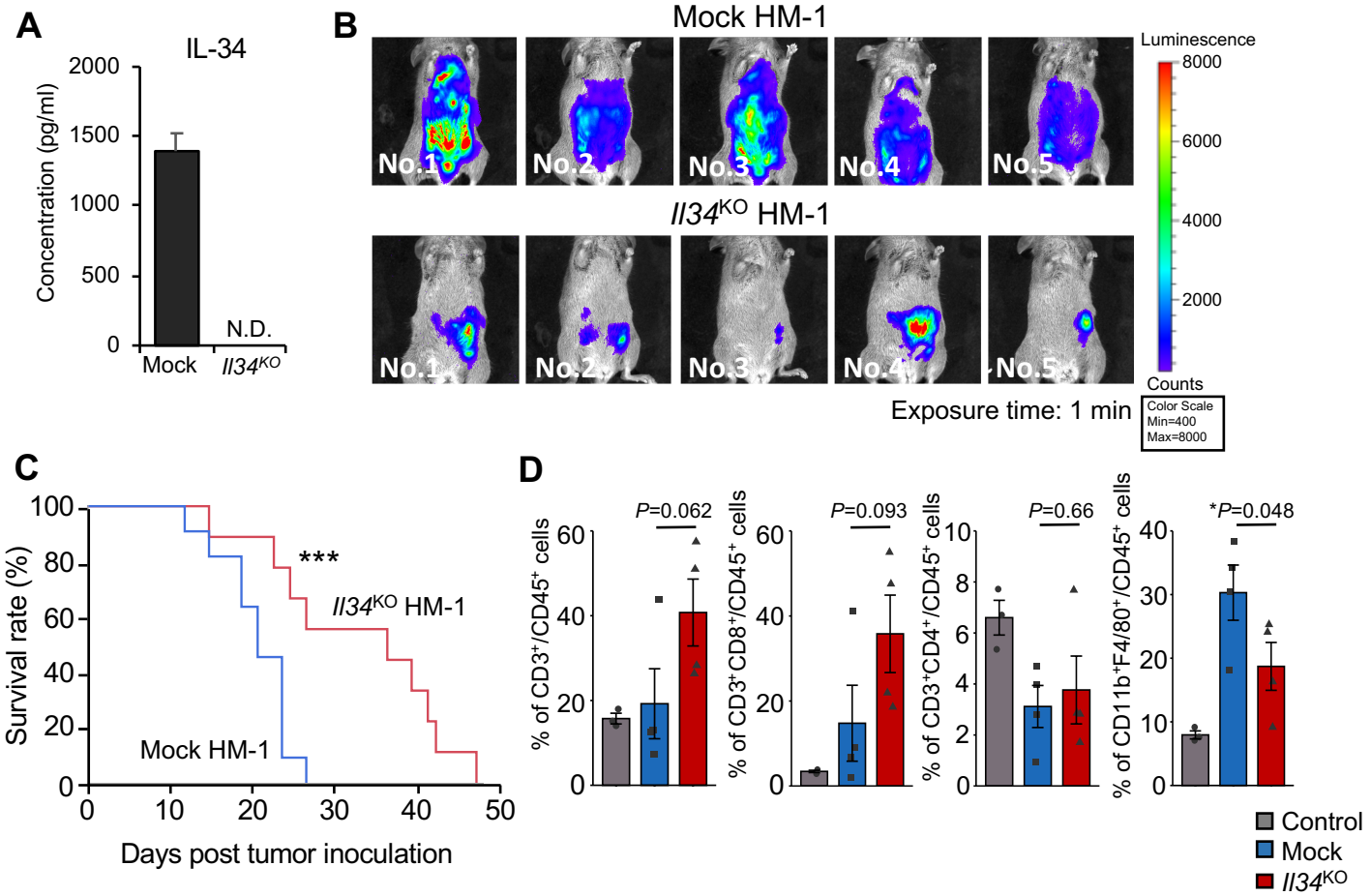
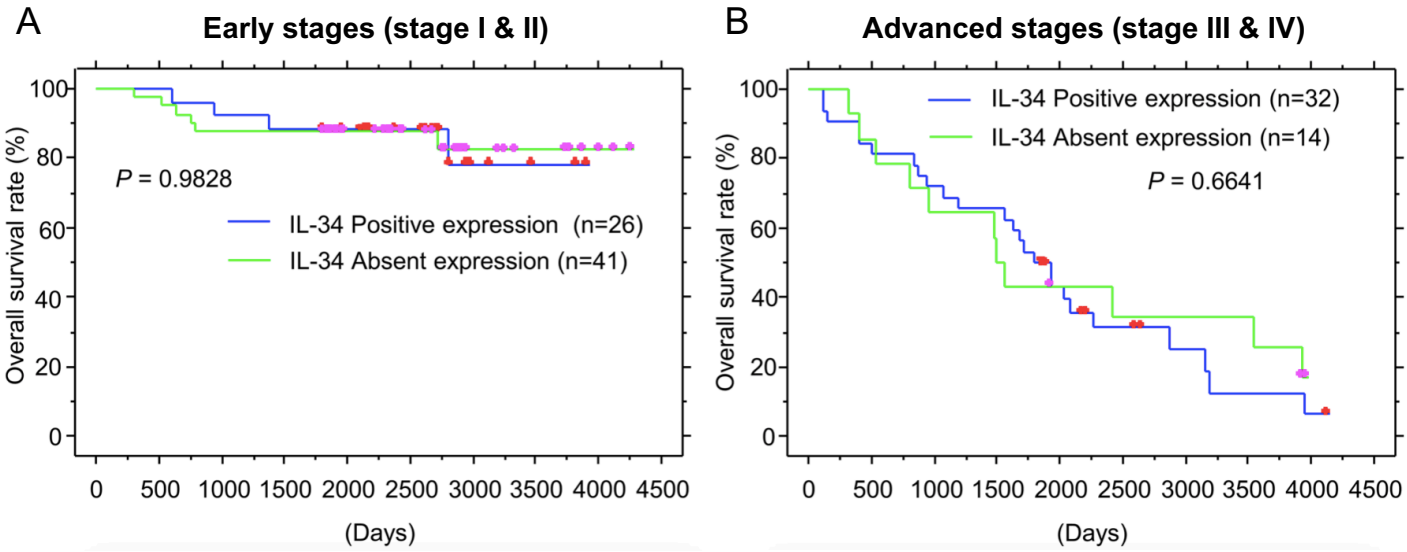
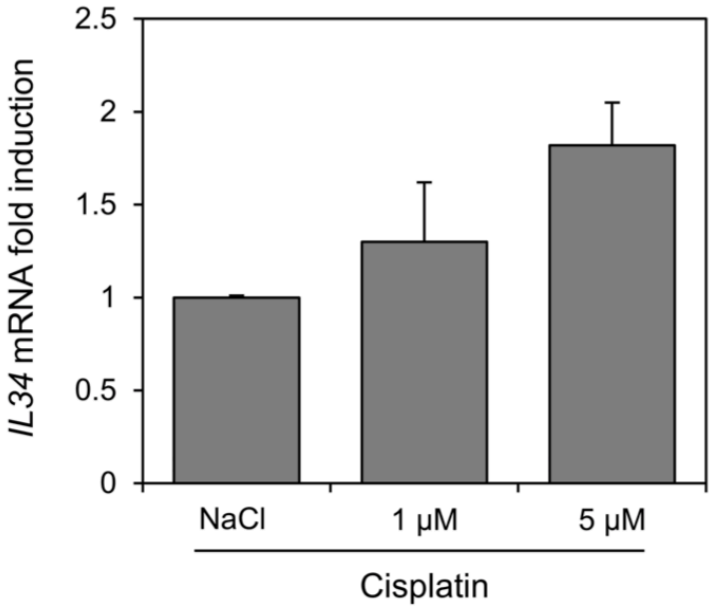

A
IL-34
Concentration (pg/ml)
2000
1500
1000
500
0
N.D.
Mock
Il34KO
B
Mock HM-1
No.1
No.2
No.3
No.4
No.5
Il34KO HM-1
No.1
No.2
No.3
No.4
No.5
Luminescence
8000
6000
4000
2000
Counts
Color Scale
Min=400
Max=8000
Exposure time: 1 min
C
Survival rate (%)
100
80
60
40
20
0
***
Il34KO HM-1
Mock HM-1
0
10
20
30
40
50
Days post tumor inoculation
D
% of CD3+/CD45+ cells
P=0.062
% of CD3+CD8+/CD45+ cells
P=0.093
% of CD3+CD4+/CD45+ cells
P=0.66
% of CD11b+F4/80+/CD45+ cells
*P=0.048
Control
Mock
Il34KO

Figure 6



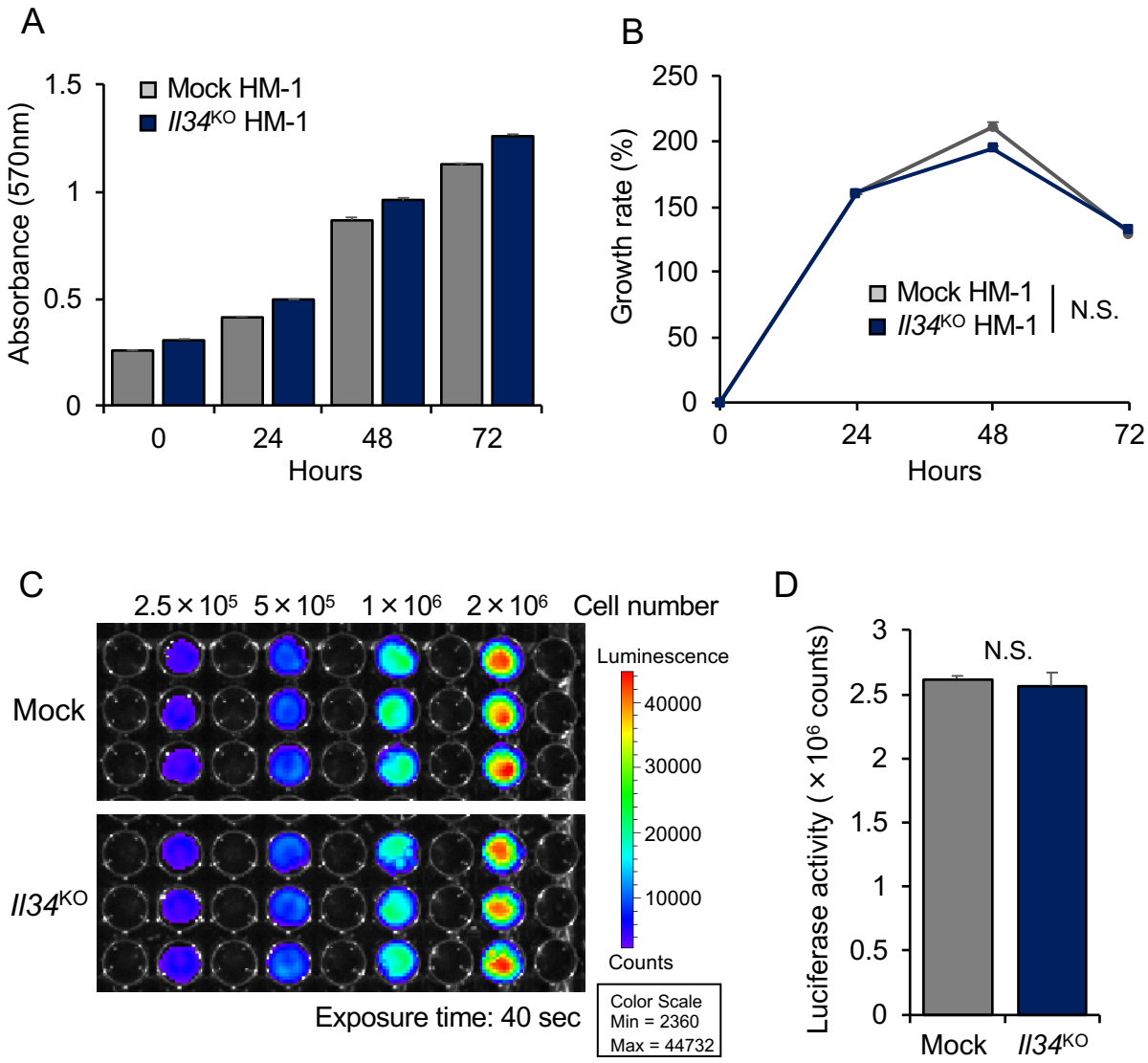
Supplementary Figure 1: Kaplan-Meier analysis showed the correlation between IL-34 expression and OS in patients at (A) early stages (I & II) or (B) advanced stages (III & IV).

Supplementary Figure 1



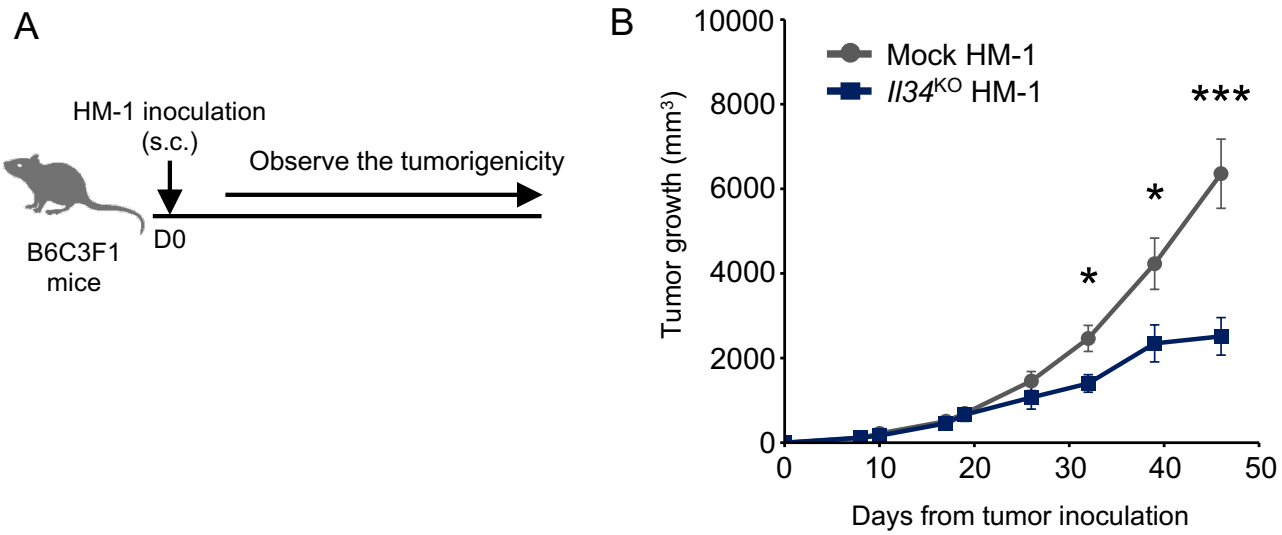
Supplementary Figure 2: *IL34* is induced by cisplatin in KF28 human ovarian cancer cell line in a dose-dependent manner. qRT-PCR analysis of *IL34* expression was evaluated 48 hours after exposure to cisplatin (1 or 5 μM) compared to vehicle control. Data represent mean ± SEM.

Supplementary Figure 2



Supplementary Figure 3: Mean cell viability of Luc^{+} Mock and *Il34*^{KO} HM-1 cells measured by MTT assay (n=3) (A). The line graph shows growth rate of each cell line (B). Luciferase expression level in each cell line was measured using IVIS imaging system (C). Luciferase activity for 2 x 10^6 cells (D). Exposure time for IVIS imaging system was 40 sec. Data represent mean ± SEM. N.S., not significant; Student's t-test.

Supplementary Figure 3



Supplementary Figure 4: Experimental model (A). Tumor growth of inoculated Luc^{+} Mock and *Il34*^{KO} HM-1 cells (B). Data represent mean ± SEM. *, P<0.05, ***, P<0.001; Student's t-test. s.c., subcutaneously.

Supplementary Figure 4