의학 박사학위논문

The role of ascites tumor-microenvironment in ovarian cancer invasion and chemoresistance

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2017 년 2 월

서울대학교 대학원

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Abstract

Ovarian cancer is the most lethal gynecologic malignancy, because of asymptomatic nature of this disease, most patients are diagnosed in late stage with peritoneal dissemination and distant metastasis. The importance of tumor microenvironment and cancer progression are increasingly recognized and the abnormal accumulation of fluid in the peritoneal cavity, called ascites is found in almost all recurrent ovarian cancer patients. Indeed, the presence of ascites correlates with peritoneal tumor spread and decreased 5-year survival in ovarian cancer. In current studies, we provide the malignant role of ascites in ovarian cancer progression. The cytokine profiles of ovarian cancer patient derived ascites demonstrated the presence of pro-inflammatory cytokines. Of those, a significantly elevated levels of interleukin 6 (IL-6), increased invasion of ovarian cancer cells. Neutralization of IL-6 in ascites reduced the stimulatory effects of ascites on ovarian cancer cell invasion. Ascites increased invasion through JAK2 and STAT3 signaling pathway, confirmed by use of selective inhibitors. Moreover, the expression of IL-6 receptor (IL-6R) on cell membrane of ovarian cancer cells correlated with ascites-induced invasion.

Cholesterol is elevated in ascites and treatment of cholesterol reduced response to cisplatin and increased membrane expression of ATP-binding cassette transporters (ABC transporters), via liver x receptor α/β (LXRα/β) in ovarian cancer cells. Similarly, ascites treatment reduced response to cisplatin and increased membrane expression of ABC transporters, with increased LXRα/β expression. Excess cellular cholesterol is toxic, a feed-forward regulatory system such as the liver x receptor

(LXR) family are activated in response to free cholesterol accumulation. Depletion of free cholesterol reduced ascites mediated increased ABC transporter expression and increased response to cisplatin. Our findings highlight the important role of ascites tumor microenvironment in ovarian cancer invasion and chemoresistance. Hence, better understanding of individual components of ascites in ovarian cancer progression will provide novel therapeutic targets as well as prognostic markers.

Keywords: Ovarian cancer, Ascites, Invasion, Chemoresistance, IL-6, Cholesterol

Student Number: 2013-30606

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Abbreviations

ABCG2: ATP-binding cassette sub-family G member 2

ABC transporters: ATP-binding cassette transporters

Anti-rh IL-6: Anti-recombinant human IL-6

CAF: Cancer associated fibroblasts

CDDP: Cisplatin

EMT: Epithelial mesenchymal transition

EOC: Epithelial ovarian cancer

IL-6: Interleukin 6

IL-6R: Interleukin 6 receptor

IOSE: Induced ovarian surface epithelial cell line

JAK2: Janus Kinase 2

LXRα/β: Liver x receptor α/β

MDR1: Multidrug resistance protein 1

MβCD: Methyl-β-cyclodextrin

STAT: Signal transducer and activator of transcription

TME: Tumor microenvironment

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Chapter 1. Introduction

1.1 Ovarian cancer

Epithelial ovarian cancer (EOC) is the most lethal gynecologic cancer and is very heterogeneous malignancy. EOCs are diverse not only in histopathology, but also in etiology, mostly diagnosed in the late stages [1]. This fatal disease is inherently silent, often present with fluid accumulation in the abdominal cavity, called ascites, at diagnosis. The standard treatment of EOC includes cytoreductive surgery followed by chemotherapy with carboplatin and paclitaxel. Despite initially high response rates to this standard treatment, most patients develop recurrent disease and the ascites is present at almost all recurrences [2]. The presence of malignant ascites correlates with deterioration in quality of life and with a poor prognosis [3, 4].

1.2 Tumor microenvironment

The importance of the tumor-microenvironment in cancer progression has been increasingly recognized and plays an essential role in mediating and sustaining the hallmarks of cancer. In particular, the ascites are gaining recognition as a unique form of the tumor-microenvironment in maintaining the hallmarks of ovarian cancer, playing an essential role in the growth, invasion, and metastasis of malignant cells. The link between the presence of ascites and ovarian cancer progression was first proposed by Lopez et al. [5]; since then, numerous studies have contributed to the characterization of the ascites components, further revealing the important role of ascites in acquiring the hallmarks of EOCs.

To overcome the limitations of current anticancer agents, better understanding of EOC and its tumor-microenvironment is needed. Remarkable progress has been made in research on malignant ascites, expanding our knowledge of both the cellular (tumor cells and stromal cells) and acellular (soluble factors) components. All of these components work in coordination to create tumor-friendly microenvironments mediating the acquisition of hallmark capabilities.

1.3 Ascites as a tumor microenvironment in ovarian cancer

Ovarian cancer is characterized by rapid growth and spread of intraperitoneal tumors and presents a huge amount of ascites in the peritoneal cavity [6]. Ascites is comprised of local tumor-microenvironment and is composed of both cellular and acellular factors, which modulates cancer cell behavior and contribute to tumor heterogeneity in ovarian cancer.

1.4 Components of ascites

The origin and phenotype of the cells in the ascites is poorly understood. Similar to other tumor-microenvironment, the cellular components of ascites contain a complex heterogeneous mixture of cell populations, including tumor cells and stromal cells, each with a defined role. The stromal cellular components of ascites include fibroblasts, endothelial or mesothelial cells, adipocytes, adipose tissue-derived stromal cells, bone marrow-derived stem cells and immune cells [7, 8]. Some

of these stromal cellular components show abnormal features, including activation of growth and angiogenesis [9, 10]. In several tumors, stromal cells play a significant role in malignant progression. In particular, malignant role of cancer-associated fibroblasts (CAF), have been highlighted, through autocrine-paracrine loops, promote proliferation, migration and invasion of cancer cells. CAFs secrete factors that can transduce signals to cancer cells as well as to themselves establishing reciprocal reinforcement of growth and migration signals as well as chemoresistance [11]. Similarly, the interactions between EOC cells and human peritoneal mesothelial cells (HPMCs) in ascites are believed to be important for tumor progression [9, 12]. Like CAFs, HPMCs secrete factors that promote tumor growth. For example, lysophosphatidic acid (LPA) produced by immortalized HPMCs and was shown to enhance adhesion, migration and invasion of ovarian cancer cells [12]. These cancer associated mesothelial cells have also been reported to produce factors that promote chemoresistance in ovarian cancer cells [10]. HPMCs also produce dipeptidyl peptidase IV and VEGF in response to malignant ascites exposure [13, 14]

In addition to the complex mixture of stromal cellular components, malignant tumor cells are found in ascites and are thought to be a major factor in disease recurrence in EOC patients [15]. Tumor cells within the ascites are present either as single cells with adherent properties or more commonly, as aggregates of non-adherent cells, also known as spheroids [16]. These cells are proposed to undergo epithelial-to-mesenchymal-transition (EMT) to a motile phenotype with low levels of E-cadherin and higher invasivity than the primary tumor cells [17, 18] Spheroids represent the invasive or metastasis-forming subpopulation leading to recurrent

disease [19, 20]. This notion has in part been supported by *in vitro* work on artificial spheroids. However, study by Wintzell 2012, showed that the spheroids freshly isolated from patients were less invasive and also low in the EOC tumor initiating cell marker CD44 and the stem cell transcription factor OCT4A [16]. But these spheroids represent a chemoresistant population since chemotherapeutic drugs do not penetrate such multicellular structures [21, 22].

Cellular components of ascites communicate with each other through soluble factors, including cytokines, proteins, and metabolites and more recently through the secretion and exchange of exosomes [23]. Thus, the heterogeneous mixture of cellular components of ascites influences the acellular components of ascites. The acellular components of ascites constitute a dynamic reservoir of both pro-tumorigenic and anti-tumorigenic factors, including cytokines, growth factors and bioactive lipids, which individually and in combination influence EOC behavior and progression [24-26].

The cytokine profiles of EOC ascites demonstrated the presence of both pro-tumorigenic and anti-tumorigenic factors in this unique tumor-microenvironment [1, 4, 27, 28]. A significantly elevated levels of pro-tumorigenic cytokines including IL-6, IL-8, IL-10, IL-15, IP-10, MCP-1, MIP-1β, and vascular endothelial growth factor (VEGF) were reported in EOC ascites. These factors cumulatively contribute to creating a pro-inflammatory and immunosuppressive tumor-microenvironment [28]. Among these factors, IL-6 and IL-10 received most attention due to their correlation with poor prognosis and response to therapy [4, 29].

The metabolomics of EOC ascites are also of interest to determine the role of metabolites in EOC ascites. Ascites metabolome results have demonstrated that the most important differences were found in fatty acids, cholesterol, ceramide, glycerol-3-phosphate, glucose, and glucose-3-phosphate in ascites derived from patients with EOC compared to those from patients with portal alcoholic cirrhosis. In addition, glycolate, glucose, furanose, and fructose were significantly decreased, whereas glycerol-3-phosphate, cholesterol, ceramide and monoacylglycerol; MAG (18:0/0:0/0:0) were significantly elevated in EOC patient derived ascites [30].

1.5 Ascites tumor-microenvironment contributing to cancer progression and chemoresistance

The progress in deciphering the cellular and acellular components of ascites has shown that ascites serves as an important tumor-microenvironment enriched in pro-tumorigenic signals that enhance hallmark capabilities, including proliferative potential, invasion and anti-apoptosis with subsequent contribution to chemoresistance [7, 8].

The contributory functions of ascites in the hallmark capabilities of EOCs are increasingly understood, where the reciprocal reprogramming of both cancer cells and ascites components occurs throughout disease progression. Ascites-derived cellular components, including stromal progenitor cells, mesothelial cells,

mesenchymal cells, and endothelial cells have been reported to enhance cancer hallmark capabilities in EOCs, enabling tumor growth and metastasis [9, 10, 31, 32].

In particular, it has been demonstrated that interactions between tumor cells and mesenchymal stem cells promote the elevation of IL-6, a proinflammatory cytokine. IL-6 acts as an oncogenic stimulus, promoting the epithelial-mesenchymal transition process that enables invasion [31]. VEGF is best known as a key regulatory molecule enabling hallmarks of cancer, including tumor growth, invasion, metastasis, and recurrence of EOC [33]. Substantial evidence also supports VEGF as a key player in the formation of ascites and ovarian cancer progression [34, 35]. Another factor, IL-8, is present in abundance and has been shown to activate the epithelial-mesenchymal transition and metastasis [36] and enables tumor growth and angiogenesis [37].

The presence of both oncogenic and tumor suppressive factors. Specifically, ascites from high-grade serous ovarian cancer patients was shown to serve as a protective tumor-microenvironment against drug-induced apoptosis through induction of survival signaling pathways such as PI3K/Akt in tumor cells [38, 39]. The extent to which ascites served as a protective tumor-microenvironment against TRAIL-induced apoptosis and chemotherapy was variable [40]. So, further research is needed to identify key players responsible for the tumoral heterogeneity by tumor-microenvironment, showing the insight in the future clinical management of ascites.

1.6 References

1. Matte I, Lane D, Laplante C, Rancourt C and Piche A. Profiling of cytokines in human epithelial ovarian cancer ascites. Am J Cancer Res. 2012; 2(5):566-580.

2. Kipps E, Tan DS and Kaye SB. Meeting the challenge of ascites in ovarian cancer: new avenues for therapy and research. Nat Rev Cancer. 2013; 13(4):273-282.

3. Ayantunde AA and Parsons SL. Pattern and prognostic factors in patients with malignant ascites: a retrospective study. Ann Oncol. 2007; 18(5):945-949.

4. Lane D, Matte I, Rancourt C and Piche A. Prognostic significance of IL-6 and IL-8 ascites levels in ovarian cancer patients. BMC Cancer. 2011; 11:210.

5. Lopez RI, Paul J, Atkinson R, Soukop M, Kitchener H, Fullerton W, Duncan I, Kennedy J, Davis J, Maclean A, Cassidy J, Pyper E and Kaye SB. Prognostic factor analysis, for patients with no evidence of disease after initial chemotherapy for advanced epithelial ovarian carcinoma. International Journal of Gynecological Cancer. 1996; 6(1):8-14.

6. Suh DH, Kim HS, Kim B and Song YS. Metabolic orchestration between cancer cells and tumor microenvironment as a co-evolutionary source of chemoresistance in ovarian cancer: a therapeutic implication. Biochem Pharmacol. 2014; 92(1):43-54.

7. Wels J, Kaplan RN, Rafii S and Lyden D. Migratory neighbors and distant invaders: tumor-associated niche cells. Genes Dev. 2008; 22(5):559-574.

8. Bhowmick NA, Neilson EG and Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature. 2004; 432(7015):332-337.

9. Pasquet M, Golzio M, Mery E, Rafii A, Benabbou N, Mirshahi P, Hennebelle I, Bourin P, Allal B, Teissie J, Mirshahi M and Couderc B. Hospicells (ascites-derived stromal cells) promote tumorigenicity and angiogenesis. Int J Cancer. 2010; 126(9):2090-2101.

10. Matte I, Lane D, Bachvarov D, Rancourt C and Piche A. Role of malignant ascites on human mesothelial cells and their gene expression profiles. BMC Cancer. 2014; 14:288.

11. Paraiso KH and Smalley KS. Fibroblast-mediated drug resistance in cancer. Biochem Pharmacol. 2013; 85(8):1033-1041.

12. Ren J, Xiao YJ, Singh LS, Zhao X, Zhao Z, Feng L, Rose TM, Prestwich GD and Xu Y. Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. Cancer Res. 2006; 66(6):3006-3014.

13. Kajiyama H, Kikkawa F, Maeda O, Suzuki T, Ino K and Mizutani S. Increased expression of dipeptidyl peptidase IV in human mesothelial cells by malignant ascites from ovarian carcinoma patients. Oncology. 2002; 63(2):158-165.

14. Stadlmann S, Amberger A, Pollheimer J, Gastl G, Offner FA, Margreiter R and Zeimet AG. Ovarian carcinoma cells and IL-1beta-activated human peritoneal mesothelial cells are possible sources of vascular endothelial growth factor in inflammatory and malignant peritoneal effusions. Gynecol Oncol. 2005; 97(3):784-789.

15. Latifi A, Luwor RB, Bilandzic M, Nazaretian S, Stenvers K, Pyman J, Zhu H, Thompson EW, Quinn MA, Findlay JK and Ahmed N. Isolation and

characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors. PLoS One. 2012; 7(10):e46858.

16. Wintzell M, Hjerpe E, Avall Lundqvist E and Shoshan M. Protein markers of cancer-associated fibroblasts and tumor-initiating cells reveal subpopulations in freshly isolated ovarian cancer ascites. BMC Cancer. 2012; 12:359.

17. Ahmed N, Thompson EW and Quinn MA. Epithelial-mesenchymal interconversions in normal ovarian surface epithelium and ovarian carcinomas: an exception to the norm. J Cell Physiol. 2007; 213(3):581-588.

18. Veatch AL, Carson LF and Ramakrishnan S. Differential expression of the cell-cell adhesion molecule E-cadherin in ascites and solid human ovarian tumor cells. Int J Cancer. 1994; 58(3):393-399.

19. Sodek KL, Ringuette MJ and Brown TJ. Compact spheroid formation by ovarian cancer cells is associated with contractile behavior and an invasive phenotype. Int J Cancer. 2009; 124(9):2060-2070.

20. Shield K, Ackland ML, Ahmed N and Rice GE. Multicellular spheroids in ovarian cancer metastases: Biology and pathology. Gynecol Oncol. 2009; 113(1):143-148.

21. Fayad W, Brnjic S, Berglind D, Blixt S, Shoshan MC, Berndtsson M, Olofsson MH and Linder S. Restriction of cisplatin induction of acute apoptosis to a subpopulation of cells in a three-dimensional carcinoma culture model. Int J Cancer. 2009; 125(10):2450-2455.

22. Tannock IF, Lee CM, Tunggal JK, Cowan DS and Egorin MJ. Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy. Clin Cancer Res. 2002; 8(3):878-884.

23. Guo L and Guo N. Exosomes: Potent regulators of tumor malignancy and potential bio-tools in clinical application. Crit Rev Oncol Hematol. 2015; 95(3):346-358.

24. Mills GB, May C, Hill M, Campbell S, Shaw P and Marks A. Ascitic fluid from human ovarian cancer patients contains growth factors necessary for intraperitoneal growth of human ovarian adenocarcinoma cells. J Clin Invest. 1990; 86(3):851-855.

25. Radke J, Schmidt D, Bohme M, Schmidt U, Weise W and Morenz J. - Cytokine level in malignant ascites and peripheral blood of patients with advanced ovarian carcinoma. Geburtshilfe Frauenheilkd. 1996; 56(2):83-87.

26. Richardson M, Gunawan J, Hatton MW, Seidlitz E, Hirte HW and Singh G. Malignant ascites fluid (MAF), including ovarian-cancer-associated MAF, contains angiostatin and other factor(s) which inhibit angiogenesis. Gynecol Oncol. 2002; 86(3):279-287.

27. Yigit R, Figdor CG, Zusterzeel PL, Pots JM, Torensma R and Massuger LF. Cytokine analysis as a tool to understand tumour-host interaction in ovarian cancer. Eur J Cancer. 2011; 47(12):1883-1889.

28. Giuntoli RL, 2nd, Webb TJ, Zoso A, Rogers O, Diaz-Montes TP, Bristow RE and Oelke M. Ovarian cancer-associated ascites demonstrates altered immune

environment: implications for antitumor immunity. Anticancer Res. 2009; 29(8):2875-2884.

29. Lane D, Matte I, Garde-Granger P, Laplante C, Carignan A, Rancourt C and Piche A. Inflammation-regulating factors in ascites as predictive biomarkers of drug resistance and progression-free survival in serous epithelial ovarian cancers. BMC Cancer. 2015; 15:492.

30. Shender VO, Pavlyukov MS, Ziganshin RH, Arapidi GP, Kovalchuk SI, Anikanov NA, Altukhov IA, Alexeev DG, Butenko IO, Shavarda AL, Khomyakova EB, Evtushenko E, Ashrafyan LA, Antonova IB, Kuznetcov IN, Gorbachev AY, et al. Proteome-metabolome profiling of ovarian cancer ascites reveals novel components involved in intercellular communication. Mol Cell Proteomics. 2014; 13(12):3558-3571.

31. So KA, Min KJ, Hong JH and Lee JK. Interleukin-6 expression by interactions between gynecologic cancer cells and human mesenchymal stem cells promotes epithelial-mesenchymal transition. Int J Oncol. 2015; 47(4):1451-1459.

32. Lane D, Matte I, Rancourt C and Piche A. Osteoprotegerin (OPG) protects ovarian cancer cells from TRAIL-induced apoptosis but does not contribute to malignant ascites-mediated attenuation of TRAIL-induced apoptosis. J Ovarian Res. 2012; 5(1):34.

33. Zhan N, Dong WG and Wang J. The clinical significance of vascular endothelial growth factor in malignant ascites. Tumour Biol. 2015.

34. Masoumi Moghaddam S, Amini A, Morris DL and Pourgholami MH. Significance of vascular endothelial growth factor in growth and peritoneal dissemination of ovarian cancer. Cancer Metastasis Rev. 2012; 31(1-2):143-162.

35. Yabushita H, Shimazu M, Noguchi M, Kishida T, Narumiya H, Sawaguchi K and Noguchi M. Vascular endothelial growth factor activating matrix metalloproteinase in ascitic fluid during peritoneal dissemination of ovarian cancer. Oncol Rep. 2003; 10(1):89-95.

36. Yin J, Zeng F, Wu N, Kang K, Yang Z and Yang H. Interleukin-8 promotes human ovarian cancer cell migration by epithelial-mesenchymal transition induction in vitro. Clin Transl Oncol. 2015; 17(5):365-370.

37. Wang Y, Xu RC, Zhang XL, Niu XL, Qu Y, Li LZ and Meng XY. Interleukin-8 secretion by ovarian cancer cells increases anchorage-independent growth, proliferation, angiogenic potential, adhesion and invasion. Cytokine. 2012; 59(1):145-155.

38. Lane D, Robert V, Grondin R, Rancourt C and Piche A. Malignant ascites protect against TRAIL-induced apoptosis by activating the PI3K/Akt pathway in human ovarian carcinoma cells. Int J Cancer. 2007; 121(6):1227-1237.

39. Lane D, Goncharenko-Khaider N, Rancourt C and Piche A. Ovarian cancer ascites protects from TRAIL-induced cell death through alphavbeta5 integrin-mediated focal adhesion kinase and Akt activation. Oncogene. 2010; 29(24):3519-3531.

40. Lane D, Matte I, Rancourt C and Piche A. The prosurvival activity of ascites against TRAIL is associated with a shorter disease-free interval in patients with ovarian cancer. J Ovarian Res. 2010; 3:1.

Chapter 2.

Malignant ascites enhances migratory and invasive properties of ovarian cancer cells with membrane bound IL-6R *in vitro*

Abstract:

Transcoelomic route is the most common and the earliest route of metastasis, causing the ascites formation in advanced epithelial ovarian cancer (EOC). The ascites forms a perfect ecosystem, creating a favorable tumor-microenvironment, promoting both invasion and chemoresistance in cancer cells. We demonstrated that interleukin 6 (IL-6) is enriched in the malignant ascites from patients with ovarian cancer, which enhanced invasive properties of EOC cells.

Interestingly, the expression of IL-6R on cell membrane of EOC cells correlated with ascites-induced invasion. Selective knockdown of IL-6R or inhibition with IL-6 neutralizing antibody, suppressed the stimulatory effects of ascites on EOC invasion. Moreover, the ascites treatment induced the phosphorylation of JAK2-STAT3 and use of selective inhibitors of JAK2 and STAT3, blocked the expression of epithelial-mesenchymal transition related proteins in parallel with the suppression of EOC invasion. Thus, IL-6/IL-6R mediated JAK2-STAT3 signaling pathway could be a promising therapeutic target for anticancer therapy in ovarian cancer patients with ascites.

2.1 Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy due to the frequent relapse and chemoresistance. Asymptomatic nature of the disease, largely contributes to the diagnosis of patients in advanced stage III/IV, causing a greater than 60% mortality rate within five years [1]. In contrast to other solid tumor, the metastatic pattern of ovarian cancer is unique. The most common and earliest route of metastasis is transcoelomic, which frequently causes the accumulation of fluid in the peritoneal cavity, called ascites [2]. Clinically, the presence of ascites is significantly associated with a decreased quality of life and a poor prognosis [3, 4].

In general, ascites formation provides a favorable environment for tumor cells. The role of ascites in ovarian cancer progression is diverse, including promotion of proliferation, spheroid formation, attenuation of TRAIL-induced apoptosis and enhanced invasive behavior [5-7]. Ascites are composed of both cellular and acellular components, each of which has a distinctive but cumulative role in disease progression. The concentration of bio-active molecules present in ascites varies between patients, according to their disease stage, grade and histological subtypes [8, 9]. Variety of soluble factors including inflammatory cytokines has been demonstrated to individually affect EOC progression through different mechanisms. Of those, high level of IL-6 in the serum and ascites of the cancer patients has been shown to be associated with worse clinical outcomes [10, 11]. The signaling pathway downstream of IL-6, especially Janus kinase (JAK) and Signal transducer and activator of transcription (STAT) pathway is aberrantly activated and is associated

with the cancer progression, though this pathway is an essential component of normal development and homeostasis [12].

Ovarian cancer cells in malignant ascites have also been characterized by epithelial mesenchymal transition (EMT), supporting the idea that ascites contains factors mediating the EMT pathway and metastasis [13]. Although previous studies have demonstrated the stimulatory role of ascites in migration and invasion of EOC cells, its exact mechanism has not been determined. Herein we report that human ovarian cancer patient derived ascites comprises pro-inflammatory tumor microenvironment (TME), of which elevated levels of IL-6 increased EOC cell invasion through JAK2-STAT3 signaling in parallel with increased expression of EMT related genes. Furthermore, our data indicated that, the expression of IL-6R on cell membrane of EOC cells is correlated with ascites induced invasion *in vitro*.

2.2 Materials and Methods

2.2.1 Cell culture, clinical samples and reagents

Human ovarian cancer cell lines, PA-1, OVCAR-3, and SKOV-3 used in this study were obtained from the American Type Culture Collection (Rockville, MD). A2780 was kindly gifted by Prof. Benjamin K. Tsang. With the exception of PA-1, these cell lines were grown in RPMI1640 (WelGENE, Seoul, Korea). PA-1 was cultured in MEM (WelGENE, Seoul, Korea).

Induced ovarian surface epithelial cell line IOSE380 used in this study was kindly gifted by Prof. Young Kee Shin. The IOSE380 cell line was maintained in MCDB105:M199, 1:1 mixture. All culture media were supplemented with 10% FBS (Gibco-BRL, Gaithersberg, MD), and 100 μg/mL penicillin-streptomycin (P/S) (Invitrogen, Carlsbard, CA).

Ascites from three serous ovarian cancer patients and peritoneal fluid from two benign conditions were collected at the time of clinical intervention at the Seoul National University Hospital (Seoul, Korea). This study was approved by the Institutional Review Board (IRB) at Seoul National University Hospital (Registration number: 1409-1540-616), and prior written and informed consent was obtained from every patient. Ascites were centrifuged at 2500 rpm for 20 minutes. The acellular fractions were filtered (70 μm), aliquoted and stored at -80℃ to minimize freeze-thaw.

2.2.2 Wound healing assay

Cells were full plated with complete media in 6-well plates and incubated at 37℃ for 24 hr. The complete medium was changed to either fresh complete media or 10% of the indicated ascites. The cell layer was scratched with a pipette tip to create an artificial wound. The wound closed area (in arbitrary unit) was measured using ImageJ software.

2.2.3 Invasion assay

To assess the influence of ascites on the invasion, the Boyden chamber assay (transparent PET membrane with 8 μm pore size, BD Biosciences) was used. Inserts were pre-coated with growth factor reduced Matrigel (30 μg/insert; BD Biosciences). Cells were serum starved overnight and 40,000 cells were loaded to the upper chamber per insert and the inserts were placed to 10% of the indicated ascites or control medium. After 24 hr, cells were washed with PBS for three times and fixed with 4% formaldehyde for 1 hr at room temperature and stained with 0.5% crystal violet. Non-invaded cells were removed from the top filter surface with a cotton swab and invaded cells were viewed and photographed under a microscope. The invaded cells were counted using ImageJ software.

2.2.4 Western Blotting

Protein lysates were prepared as described previously [14]. In brief, after cell extraction, proteins were separated by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE), (6-15% gel, depending on specific protein assessed) followed by electrotransfer onto nitrocellulose membranes and probed with the indicated antibodies.

2.2.5 Reagents and Antibodies

WP1066 and TG101348 (Selleckchem, Houston, USA) were used in this study. Anti-recombinant human interleukin-6 (anti-rh IL-6), rh interleukin-6 (rh IL-6) and normal goat IgG were purchased from R&D Systems (R&D Systems, Minneapolis, MN). JAK2, p-JAK2 (Y1007), STAT3, p-STAT3 (Y705) and N-cadherin were purchased from Cell signaling (Danvers, MA). Snail, E-cadherin, vimentin, GAPDH and IL-6R were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-tubulin antibody was obtained from Sigma-Aldrich. Alexa Fluor conjugated anti-rabbit antibody was obtained from Invitrogen (Carlsbad, CA).

2.2.6 Ascites analysis using Proteome Profiler cytokine array

Measured by the Human Cytokine Array Kit (R&D Systems, Minneapolis, MN), according to the manufacturing protocols. Ascites from two ovarian cancer patients

and peritoneal fluid from two benign conditions were subjected to human cytokine array to detect the relative expression levels of 36 different cytokines.

2.2.7 Determination of IL-6 concentration by ELISA

IL-6 levels in ascites samples were determined by ELISA using the commercially available human Quantikine HS ELISA Kit (R&D Systems, Minneapolis, MN). The assays were performed in duplicate according to the manufacturer's protocols. The detection threshold was 0.156 pg/ml. A quantity of 50 μl of undiluted ascites was added in each well.

2.2.8 Depletion of soluble interleukin-6

10% ascites diluted in complete medium were incubated with 50 ng/ml of a goat polyclonal anti-rh interleukin-6 (anti-rh IL-6; R&D Systems, Minneapolis, MN) or a normal goat IgG control (R&D Systems, Minneapolis, MN) at 37℃ for 6 hr. The media were used in invasion assay as described above.

2.2.9 RT-PCR

Total RNA was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD). Single-stranded cDNA was constructed by PrimeScript Reverse Transcriptase

(Takara, Japan). PCR was performed with specific primers IL-6R sense 5'-TCC ACC CCC ATG CAG GCA CT-3', antisense 5'-GTGCCACCCAGCCAGCTATC-3'(size, 441 bp); IL-6 sense 5'-TAG CCG CCC CAC ACA GAC AG-3', antisense 5'-GGC TGG CAT TTG TGG TTG GG-3' (size, 408 bp); and β–actin sense 5'-ACA CTG CCA TCT ACG AGC-3', antisense 5'-AGG GGC CGG ACT CGT CAT ACT-3' (size, 480 bp) using the following amplification conditions: denaturation (95℃, 30 sec), annealing (60℃, 30 sec), and extension (72℃, 1 min) followed by 35 cycles (72℃, 10 min).

2.2.10 Small interfering RNA transfection

The siRNA-targeting IL-6R (100 nM) gene corresponds to sequence was CGA CUC UGG AAA CUA UUC ATT and scrambled RNA (100 nM) was used as a negative control (mBioTech, Gyeonggido, Korea). All cancer cells were transfected with siRNA oligonucleotides with Lipofectamine® 2000 Invitrogen (Carlsbad, CA). At 24 hr post-transfection cells were harvested and subsequently used for invasion assay.

2.2.11 Immunofluorescence Microscopy

Cells were plated onto a cover-slip and grown overnight in normal cell culture condition. The cells were fixed with 4% formaldehyde and blocked with 5% of goat serum. The anti-IL-6R antibody (1:500), Alexa Fluor conjugated anti-rabbit antibody

(1:1000) and the membrane specific dye (CellMask™ Deep Red Plasma Membrane Stain, Invitrogen, Carlsbad, CA) were used for IL-6R membrane localization. Imaging was performed using a Confocal-A1 imaging system (Nikon, Japan). Original magnification was 400x for all panels, respectively.

2.2.12 Statistical Analysis

Data were presented as mean ± SD of triplicate experiments. One-way ANOVA and, when appropriate, Student's t-test were used for statistical analyses. Significant difference among experimental groups was analyzed by Scheffe's post hoc test. All analyses were conducted using IBM SPSS statistics 21 (SPSS Inc., Chicago, IL). *P* values of < 0.05 were considered statistically significant.

2.3 Results

2.3.1 Ascites promotes migration and invasion of EOC cells

To assess the role of ascites in the migration and invasion of ovarian cancer cells, three ascites were randomly selected from women with advanced serous ovarian carcinoma, which is the most commonly encountered ovarian cancer subtype in clinical intervention. SKOV-3 cancer cell line, the most commonly used cellular models of ovarian cancer were used to confirm the effect of ascites on EOC cell migration and invasion. Using *in vitro* migration (wound healing) and invasion (Matrigel-coated transwell) assay, we found all three ascites from ovarian cancer patients increase migration and invasion in SKOV-3 (Fig. 1A and 1B). However, this phenomenon was only confined to SKOV-3 ovarian cancer cells, and did not occur in normal immortalized ovarian surface epithelial cells (IOSE380) (Fig. 1C). It has been established that cells with mesenchymal phenotype are endowed with enhanced migration and invasive capabilities [15] and EMT-dependent invasion and metastasis programs are strongly responsive to microenvironment changes [16, 17]. Therefore, we determined the effect of ascites on the expression of EMT related proteins. We found all three ascites from ovarian cancer patients reduced the expression of an epithelial marker (E-cadherin), and increased the expression of mesenchymal markers (Snail and Vimentin) (Fig. 1D and 1E) and these changes were statistically significant (Fig. S1A and S1B). Although, the expression of N-cadherin is induced in the first 30 min of ascites treatment and decreased thereafter, ascites treatment decreased overall E-/N-cadherin ratio (Fig. S1C and S1D).

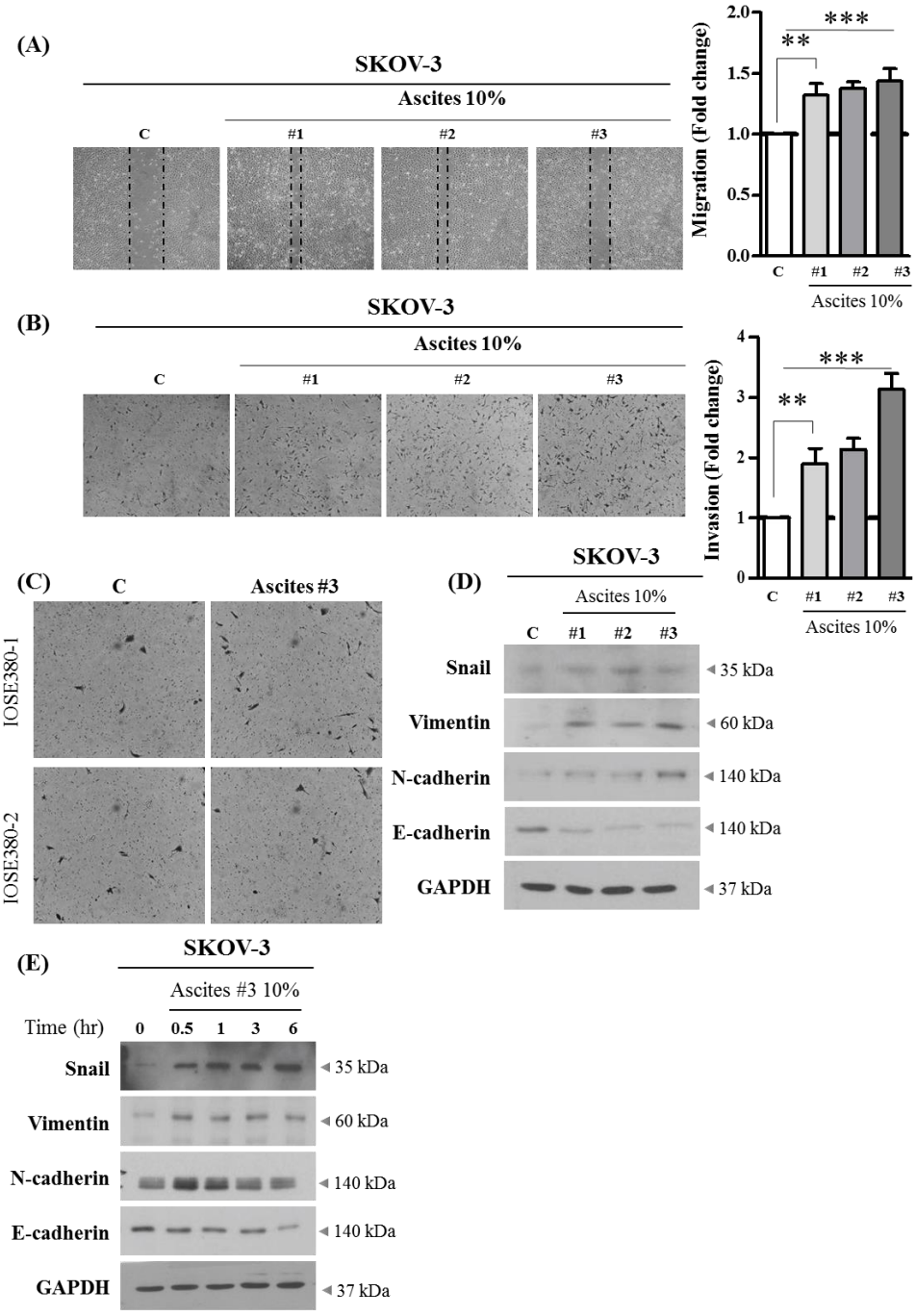

(A)
SKOV-3
Ascites 10%
C
#1
#2
#3
Migration (Fold change)
2.0
1.5
1.0
0.5
0.0
**
***
C
#1
#2
#3
Ascites 10%
(B)
SKOV-3
Ascites 10%
C
#1
#2
#3
Invasion (Fold change)
4
3
2
1
0
**
***
C
#1
#2
#3
Ascites 10%
(C)
C
Ascites #3
IOSE380-1
IOSE380-2
(D)
SKOV-3
Ascites 10%
C
#1
#2
#3
Snail
Vimentin
N-cadherin
E-cadherin
GAPDH
35 kDa
60 kDa
140 kDa
140 kDa
37 kDa
(E)
SKOV-3
Ascites #3 10%
Time (hr)
0
0.5
1
3
6
Snail
Vimentin
N-cadherin
E-cadherin
GAPDH
35 kDa
60 kDa
140 kDa
140 kDa
37 kDa

Fig. 1. Effect of ovarian cancer patient derived ascites on SKOV-3 cell migration and invasion

(A) SKOV-3 cancer cells were treated with or without 10% ascites. After 24 hr, wound healing ability was verified by measuring wound closed area under a light microscope (magnification x 40).

(B) SKOV-3 cancer cells were seeded into the upper chamber of Matrigel-coated membrane in transwells. Cell invasion were induced with or without 10% ascites. After 24 hr, invaded cells at the bottom of the transwell were stained with 0.5% crystal violet and were counted under a light microscope (magnification x 200).

(C) IOSE380 cells were seeded into the upper chamber of Matrigel-coated membrane in transwells. Cell invasion were induced and counted as above.

(D) SKOV-3 cancer cells were treated with or without 10% ascites. After 24 hr, the expression levels of EMT molecular markers, Snail, Vimentin, N-cadherin and E-cadherin were examined by western blot. GAPDH was used as an internal control.

(E) SKOV-3 cancer cells were treated with or without 10% ascites for 0 – 6 hr. Total cell lysates were extracted and subjected to western blot as above. ** and *** represent $P < 0.01$ and $P < 0.001$, respectively.

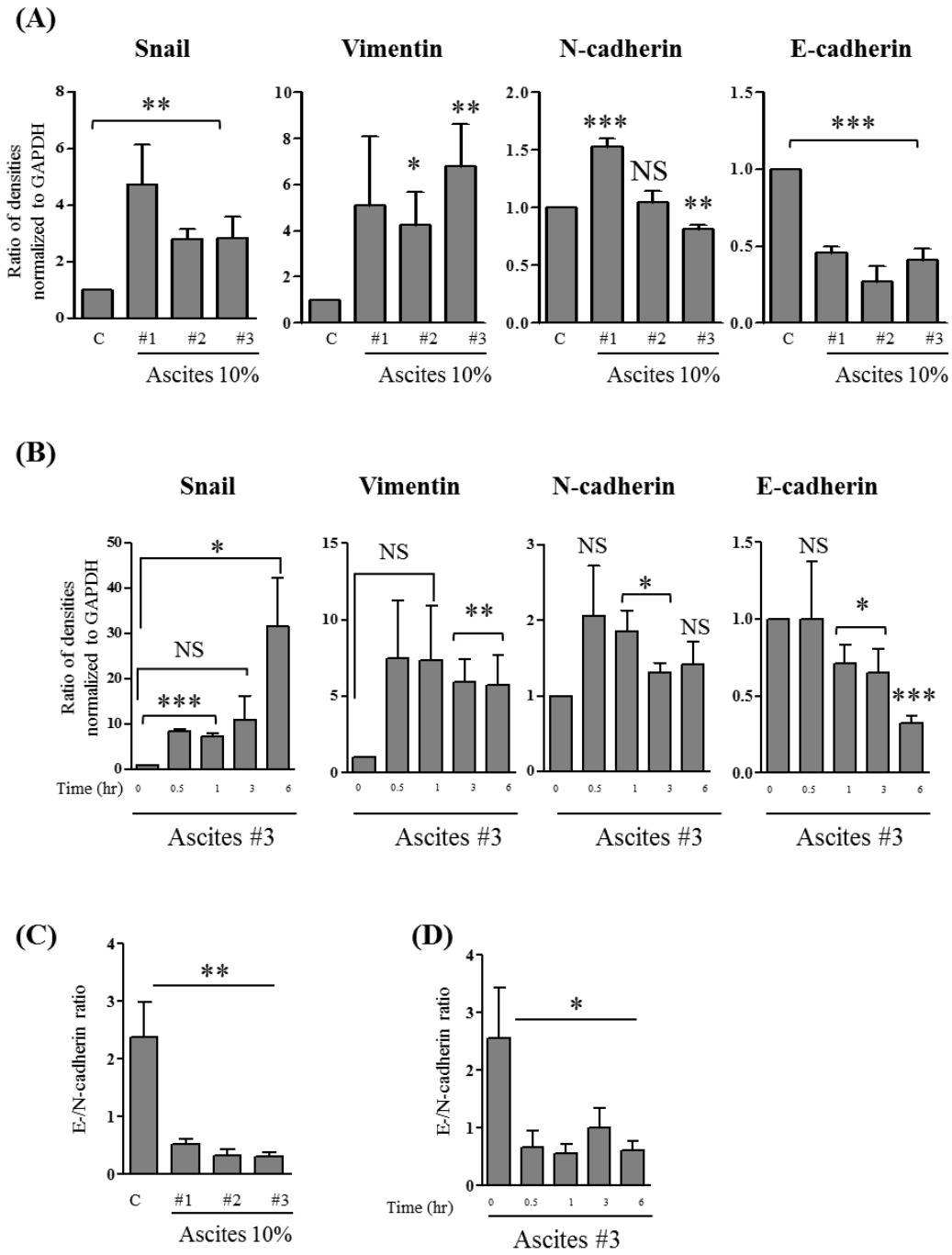

(A)
Snail
Vimentin
N-cadherin
E-cadherin
Ratio of densities normalized to GAPDH
**
*
**
***
NS
**
***
C
#1
#2
#3
Ascites 10%
(B)
Snail
Vimentin
N-cadherin
E-cadherin
Ratio of densities normalized to GAPDH
*
NS
***
NS
**
NS
*
NS
NS
*
***
Time (hr)
0
0.5
1
3
6
Ascites #3
(C)
E-/N-cadherin ratio
**
C
#1
#2
#3
Ascites 10%
(D)
E-/N-cadherin ratio
*
Time (hr)
Ascites #3

Fig. S1. Statistical analysis of EMT related protein expression, related to Fig 1

(A) Statistical analysis of quantification of western blot in Fig 1D. EMT related proteins levels were quantitated by densitometry and normalized to GAPDH. Graph represents the fold induction upon treatment of ovarian cancer patient derived ascites collected from three patients, compared to complete media.

(B) Statistical analysis of quantification of western blot in Fig 1E. EMT related protein levels were quantitated as above. Graph represents the fold-induction upon treatment of ascites for 0 to 6 hr.

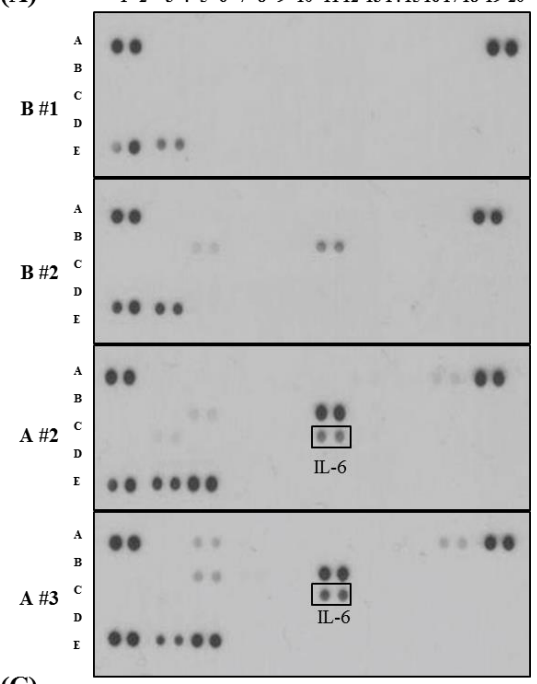
(C) Statistical analysis of quantification of N-cadherin protein densitometry normalized to E-cadherin in S1A.

(D) Statistical analysis of quantification of N-cadherin protein densitometry normalized to E-cadherin in S1B. Graph represents the fold-induction upon treatment of ascites. SD is calculated from the mean of three independent experiments. * , ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

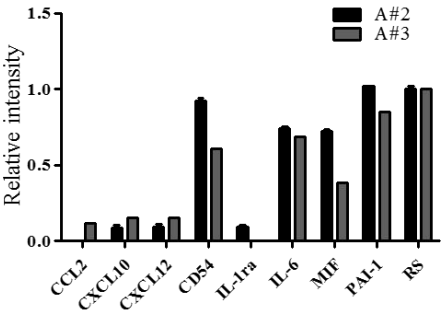
2.3.2 High levels of pro-inflammatory cytokines in malignant ascites from patients with ovarian cancer

Ascites constitutes a dynamic reservoir of soluble factors, which individually and in a combined fashion may affect tumor cells behavior [18]. To determine the cytokine(s) in ascites that are associated with EMT-dependent invasion of SKOV-3 cells, we evaluated a panel of cytokines using a cytokine array. Using two peritoneal fluids as benign control (Table 1, description of patients), the presence of pro-inflammatory cytokines in ovarian cancer patient derived ascites were compared. From relative comparison, we found IL-6 expression only in ovarian cancer patient derived ascites (Fig 2A and 2B). Then we applied enzyme-linked immunosorbent assay (ELISA), to measure the IL-6 levels. IL-6 was present at high levels (> 3 ng/ml) in all three tested ascites (Fig 2D).

(A)



(B)



(C)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	RS	RS	CCL 1	CCL 1	CCL 2	CCL 2	MIP-1α	MIP-1α	CCL 5	CCL 5	CD15 4	CD15 4	C5/C 5a	C5/C 5a	CXC L1	CXC L1	CXC L10	CXC L10	RS	RS
B	Blank	Blank	CXC L11	CXC L11	CXC L12	CXC L12	G-CSF	G-CSF	GM-CSF	GM-CSF	CD54	CD54	IFN-γ	IFN-γ	IL-1α	IL-1α	IL-1β	IL-1β	Blank	Blank
C	Blank	Blank	IL-1ra	IL-1ra	IL-2	IL-2	IL-4	IL-4	IL-5	IL-5	IL-6	IL-6	IL-8	IL-8	IL-10	IL-10	IL-12 p70	IL-12 p70	Blank	Blank
D	Blank	Blank	IL-13	IL-13	IL-16	IL-16	IL-17A	IL-17A	IL-17E	IL-17E	IL-18	IL-18	IL-21	IL-21	IL-27	IL-27	IL-32α	IL-32α	Blank	Blank
E	RS	RS	MIF	MIF	PAI-1	PAI-1	TNF-α	TNF-α	TREM-1	TREM-1	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	NegC	NegC

(D)

Sample	IL-6 (ng/ml)
A#1	11.34
A#2	10.98
A#3	43.4

Fig. 2. Pro-inflammatory cytokines enriched in ovarian cancer patient derived ascites

(A) Human cytokine array. The array key represents the location of each antibody in duplicate on the membrane. Peritoneal fluid collected from patients with benign condition (**top two panels**) and ovarian cancer patient derived ascites (**bottom two panels**).

(B) Relative intensity of each spot on the membranes were measured and normalized to the reference spot (RS). Representative cytokines enriched in ovarian cancer patient derived ascites are listed on the graph.

(C) Human cytokine array panel.

(D) IL-6 concentration in ovarian cancer patient derived ascites. The concentration of IL-6 was determined by ELISA.

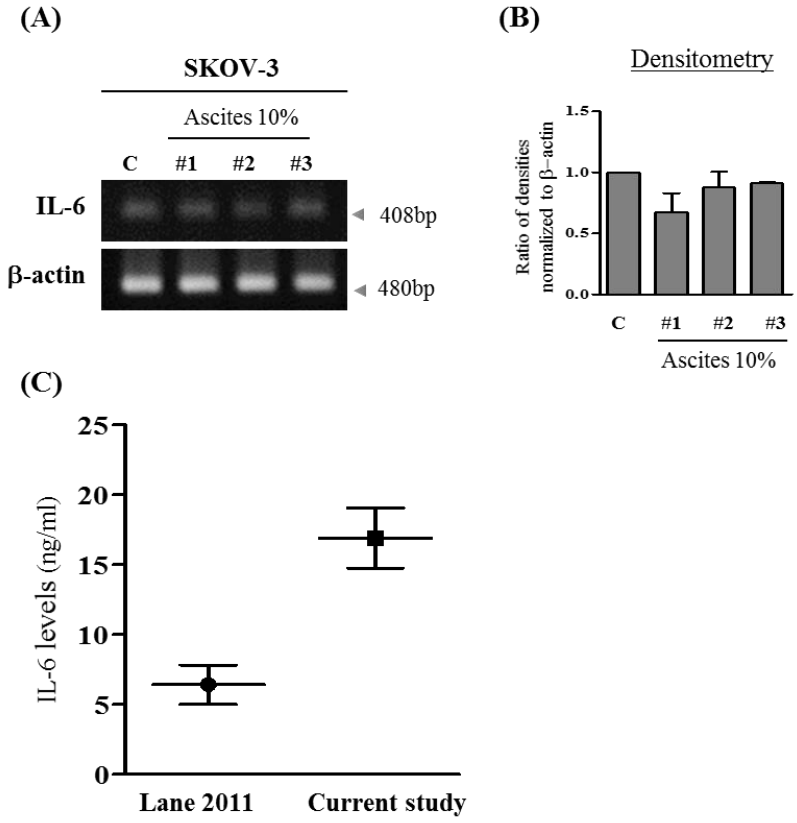

(A)
SKOV-3
Ascites 10%
C
#1
#2
#3
IL-6
β-actin
408bp
480bp
(B)
Densitometry
Ratio of densities normalized to β-actin
1.5
1.0
0.5
0.0
C
#1
#2
#3
Ascites 10%
(C)
IL-6 levels (ng/ml)
25
20
15
10
5
0
Lane 2011
Current study

Fig. S2. Effect of ovarian cancer patient derived ascites on IL-6 expression in SKOV-3, related to Fig. 2

(A) Expression of IL-6 in SKOV-3 cells were examined by RT-PCR upon treatment of ovarian cancer patient derived ascites collected from three patients, compared to complete media.

(B) The quantification of RT-PCR in S2A. IL-6 expression levels were quantitated by densitometry and normalized to β–actin.

(C) The concentration of IL-6 was determined by ELISA in ovarian cancer patient derived ascites (n = 34) compared to Lane et al. 2011 [10] Horizontal bar represents median value.

2.3.3 IL-6 in ascites increases migration and invasion via JAK2-STAT3 signaling

To investigate whether IL-6 enriched in ascites increased SKOV-3 cell migration and invasion, we applied neutralization approach. Ascites were pre-treated with neutralizing IL-6 antibody for 6 hr. This process suppressed the enhanced migration and invasion in SKOV-3 cells (Fig. 3A and 3B). Importantly, we also found that neutralization of IL-6 in ascites suppressed the effect of ascites on the expression of EMT related proteins (Fig. 3C and S3A). Moreover, ascites treatment did not affect IL-6 autocrine expression in SKOV-3 cells (Fig. S2A and S2B). Perturbed JAK2-STAT3 signaling pathway is implicated in a range of cancers and influences various cellular processes including invasion [19, 20]. To investigate this point, we first examined the effect of ascites on tyrosine phosphorylation of JAK2 and STAT3 in SKOV-3 cells. As expected, we found that ascites treatment induced both JAK2 and STAT3 phosphorylation on Y1007 and Y705, respectively. STAT3 was rapidly phosphorylated upon ascites treatment, reaching the highest level at the 30 min time point, and declined rapidly (Fig. 3D and S3B). However, this phenomenon was only confined to ovarian cancer cells, SKOV-3 cells, and did not occur in normal IOSE380 cells (Fig. 3E and S3C). Moreover, pre-treatment with neutralizing IL-6 antibody as above, suppressed the ascites induced JAK2 and STAT3 phosphorylation (Fig. 3F and S3D). To further validate the necessity of JAK2-STAT3 in ascites enhanced invasion in SKOV-3 cells, we used WP1066 (an inhibitor of JAK2 and STAT3, 2 μM) and TG101348 (an inhibitor for JAK2, 1 μM) to expectably suppress the JAK2 and

STAT3 signaling activity. Both inhibitors significantly suppressed the enhanced migration and invasion in SKOV-3 cells (Fig. 4A and 4B). Importantly, co-treatment with these inhibitors markedly reversed the acquisition of mesenchymal cell markers upon ascites treatment (Fig. 4C and S4A) and also reversed the ascites induced JAK2 and STAT3 phosphorylation (Fig. 4D and S4B). These results suggest that ascites activate JAK2-STAT3 signaling pathway via IL-6 and increase SKOV-3 cell invasion.

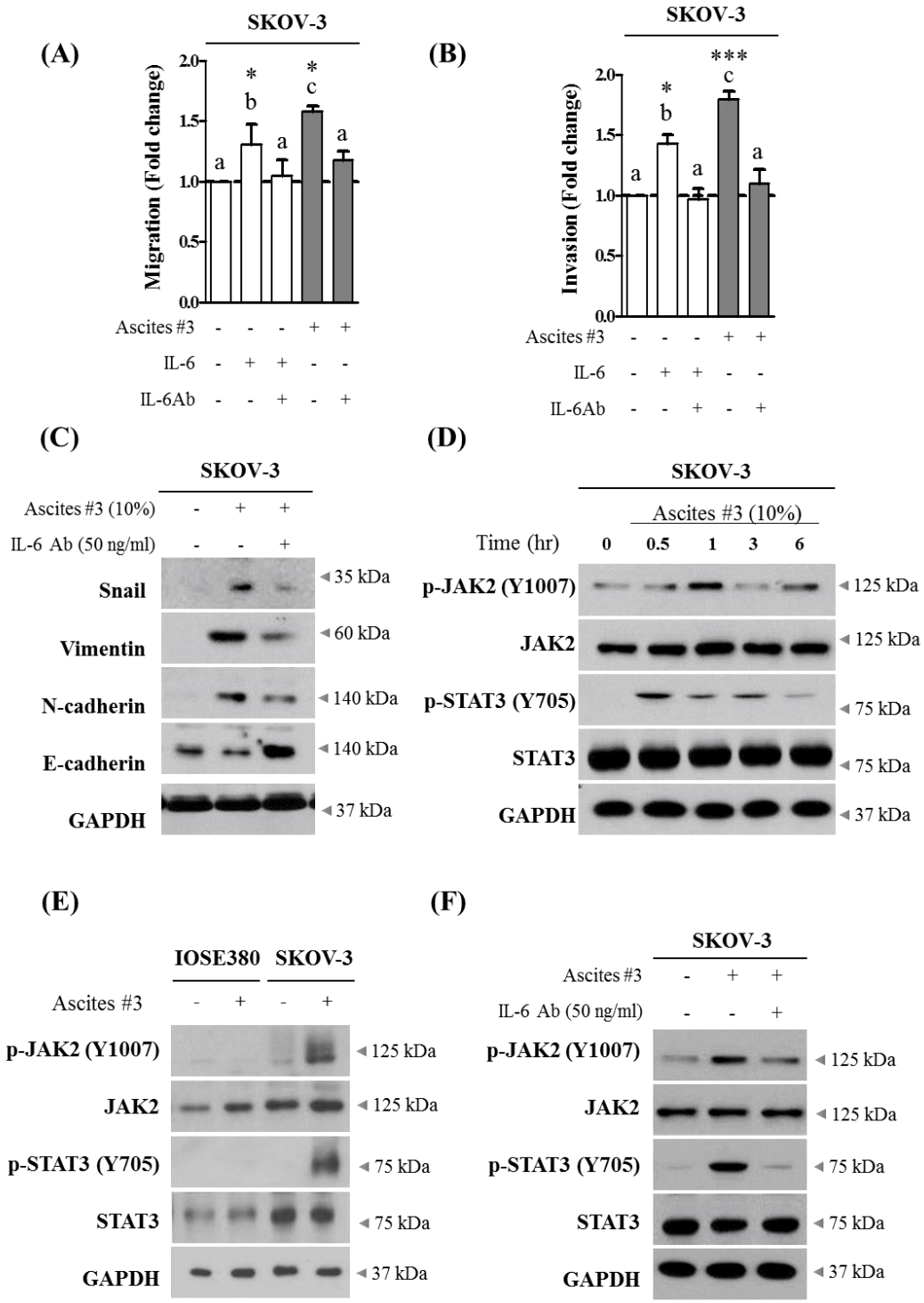

(A)
SKOV-3
Migration (Fold change)
2.0
1.5
1.0
0.5
0.0
a
b
a
c
a
*
*
Ascites #3 - - - + +
IL-6 - + + - -
IL-6Ab - - + - +
(B)
SKOV-3
Invasion (Fold change)
2.0
1.5
1.0
0.5
0.0
a
b
a
c
a
*
***
Ascites #3 - - - + +
IL-6 - + + - -
IL-6Ab - - + - +
(C)
SKOV-3
Ascites #3 (10%) - + +
IL-6 Ab (50 ng/ml) - - +
Snail
Vimentin
N-cadherin
E-cadherin
GAPDH
35 kDa
60 kDa
140 kDa
140 kDa
37 kDa
(D)
SKOV-3
Ascites #3 (10%)
Time (hr) 0 0.5 1 3 6
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
GAPDH
125 kDa
125 kDa
75 kDa
75 kDa
37 kDa
(E)
IOSE380
SKOV-3
Ascites #3 - + - +
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
GAPDH
125 kDa
125 kDa
75 kDa
75 kDa
37 kDa
(F)
SKOV-3
Ascites #3 - + +
IL-6 Ab (50 ng/ml) - - +
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
GAPDH
125 kDa
125 kDa
75 kDa
75 kDa
37 kDa

Fig. 3. IL-6 in ovarian cancer patient derived ascites increases migration and invasion of SKOV-3 cells via JAK2-STAT3 signaling

(A) SKOV-3 cancer cells were treated with 10% ascites that were either pre-treated with or without 50 ng/ml of IL-6 antibody (IL-6 Ab) for 6 hr. Recombinant IL-6 (10 ng/ml) was used as a positive control. After 24 hr, wound healing ability was verified by measuring wound closed area under a light microscope (magnification x40).

(B) SKOV-3 cancer cells were seeded into the upper chamber of Matrigel-coated membrane in transwells. Cell invasion were induced by ascites as above. After 24 hr, invaded cells at the bottom of the transwell were stained with 0.5% crystal violet and counted under a light microscope (magnification x200).

(C) SKOV-3 cancer cells were treated with IL-6 Ab as above. After 24 hr, the expression of snail, vimentin, N-cadherin and E-cadherin were examined by western blot. GAPDH was used as an internal control.

(D) SKOV-3 cancer cells were treated with or without 10% ascites for 0 - 6 hr. The expression of p-JAK2 (Y1007), JAK2, p-STAT3 (Y705) and STAT3 were examined by western blot. GAPDH was used as an internal control.

(E) IOSE380 and SKOV-3 cells were treated with or without 10% ascites. After 0.5 hr, the expression of p-JAK2 (Y1007), JAK2, p-STAT3 (Y705) and STAT3 were examind by western blot. GAPDH was used as an internal control.

(F) SKOV-3 cancer cells were treated with IL-6 Ab as above.The expression of p-JAK2, JAK2, p-STAT3 and STAT3 were examined by western blot.

GAPDH was used as an internal control. * and *** represent $P < 0.05$ and $P < 0.001$, respectively.

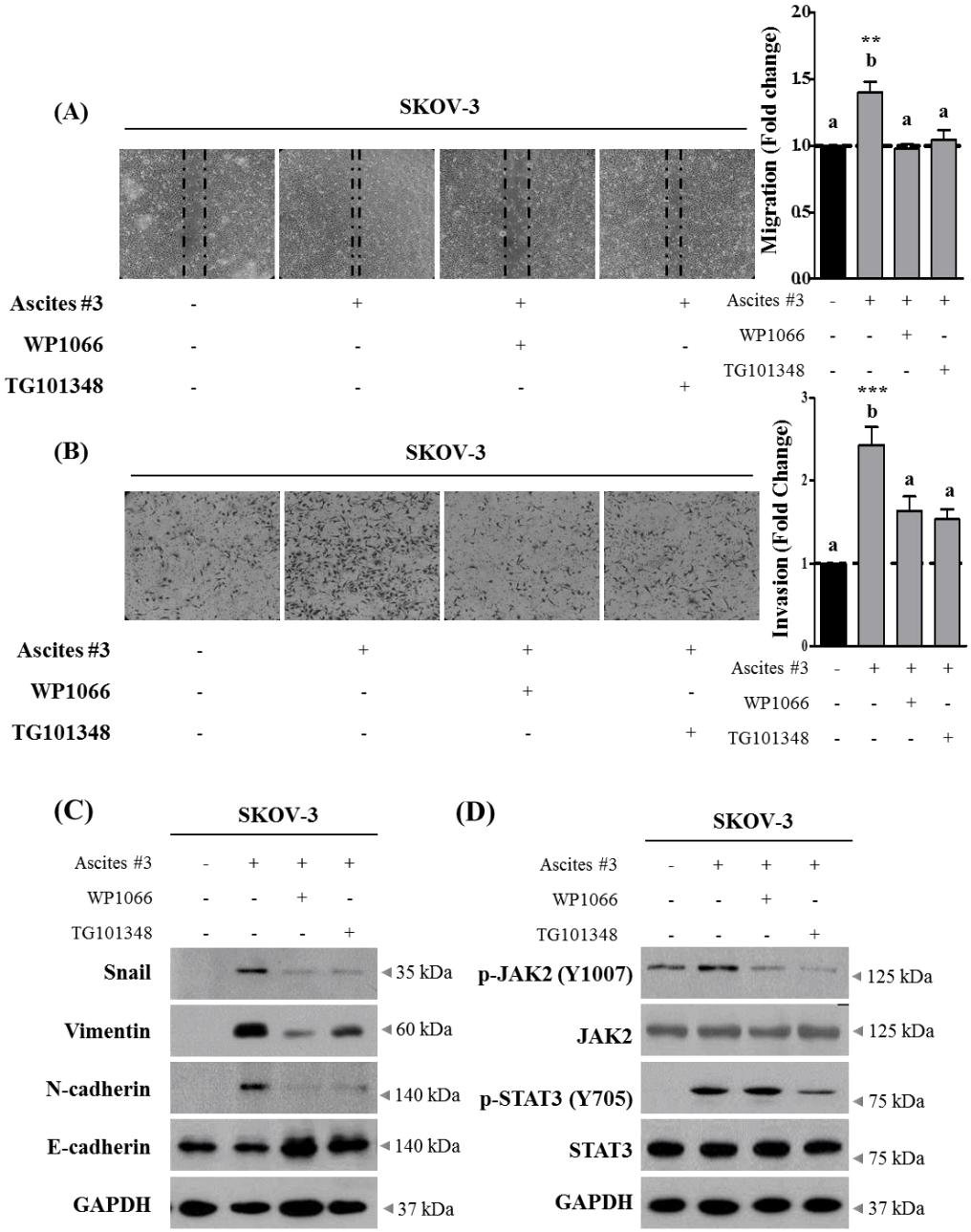


Fig. 4. Inhibition of JAK2-STAT3 signaling suppresses ascites-induced migration and invasion in SKOV-3 cells.

(A) SKOV-3 cancer cells were treated with 10% ascites, with or without JAK2 and STAT3 inhibitors, WP1066 and TG101348. After 24 hr, wound healing ability was verified by measuring wound closed area under a light microscope (magnification x40).

(B) SKOV-3 cancer cells were seeded into the upper chamber of Matrigel-coated membrane in transwells. Cell invasion were induced by ascites with or without JAK2 and STAT3 inhibitors. After 24 hr, invaded cells at the bottom of the transwell were stained with 0.5% crystal violet and counted under a light microscope (magnification x200).

(C) SKOV-3 cancer cells were treated with JAK2 and STAT3 inhibitors as above. After 24 hr, the expression of snail, vimentin**,** N-cadherin and E-cadherin were examined by western blot. GAPDH was used as an internal control.

(D) SKOV-3 cancer cells were treated as above. The expression of p-JAK2 (Y1007), JAK2, p-STAT3 (Y705) and STAT3 were examined by western blot. GAPDH was used as an internal control. Means without a common letter are significantly different: ** and *** represent $P < 0.01$ and $P < 0.001$, respectively.

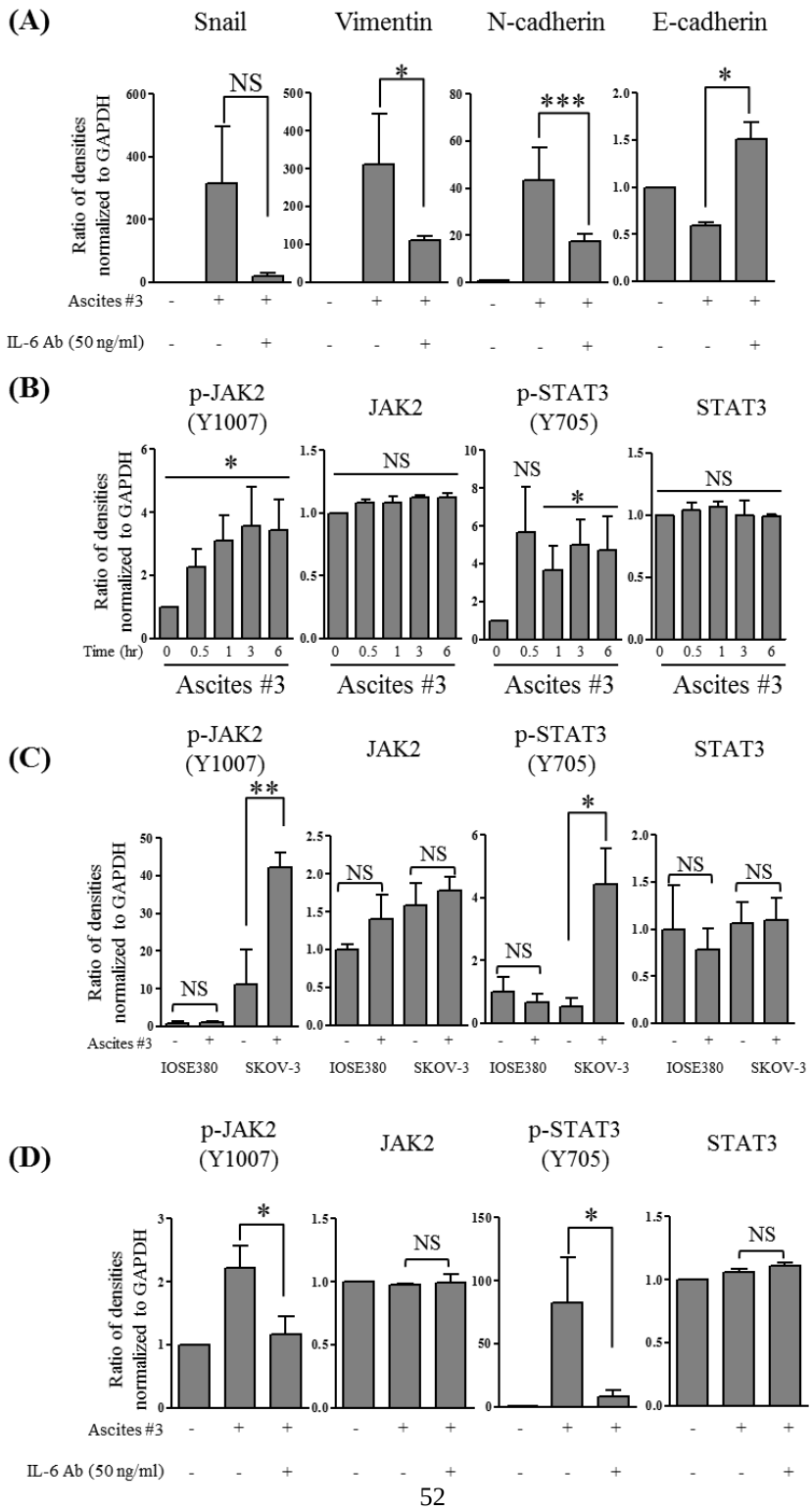

(A)
Snail
Vimentin
N-cadherin
E-cadherin
Ratio of densities normalized to GAPDH
NS
*
***
*
Ascites #3
IL-6 Ab (50 ng/ml)
(B)
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
Ratio of densities normalized to GAPDH
Time (hr)
0 0.5 1 3 6
Ascites #3
(C)
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
Ratio of densities normalized to GAPDH
**
*
NS
Ascites #3
IOSE380
SKOV-3
(D)
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
Ratio of densities normalized to GAPDH
Ascites #3
IL-6 Ab (50 ng/ml)

Fig. S3. Statistical analysis of EMT related protein expression and JAK2-STAT3 signaling, related to Fig. 3

(A) Statistical analysis of quantification of western blot in Fig. 3C. EMT related proteins level were quantitated by densitometry and normalized to GAPDH. Graph represents the fold induction upon treatment of ascites with or without neutralizing IL-6 antibody.

(B) Statistical analysis of quantification of western blot in Fig. 3D. JAK2-STAT3 phosphorylation were quantitated by densitometry and normalized to GAPDH. Graph represents the fold induction upon treatment of ovarian cancer patient derived ascites for 0 to 6 hr.

(C) Statistical analysis of quantification of western blot in Fig. 3E. JAK2-STAT3 phosphorylation were quantitated by densitometry as above. Graph represents the fold induction upon treatment of ovarian cancer patient derived ascites for 0.5 hr in IOSE380 and SKOV-3.

(D) Statistical analysis of quantification of western blot in Fig. 3F. JAK2-STAT phosphorylation were quantitated by densitometry as above. Graph represents the fold induction upon treatment of ascites with or without neutralizing IL-6 antibody. SD is calculated from the mean of three independent experiments. * , ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

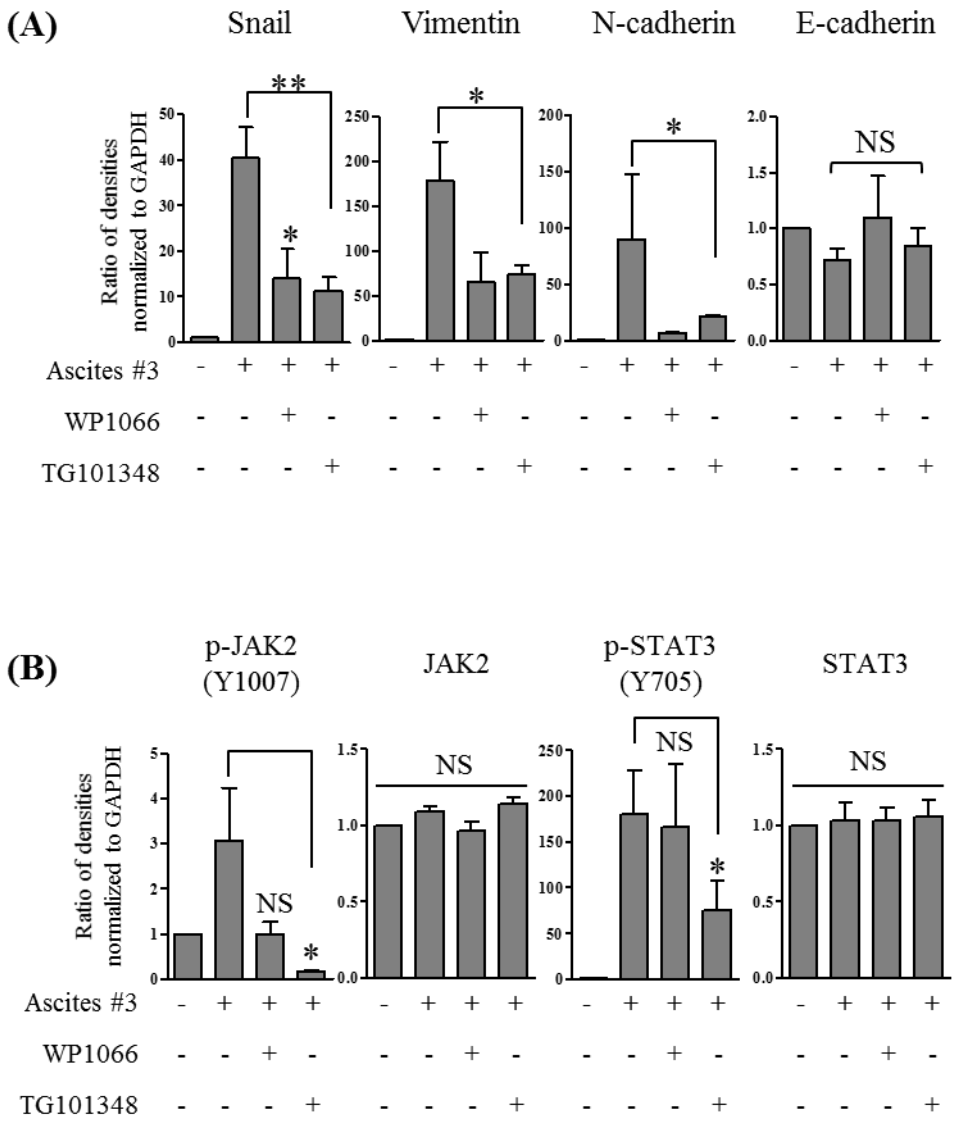

(A)
Snail
Vimentin
N-cadherin
E-cadherin
Ratio of densities normalized to GAPDH
**
*
*
*
NS
Ascites #3
WP1066
TG101348
(B)
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
Ratio of densities normalized to GAPDH
NS
NS
NS
NS
*
*
Ascites #3
WP1066
TG101348

Fig. S4. Statistical analysis of EMT related protein expression and JAK2-STAT3 signaling, related to Fig. 4

(A) Statistical analysis of quantification of western blot in Fig. 4C. EMT related proteins level were quantitated by densitometry and normalized to GAPDH. Graph represents the fold induction upon treatment of ascites with or without JAK2 and STAT3 inhibitors.

(B) Statistical analysis of quantification of western blot in Fig. 4D. JAK2-STAT3 phosphorylation were quantitated by densitometry and normalized to GAPDH. Graph represents the fold induction upon treatment of ovarian cancer patient derived ascites with or without JAK2 and STAT3 inhibitors. SD is calculated from the mean of three independent experiments. * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

2.3.4 Ascites increase invasion only in ovarian cancer cells with IL-6R expression on cell membrane

To determine whether ascites increase invasion in ovarian cancer cells in general or in a selective subset, additional ovarian cancer cell lines were tested. Four ovarian cancer cell lines including SKOV-3 were tested for expression of IL-6R at mRNA and protein levels. IL-6R were expressed in the 4 cell lines at both mRNA and protein levels (Fig. 5A-B and S5A-B). We found that that ascites increase invasion only in PA-1 and SKOV-3 cells, but had no significant effect on OVCAR-3 and A2780 cell lines (Fig. 5C). Interestingly, the effect of ascites treatment was not associated with endogenous IL-6 mRNA levels in ovarian cancer cell lines (Fig. S5C), but the expression of IL-6R on plasma membrane but not in cytosol of PA-1 and SKOV-3 cells correlate with ascites induced invasion (Fig. 5D-E and S5D). Finally, to link the IL-6R expression is critical for ascites mediated increased invasion in PA-1 and SKOV-3 cells, IL-6R was silenced and this process suppressed the ascites mediated enhanced invasion in these two cell lines (Fig. 6B1 and 6B2). In addition, ascites induced JAK2 and STAT3 phosphorylation only in PA-1 and SKOV-3 cells (Fig. 6A). These results demonstrate that IL-6 in ascites increase invasion via IL-6R on cell membrane and thus increase invasive properties only in a selective subset of ovarian cancer cells.

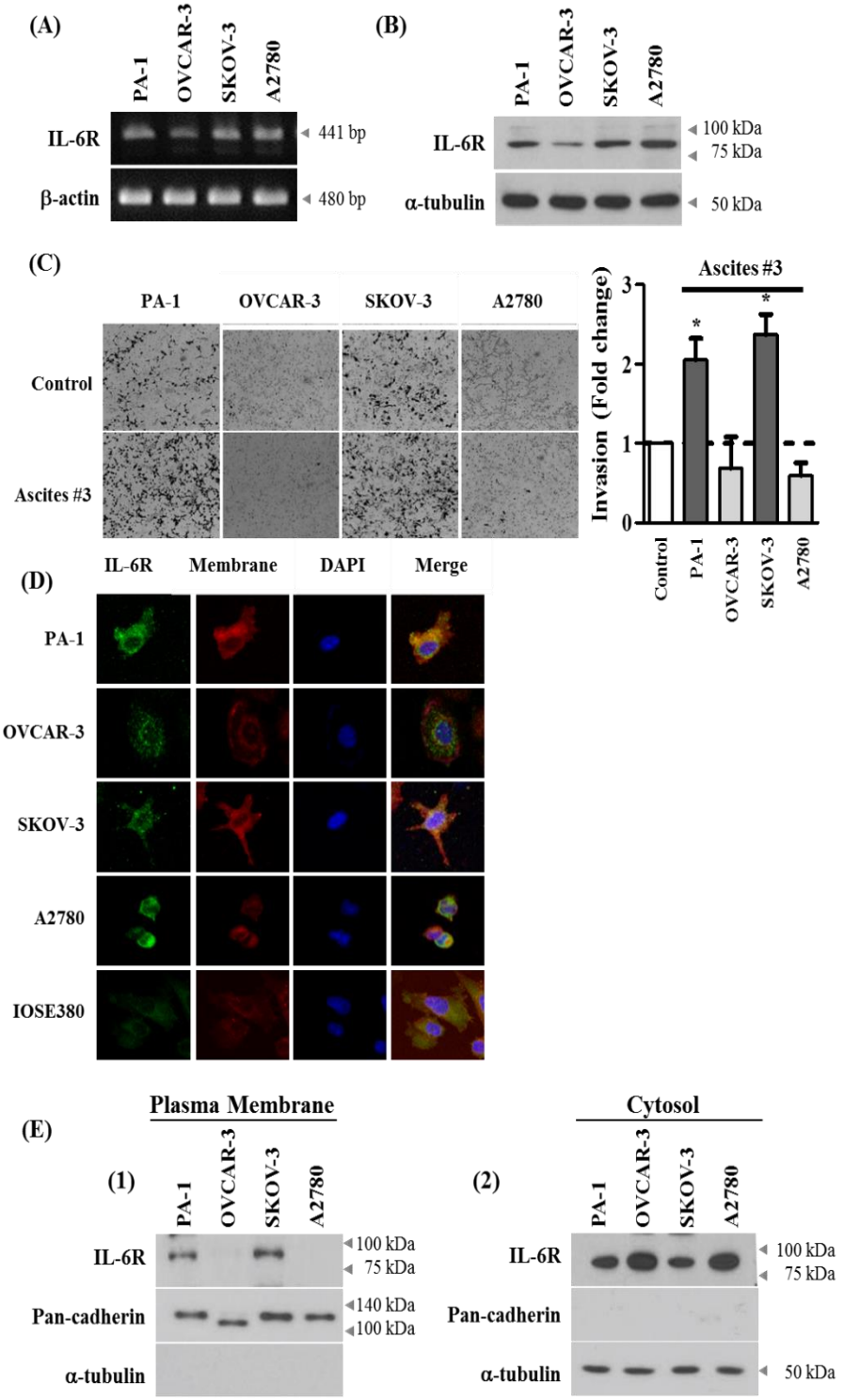

(A)
PA-1
OVCAR-3
SKOV-3
A2780
IL-6R
441 bp
β-actin
480 bp
(B)
PA-1
OVCAR-3
SKOV-3
A2780
IL-6R
100 kDa
75 kDa
α-tubulin
50 kDa
(C)
PA-1
OVCAR-3
SKOV-3
A2780
Control
Ascites #3
Ascites #3
Invasion (Fold change)
3
2
1
0
*
*
Control
PA-1
OVCAR-3
SKOV-3
A2780
(D)
IL-6R
Membrane
DAPI
Merge
PA-1
OVCAR-3
SKOV-3
A2780
IOSE380
(E)
Plasma Membrane
(1)
PA-1
OVCAR-3
SKOV-3
A2780
IL-6R
100 kDa
75 kDa
Pan-cadherin
140 kDa
100 kDa
α-tubulin
Cytosol
(2)
PA-1
OVCAR-3
SKOV-3
A2780
IL-6R
100 kDa
75 kDa
Pan-cadherin
α-tubulin
50 kDa

Fig. 5. Expression of IL-6R on cell membrane correlates with ascites-induced invasion

(A) Basal mRNA expression of IL-6 receptor (IL-6R) in ovarian cancer cell lines were examined by RT-PCR.

(B) Basal protein expression of IL-6R in ovarian cancer cell lines was analyzed by western blot. α-tubulin was used as an internal control.

(C) Ovarian cancer cells were seeded into the upper chamber of Matrigel-coated membrane in transwells. Cell invasion were induced by ascites. After 24 hr, invaded cells at the bottom of the transwell were stained with 0.5% crystal violet and counted under a light microscope (magnification x200).

(D) Expression of IL-6R on cell membrane of ovarian cancer cells were examined by immunocytochemistry. The representative confocal microscopy figure (Green, IL-6R; red, plasma membrane; blue, DAPI) are shown. Original magnification 400x for all panels.

(E) Expression of IL-6R on cell membrane of ovarian cancer cell lines were examined by isolation of proteins from the plasma membrane and the cytosol fraction. The expression level of IL-6R was examined by western blot. Pan-cadherin and α-tubulin were used as an internal control. * , ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

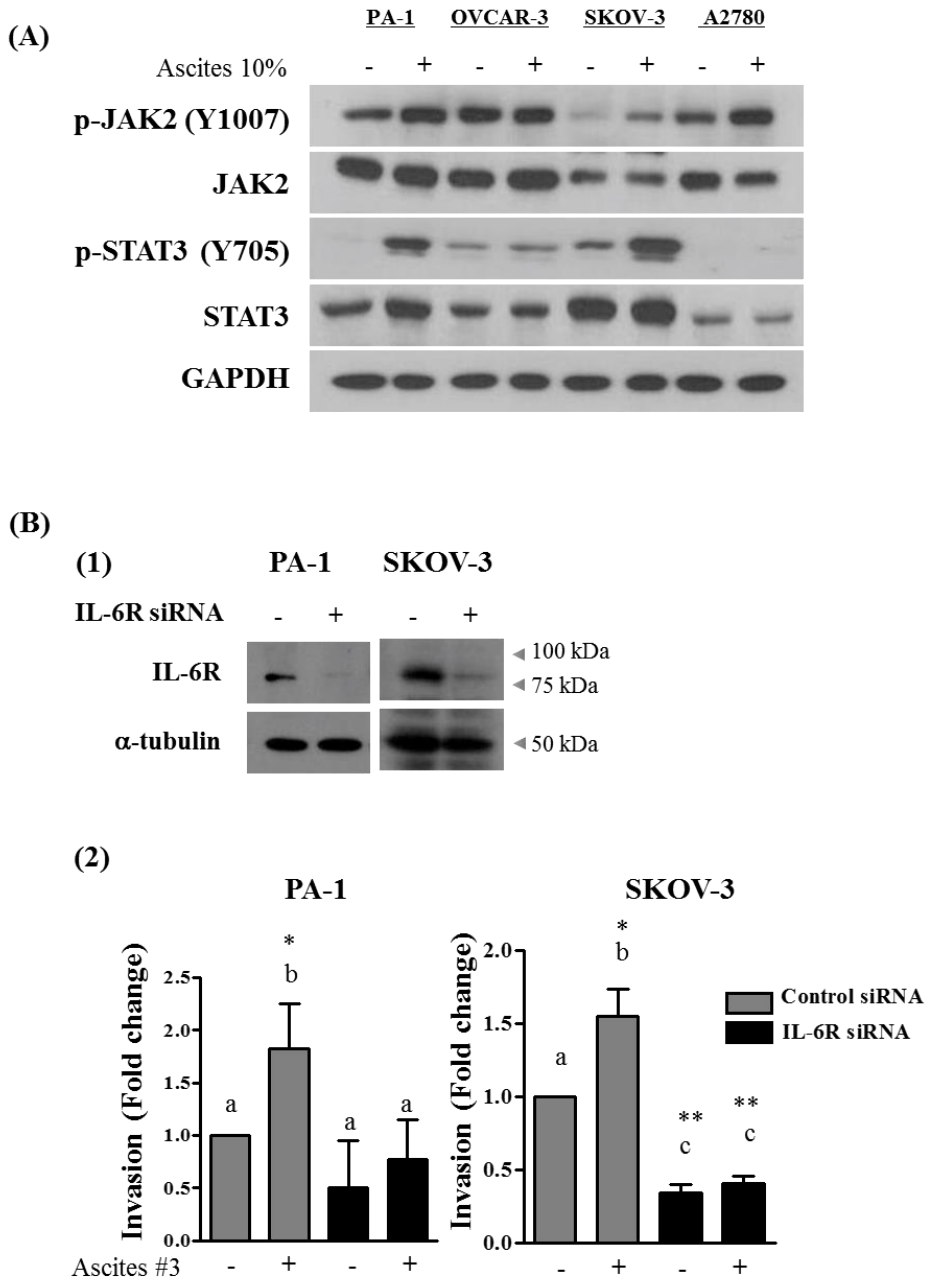

(A)
PA-1
OVCAR-3
SKOV-3
A2780
Ascites 10%
- + - + - + - +
p-JAK2 (Y1007)
JAK2
p-STAT3 (Y705)
STAT3
GAPDH
(B)
(1)
PA-1
SKOV-3
IL-6R siRNA
- + - +
IL-6R
α-tubulin
100 kDa
75 kDa
50 kDa
(2)
PA-1
SKOV-3
Invasion (Fold change)
Control siRNA
IL-6R siRNA
Ascites #3
- + - +

Fig. 6. Silencing of IL-6R impairs ascites-induced invasion

(A) PA-1 and SKOV-3 cells were transfected with IL-6R targeted siRNA (100 nM) or scrambled RNA (100 nM) as a negative control. Silencing of IL-6R expression was examined by western blot.

(B) PA-1 and SKOV-3 cells were transfected with IL-6R targeted siRNA (100 nM) or negative control scrambled RNA (100 nM) for 24 hr. Cells were seeded into the upper chamber of Matrigel-coated membrane in transwells. Cell invasion were induced by 10% ascites. After 24, invaded cells at the bottom of the transwell were stained with 0.5% crystal violet and were counted under a light microscope (magnification x200). The data summarized in the bar charts are presented as mean ± SD of three independent fields. Means without a common letter are significantly different: * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

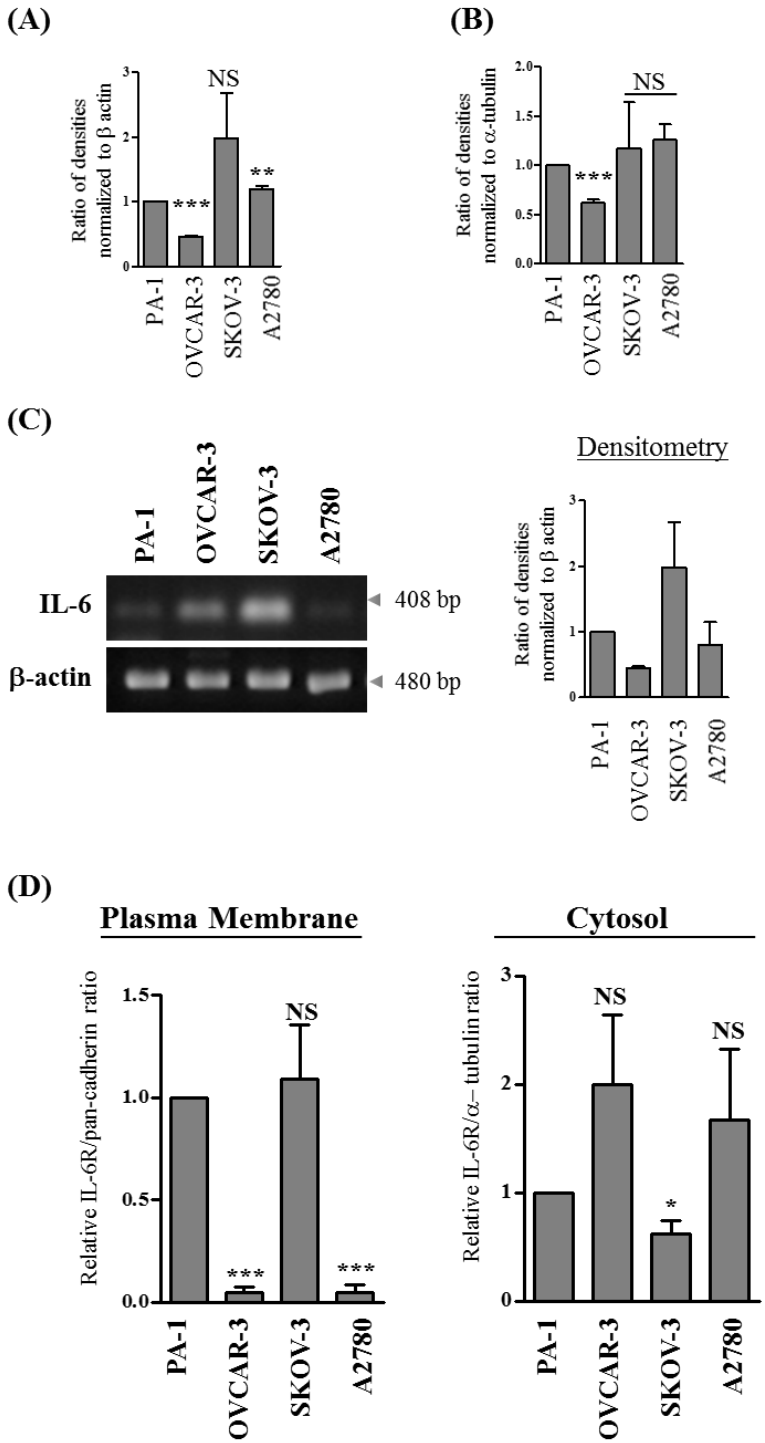

(A)
Ratio of densities normalized to β actin
NS
***
**
PA-1
OVCAR-3
SKOV-3
A2780
(B)
Ratio of densities normalized to α-tubulin
NS
***
PA-1
OVCAR-3
SKOV-3
A2780
(C)
PA-1
OVCAR-3
SKOV-3
A2780
IL-6
408 bp
β-actin
480 bp
Densitometry
Ratio of densities normalized to β actin
PA-1
OVCAR-3
SKOV-3
A2780
(D)
Plasma Membrane
Relative IL-6R/pan-cadherin ratio
NS
***
***
PA-1
OVCAR-3
SKOV-3
A2780
Cytosol
Relative IL-6R/α– tubulin ratio
NS
NS
*
PA-1
OVCAR-3
SKOV-3
A2780

Fig. S5. IL-6 and IL-6R expression in ovarian cancer cell lines, related to Fig. 5

(A) Statistical analysis of quantitation of RT-PCR in Fig. 5A. IL-6R mRNA expression levels were quantitated by densitometry and normalized to β–actin. SD is calculated from the mean of three independent experiments.

(B) Statistical analysis of quantitation of western blot in Fig. 5B. IL-6R expression levels were quantitated by densitometry and normalized to α–tubulin. SD is calculated from the mean of three independent experiments.

(C) Expression of IL-6 in ovarian cancer cell lines were examined by RT-PCR. The representative figure and quantified data from three independent experiments were shown.

(D) Statistical analysis of quantitation of western blot in Fig. 5E. SD is calculated from the mean of three independent experiments. ** and *** represent $P < 0.01$ and $P < 0.001$, respectively.

Table 1. Description of patients recruited in the study

	Patient ID	Disease	Histopathology	STAGE	Age at diagnosis	Survival	Survival status	Tumor spread
Cancer	A#1	Ovarian cancer	Serous	IV	79	2 years as of 09/06/16	Alive	Ovary, omentum, pelvic lymph node, colon serosa, diaphragm
	A#2	Ovarian cancer	Serous	IIIC	72	2 years as of 27/06/16	Alive	Ovary omentum, pelvic lymph node, colon serosa, appendix, diaphragm
	A#3	Ovarian cancer	Serous	IV	68	1 year 11 months as of 19/07/16	NA	Ovary, omentum, bilateral sub-diaphragmatic, gallbladder, left obturator
Benign	B#1	Para-tubal cyst	NA	NA	76		Alive	
	B#2	Ovarian mucinous cystadenoma	NA	NA	55		Alive	

***NA**: Not available

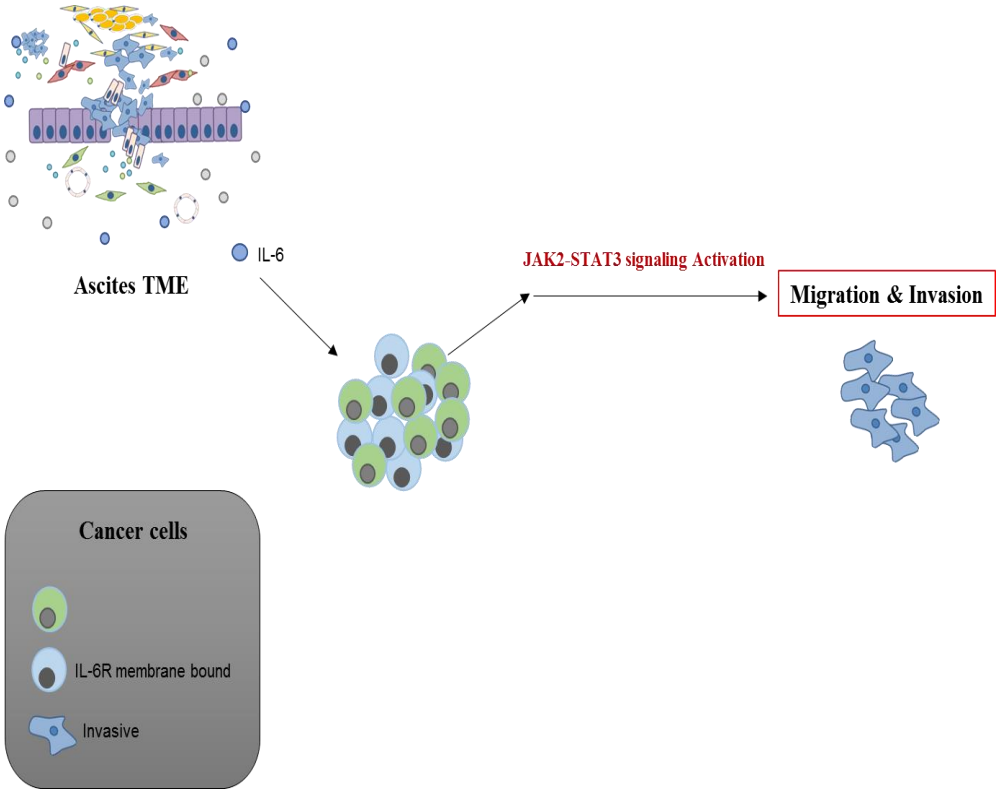


Fig. 7. IL-6 enriched in ascites tumor-microenvironment (TME) enhances migration and invasion of ovarian cancer cells with membrane bound IL-6R

2.4. Discussion

Although the presence of ascites correlates with a poor prognosis in ovarian cancer patients, underlying molecular mechanism that lead to disease progression is poorly defined. Interestingly, the ascites presented in EOC patients constitute a dynamic reservoir of both pro-tumorigenic and anti-tumorigenic factors [21]. Among several factors accumulated in ascites, we found that IL-6 is enriched in ascites and is responsible for increased invasion, suggesting that suppressing its downstream signals might decrease migration and invasion.

Here we screened 36 pro-inflammatory cytokines and found IL-6 expression only in ovarian cancer patient derived ascites but absent in peritoneal fluids from benign condition (Fig. 2A). Early studies have also reported enrichment of pro-inflammatory cytokines such as IL-6, CXCL10, and CD54 in ascites and have a pro-metastatic role in ovarian cancer progression [7, 21-24]. IL-6 signals through binding to heterodimer of IL-6R and gp130 [25, 26] and is associated with the metastatic phenotype in a range of cancers [12], and Lane et al. reported that IL-6 levels above 3 ng/ml in ascites is associated with poor prognosis in patients with ovarian cancer [10]. In line with this previous report, ascites collected in our study setting had higher IL-6 levels compared to reports by Lane et al. (Fig. S2C). Interestingly, early studies suggest that ascites in ovarian cancer patients forms a protective TME against drug-induced apoptosis by inducing survival signaling pathways such as PI3K/Akt [27, 28]. Moreover, the extent to which ascites served as protective TME was dependent on patients [9], which highlights inter-patient variability in the components of ascites.

Similarly, although all three ascites tested in study increased invasion, we also observed that the magnitude of increased migration and invasion by ascites were variable among the patients (Fig. 1A and 1B).

The metastasis is a critical step in determining the outcome of ovarian cancer patients, therefore preventing metastasis would improve prognosis in ovarian cancer patients [29]. Although a number of biological molecules related to metastasis in ovarian tumors have been elucidated [30], the precise role of IL-6R expression on cell membrane is undefined. Accumulating evidence suggests a rationale for anti- IL-6/IL-6R therapy for ovarian cancer treatment [31-33]. The inhibition of IL-6R using tocilizumab almost completely inhibited invasion promoted by the microenvironment [31]. Similarly, neutralization of IL-6 significantly enhanced the therapeutic efficacy of paclitaxel in mouse models of EOC by reducing tumor growth [34].

Our results demonstrate that IL-6R protein expression on plasma membrane of PA-1 and SKOV-3 cancer cells but not in cytosol predicts responsiveness to ascites in cell-based models of EOC invasion (Fig. 5E1-2). Further study across a broader spectrum of patient-derived ascites and tumor samples is needed to fully investigate this hypothesis.

Collectively, the findings presented herein demonstrate that IL-6 in ascites function through membrane-bound IL-6R expressed in cancer cells and increase the EOC cell invasion via JAK2-STAT3 signaling. Targeting IL-6, IL-6R or JAK2-STAT3 may offer an efficient management of ascites induced migration and invasion in ovarian cancer patients**.**

2.4 References

1. Narod S. Can advanced-stage ovarian cancer be cured? Nat Rev Clin Oncol. 2016; 13(4):255-261.

2. Tan DS, Agarwal R and Kaye SB. Mechanisms of transcoelomic metastasis in ovarian cancer. Lancet Oncol. 2006; 7(11):925-934.

3. Parsons SL, Lang MW and Steele RJ. Malignant ascites: a 2-year review from a teaching hospital. Eur J Surg Oncol. 1996; 22(3):237-239.

4. Ayantunde AA and Parsons SL. Pattern and prognostic factors in patients with malignant ascites: a retrospective study. Ann Oncol. 2007; 18(5):945-949.

5. Puiffe ML, Le Page C, Filali-Mouhim A, Zietarska M, Ouellet V, Tonin PN, Chevrette M, Provencher DM and Mes-Masson AM. Characterization of ovarian cancer ascites on cell invasion, proliferation, spheroid formation, and gene expression in an in vitro model of epithelial ovarian cancer. Neoplasia. 2007; 9(10):820-829.

6. Goncharenko-Khaider N, Matte I, Lane D, Rancourt C and Piche A. Ovarian cancer ascites increase Mcl-1 expression in tumor cells through ERK1/2-Elk-1 signaling to attenuate TRAIL-induced apoptosis. Mol Cancer. 2012; 11:84.

7. Carduner L, Leroy-Dudal J, Picot CR, Gallet O, Carreiras F and Kellouche S. Ascites-induced shift along epithelial-mesenchymal spectrum in ovarian cancer cells: enhancement of their invasive behavior partly dependant on alphav integrins. Clin Exp Metastasis. 2014; 31(6):675-688.

8. Kim S, Kim B and Song YS. Ascites modulates cancer cell behavior, contributing to tumor heterogeneity in ovarian cancer. Cancer Sci. 2016.

9. Lane D, Matte I, Rancourt C and Piche A. The prosurvival activity of ascites against TRAIL is associated with a shorter disease-free interval in patients with ovarian cancer. J Ovarian Res. 2010; 3:1.

10. Lane D, Matte I, Rancourt C and Piche A. Prognostic significance of IL-6 and IL-8 ascites levels in ovarian cancer patients. BMC Cancer. 2011; 11:210.

11. Lane D, Matte I, Garde-Granger P, Laplante C, Carignan A, Rancourt C and Piche A. Inflammation-regulating factors in ascites as predictive biomarkers of drug resistance and progression-free survival in serous epithelial ovarian cancers. BMC Cancer. 2015; 15:492.

12. Kumar J and Ward AC. Role of the interleukin 6 receptor family in epithelial ovarian cancer and its clinical implications. Biochim Biophys Acta. 2014; 1845(2):117-125.

13. Ho CM, Chang SF, Hsiao CC, Chien TY and Shih DT. Isolation and characterization of stromal progenitor cells from ascites of patients with epithelial ovarian adenocarcinoma. J Biomed Sci. 2012; 19:23.

14. Kim SH, Kim SH, Kim YB, Jeon YT, Lee SC and Song YS. Genistein inhibits cell growth by modulating various mitogen-activated protein kinases and AKT in cervical cancer cells. Annals of the New York Academy of Sciences. 2009; 1171:495-500.

15. Vergara D, Merlot B, Lucot JP, Collinet P, Vinatier D, Fournier I and Salzet M. Epithelial-mesenchymal transition in ovarian cancer. Cancer Lett. 2010; 291(1):59-66.

16. De Craene B and Berx G. Regulatory networks defining EMT during cancer initiation and progression. Nature reviews Cancer. 2013; 13(2):97-110.

17. Matte I, Lane D, Laplante C, Garde-Granger P, Rancourt C and Piche A. Ovarian cancer ascites enhance the migration of patient-derived peritoneal mesothelial cells via cMet pathway through HGF-dependent and -independent mechanisms. Int J Cancer. 2014.

18. Cohen M and Petignat P. The bright side of ascites in ovarian cancer. Cell Cycle. 2014; 13(15):2319.

19. Malek JA, Mery E, Mahmoud YA, Al-Azwani EK, Roger L, Huang R, Jouve E, Lis R, Thiery JP, Querleu D and Rafii A. Copy number variation analysis of matched ovarian primary tumors and peritoneal metastasis. PloS one. 2011; 6(12):e28561.

20. Teng Y, Ross JL and Cowell JK. The involvement of JAK-STAT3 in cell motility, invasion, and metastasis. JAKSTAT. 2014; 3(1):e28086.

21. Ahmed N and Stenvers KL. Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research. Front Oncol. 2013; 3:256.

22. Matte I, Lane D, Laplante C, Rancourt C and Piche A. Profiling of cytokines in human epithelial ovarian cancer ascites. Am J Cancer Res. 2012; 2(5):566-580.

23. Giuntoli RL, 2nd, Webb TJ, Zoso A, Rogers O, Diaz-Montes TP, Bristow RE and Oelke M. Ovarian cancer-associated ascites demonstrates altered immune environment: implications for antitumor immunity. Anticancer Res. 2009; 29(8):2875-2884.

24. Yigit R, Figdor CG, Zusterzeel PL, Pots JM, Torensma R and Massuger LF. Cytokine analysis as a tool to understand tumour-host interaction in ovarian cancer. Eur J Cancer. 2011; 47(12):1883-1889.

25. Lutticken C, Wegenka UM, Yuan J, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yasukawa K, Taga T and et al. Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. Science. 1994; 263(5143):89-92.

26. Zhong Z, Wen Z and Darnell JE, Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science. 1994; 264(5155):95-98.

27. Lane D, Robert V, Grondin R, Rancourt C and Piche A. Malignant ascites protect against TRAIL-induced apoptosis by activating the PI3K/Akt pathway in human ovarian carcinoma cells. Int J Cancer. 2007; 121(6):1227-1237.

28. Lane D, Goncharenko-Khaider N, Rancourt C and Piche A. Ovarian cancer ascites protects from TRAIL-induced cell death through alphavbeta5 integrin-mediated focal adhesion kinase and Akt activation. Oncogene. 2010; 29(24):3519-3531.

29. Li J, Zhou J, Chen G, Wang H, Wang S, Xing H, Gao Q, Lu Y, He Y and Ma D. Inhibition of ovarian cancer metastasis by adeno-associated virus-mediated gene transfer of nm23H1 in an orthotopic implantation model. Cancer Gene Ther. 2006; 13(3):266-272.

30. Zhao H, Jhanwar-Uniyal M, Datta PK, Yemul S, Ho L, Khitrov G, Kupershmidt I, Pasinetti GM, Ray T, Athwal RS and Achary MP. Expression profile

of genes associated with antimetastatic gene: nm23-mediated metastasis inhibition in breast carcinoma cells. Int J Cancer. 2004; 109(1):65-70.

31. Isobe A, Sawada K, Kinose Y, Ohyagi-Hara C, Nakatsuka E, Makino H, Ogura T, Mizuno T, Suzuki N, Morii E, Nakamura K, Sawada I, Toda A, Hashimoto K, Mabuchi S, Ohta T, et al. Interleukin 6 receptor is an independent prognostic factor and a potential therapeutic target of ovarian cancer. PloS one. 2015; 10(2):e0118080.

32. Colomiere M, Ward AC, Riley C, Trenerry MK, Cameron-Smith D, Findlay J, Ackland L and Ahmed N. Cross talk of signals between EGFR and IL-6R through JAK2/STAT3 mediate epithelial-mesenchymal transition in ovarian carcinomas. Br J Cancer. 2009; 100(1):134-144.

33. Guo Y, Xu F, Lu T, Duan Z and Zhang Z. Interleukin-6 signaling pathway in targeted therapy for cancer. Cancer Treat Rev. 2012; 38(7):904-910.

34. Coward J, Kulbe H, Chakravarty P, Leader D, Vassileva V, Leinster DA, Thompson R, Schioppa T, Nemeth J, Vermeulen J, Singh N, Avril N, Cummings J, Rexhepaj E, Jirstrom K, Gallagher WM, et al. Interleukin-6 as a therapeutic target in human ovarian cancer. Clin Cancer Res. 2011; 17(18):6083-6096.

Chapter 3.

Cholesterol in malignant ascites enhances chemoresistance via LXRα/β in ovarian cancer cells

Abstract:

Resistance to chemotherapy is the major therapy failure in ovarian cancer treatment. Increased expression of ATP-binding cassette transporters (ABC transporter proteins), ATP-binding cassette sub-family G member 2 (ABCG2) and multidrug resistance protein 1 (MDR1) are one of the commonly known mechanism behind platinum-based drug resistance in ovarian cancer. Due to lack of symptoms, most ovarian cancer patients are diagnosed in late stage of the disease and the accumulation of fluid in the abdominal cavity are frequently encountered in clinic and are associated with poor prognosis. Here, we demonstrate that cholesterol pre-treatment, ovarian cancer cells became less sensitive to cisplatin (CDDP). Moreover, cholesterol pre-treatment increased ABC transporters expression via Liver x Receptor α/β (LXRα/β). To demonstrate relevance of our findings to ovarian cancer patients with ascites, we collected ascites from eight ovarian cancer patients and found cholesterol is elevated in ascites and promote ABC transporter protein expression. Collectively, our findings identify that cholesterol promote ovarian cancer multidrug resistance via increasing ABCG2 and MDR1 expression.

3.1 Introduction

Ovarian cancer is the most lethal cancer in women, due in part, to the asymptomatic nature of the disease, causing late diagnosis and development of resistance to chemotherapy in recurrent disease. Standard treatment of ovarian cancer includes, maximal cytoreductive surgery and adjuvant taxane and platinum based therapy. Initial response rate is high 70-80%, but majority of patients relapse within 2 years with subsequent resistance to chemotherapy [1, 2]. Response to cancer therapy has been hindered by the development of drug resistance in recurrent disease. Despite recent advances in our understanding of ovarian cancer, ovarian cancer survival remains poor and our understanding of ovarian cancer progression and chemoresistance is still very limited.

There is growing evidence indicating the importance of tumor microenvironment mediated chemoresistance mechanisms in ovarian cancer [3]. In particular, ascites forms a unique tumor microenvironment, which is present in almost all recurrent ovarian cancer patients [2]. Recent progress in deciphering the cellular and acellular components of ascites has shown that ascites serves as an important tumor-microenvironment enriched in pro-tumorigenic signals that contribute to chemoresistance in ovarian cancer [4]. Moreover, the presence of ascites correlates with poor prognosis [5, 6].

Previous studies suggested that cholesterol is significantly elevated in ascites and could be used as a marker for malignant ascites [7, 8]. Indeed, cholesterol have been shown to be involved in the regulation of drug response in a number of cancer models

[9, 10]. However, underlying molecular mechanism of acquired chemoresistance in ascites tumor microenvironment is poorly defined. Herein, we demonstrate that the cholesterol is enriched in ascites and reduces response to cisplatin (CDDP) in ovarian cancer cells via Liver x receptor α/β (LXRα/β) mediated induction of multidrug resistance protein expression including ABCG2 and MDR1 protein *in vitro*.

3.2 Materials and Methods

3.2.1 Cell culture, clinical samples and reagents

PA-1, OVCAR-3, and SKOV-3 used in this study were obtained from the American Type Culture Collection (Rockville, MD). With the exception of PA-1, these cell lines were grown in RPMI1640 (WelGENE, Seoul, Korea). PA-1 was cultured in MEM (WelGENE, Seoul Korea). All culture media were supplemented with 10% FBS (Gibco-BRL, Gaithersberg, MD), and 100 μg/mL penicillin-streptomycin (P/S) (Invitrogen, Carlsbard, CA).

Ascites from seven serous and one clear cell ovarian cancer patients were collected at the time of clinical intervention at the Seoul National University Hospital (Seoul, Korea). This study was approved by the Institutional Review Board (IRB) at Seoul national University Hospital (Registration number: 1409-1540-616), and prior written and informed consent was obtained from every patient. Ascites were centrifuged at 2500 rpm for 20 minutes. The acellular fractions were filtered (70 μm), aliquoted and stored at -80℃ to minimize freeze-thaw.

3.2.2 Cell viability assay

Cell viabilities were evaluated by the Thiazolyl blue tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plate. After overnight incubation, an increasing cisplatin concentration from 0 to 20 μM were adjusted to a final volume of 100

μl/well for indicated time. The cells were incubated with 50 μL MTT (2 mg/ml; 3 hr, 37℃) in 5% CO_2 in humidified atmosphere and subsequently solubilized in DMSO 100 μl/well. The optical density at 540 nm was determined using an enzyme linked immunosorbent assay reader.

3.2.3 Cell death analysis

Using flow cytometry analysis, apoptotic cell death was determined via Annexin-V and PI staining (BD Pharmingen, CA) according to the manufacturer's protocol.

3.2.4 Western Blotting

Protein lysates were prepared as described previously [11]. In brief, after cell extraction, proteins were separated by SDS/PAGE (6-15% gel, depending on specific protein assessed) followed by electrotransfer onto nitrocellulose membranes and probed with the indicated antibodies.

3.2.5 Reagents and Antibodies

Stock solutions of cisplatin (Enzo life science) were prepared in Dimethylformamide; 0.001% and water soluble cholesterol (Sigma-Aldrich, St. Louis, MO), prepared in DEPC water and used at the final concentration indicated.

MTT was from Amresco (Olon, OH). Antibodies to ABCG2, PARP, LXRα/β and Lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to MDR1, pan Cadherin were purchased from Cell Signaling (Danvers, MA). GAPDH (AB frontier) and α tubulin (Santa Cruz, CA) were used as a loading control.

3.2.6 Nuclear and cytoplasmic protein extraction

Using NE-PER nuclear and cytoplasmic extraction reagents from Pierce Biotechnology (Rockford, USA), cytoplasmic and nuclear proteins were separated and extracted from cancer cells, according to the manufacturer's protocol.

3.2.7 Ascites cholesterol quantitation

Ascites cholesterol level were quantitated using cholesterol quantitation kit (Sigma-Aldrich, St. Louis, MO). Ascites were diluted 5-10% to that of final volume, and the cholesterol level were quantitated according to the manufacturer's protocol.

3.2.8 Statistical Analysis

Data were presented as mean ± SEM of triplicate experiments. One-way ANOVA and, when appropriate, Student's t-test were used for statistical analyses. Significant

difference among experimental groups was analyzed by Scheffe's post hoc test. All analyses were conducted using IBM SPSS statistics 21 (SPSS Inc., Chicago, IL). P values of < 0.05 were considered statistically significant.

3.3 Results

3.3.1 Response of ovarian cancer cell lines to cisplatin is associated with ABC transporter protein expression

Basal CDDP response were measured in three ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3. Using MTT assay, treatment cisplatin reduced cell viability in all three ovarian cancer cell lines in a time and dose dependent manner (Fig. 1A). In ovarian cancer, ATP-binding cassette transporters (ABC transporter) proteins including ABCG2 and MDR1 are closely related with platinum-based drug resistance [12, 13]. Interestingly, the expression levels of these ABC transporter proteins were correlated with increased IC50 to CDDP in ovarian cancer cell lines (Fig. 1B and 1C).

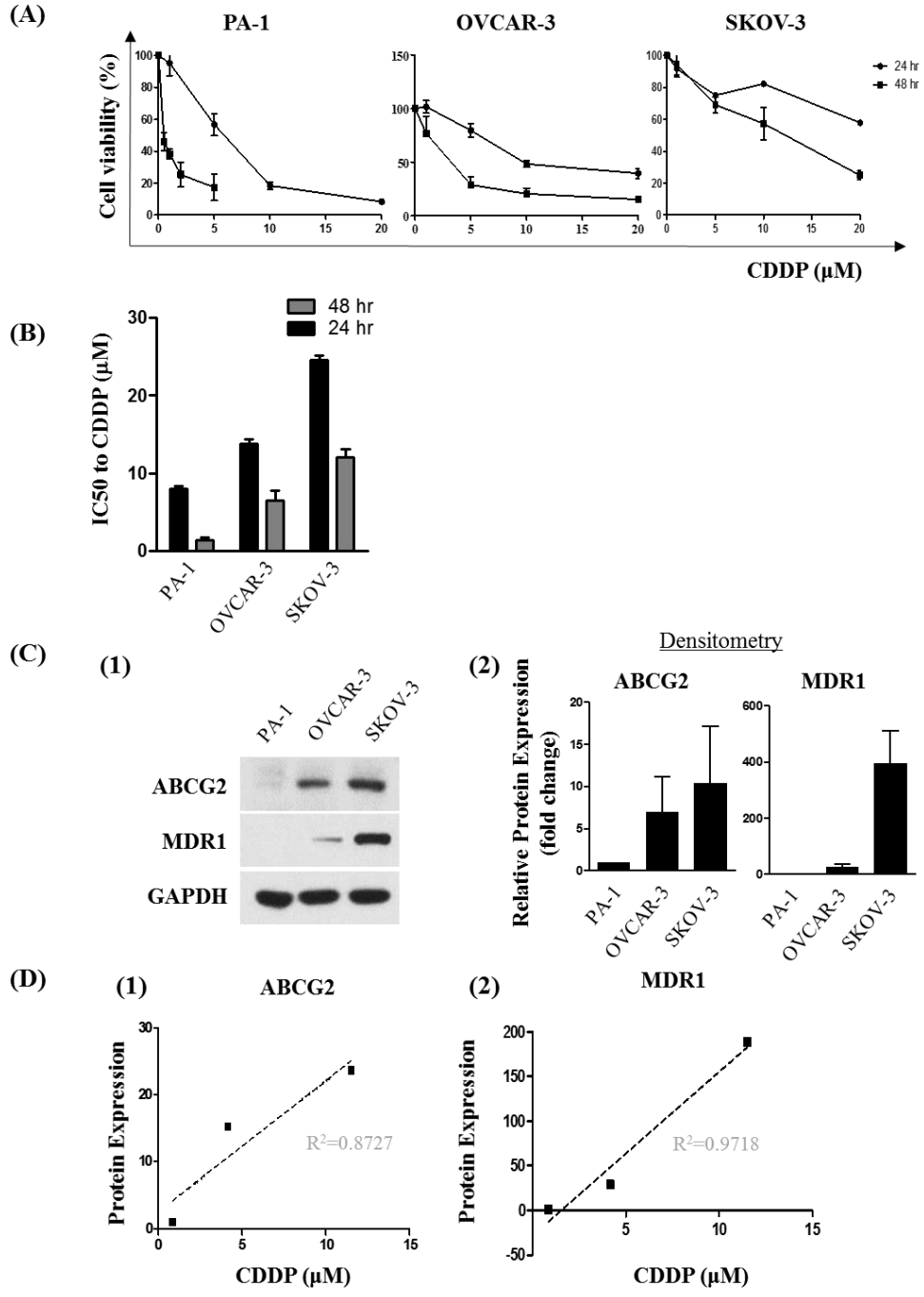

(A)
PA-1
OVCAR-3
SKOV-3
Cell viability (%)
24 hr
48 hr
CDDP (μM)
(B)
48 hr
24 hr
IC50 to CDDP (μM)
PA-1
OVCAR-3
SKOV-3
(C)
(1)
PA-1
OVCAR-3
SKOV-3
ABCG2
MDR1
GAPDH
(2)
Densitometry
ABCG2
MDR1
Relative Protein Expression (fold change)
PA-1
OVCAR-3
SKOV-3
(D)
(1)
ABCG2
Protein Expression
R²=0.8727
CDDP (μM)
(2)
MDR1
Protein Expression
R²=0.9718
CDDP (μM)

Fig. 1. CDDP sensitivity correlates with ABC transporter protein expression

(A) Effect of CDDP in the ovarin cancer cell viabilities. Ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3 were treated with an increasing CDDP concentration from 0 to 20 μM for 24 and 48 hr. Cell vibility were measured using MTT assay.

(B) IC50 to CDDP were calculated from Fig. 1A.

(C) The basal protein expressions of ABCG2 and MDR1 in ovarian cancer cell lines (1) and quantitated using densitometry (2).

(D) Correlation between IC50 to CDDP and ABC transporter protein expression from Fig. 1B and 1C. The correlation coefficient square (R2) was determined by Pearson's correlation coefficient test.

3.3.2 Cholesterol pre-treatment increase chemoresistance of ovarian cancer cells

Circulating free cholesterol levels are tightly regulated and cholesterol have been shown to be involved in the regulation of various membrane proteins, including ABCG2. Moreover, lowering cholesterol synthesis reduced ovarian cancer risk [14-16]. Therefore, we applied various concentration of cholesterol containing media (0, 5, 10, 20 μg/ml) and assessed the role of cholesterol on chemosensitivity in ovarian cancer cell *in vitro*. Ovarian cancer cells were pre-treated with an indicated cholesterol concentration for 24 hr and cell viability to CDDP were measured using MTT assay after another 48 hr. As shown in Fig. 2A, cholesterol treatment significantly reduced chemosensitivity to CDDP in PA-1 and SKOV-3 cell lines when these cell lines were pre-treated with cholesterol (5 and 10 μg/ml). For water solubility, cholesterol used in this study, is loaded with methyl-β-cyclodextrin (MβCD). An increasing concentration of MβCD containing media (0, 5, 10, 20 μg/ml) were tested for a possible toxic effect on cancer cell viability. As shown in Fig. 2B, MβCD alone has no additional effect on cell viability on all three ovarian cancer cell lines tested. Our data showed that response to CDDP depended upon the cholesterol levels in relatively chemosenstivity ovarian cancer cells.

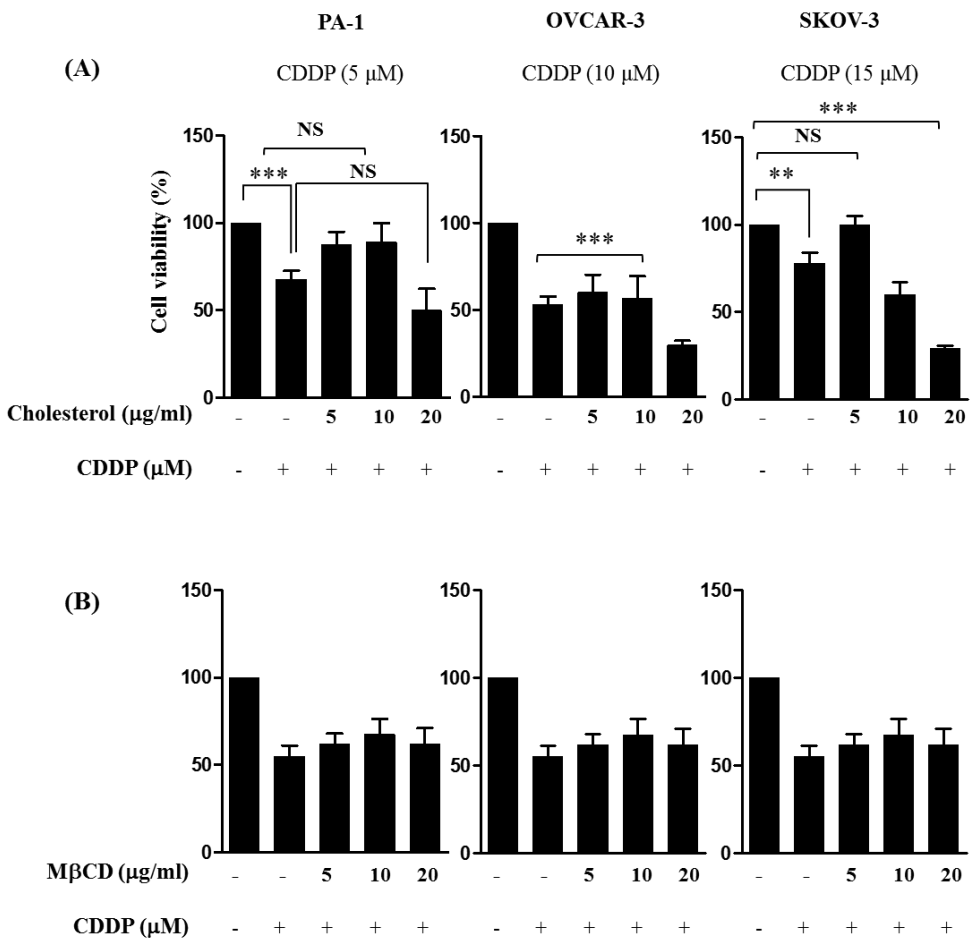

(A)
PA-1
OVCAR-3
SKOV-3
CDDP (5 μM)
CDDP (10 μM)
CDDP (15 μM)
Cell viability (%)
150
100
50
0
NS
***
NS
***
**
NS
***
Cholesterol (μg/ml)
- - 5 10 20 - - 5 10 20 - - 5 10 20
CDDP (μM)
- + + + + - + + + + - + + + +
(B)
MβCD (μg/ml)
- - 5 10 20 - - 5 10 20 - - 5 10 20
CDDP (μM)
- + + + + - + + + + - + + + +

Fig. 2. Cholesterol increase ovarian cancer cell viability

(A) Effect of cholesterol pre-treatment in the ovarian cancer cell viability. Ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3 were pre-treated with an increasing cholesterol concentration from 0 to 20 μg/ml for 24 and were treated with an indicated CDP concentration per cell line for 48 hr. Cell vibility were measured using MTT assay. ** and *** represent $P < 0.01$ and $P < 0.001$, respectively.

(B) Effect of loading control on ovarian cancer cell viability. Ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3 were pre-treated with an increasing concentration of methyl-β-cyclodextrin from 0 to 20 μM for 24 and were treated with an indicated CDDP concentration per cell line for 48 hr. Cell vibility were measured using MTT assay.

3.3.3 Cholesterol increase ABC transporter protein expression

In previous report, cholesterol increase resistance to platinum-based drugs via upregulating ABCG2 and also via modulating ABCG2 activity [9, 10]. As mentioned above, ABC transporter protein expression are associated with resistance to platinum-based drugs, we postulated that cholesterol will increase ABCG2 and MDR1 protein expression. When exposed to cholesterol containing media (5 and 10 μg/ml) IC50 at 48 hr to CDDP increased significantly in all three ovarian cancer cell lines (Fig. 3A). Associated with these findings, cholesterol treatment increased expression of both ABCG2 and MDR1 protein in PA-1 and SKOV-3 (Fig. 3B). Moreover, apoptotic cell death induced upon CDDP treatment were reversed when cells were pre-treated with cholesterol (5 μg/ml) for 24 hr prior to CDDP treatment (Fig. 3C). In line with apoptotic cell death reduction, PARP cleavage were also greatly reduced when cells were pre-treated with cholesterol (5 μg/ml) for 24 hr prior to CDDP treatment (Fig. 3D). For further evaluation of the role of cholesterol on the CDDP resistance, we used PA-1 cells.

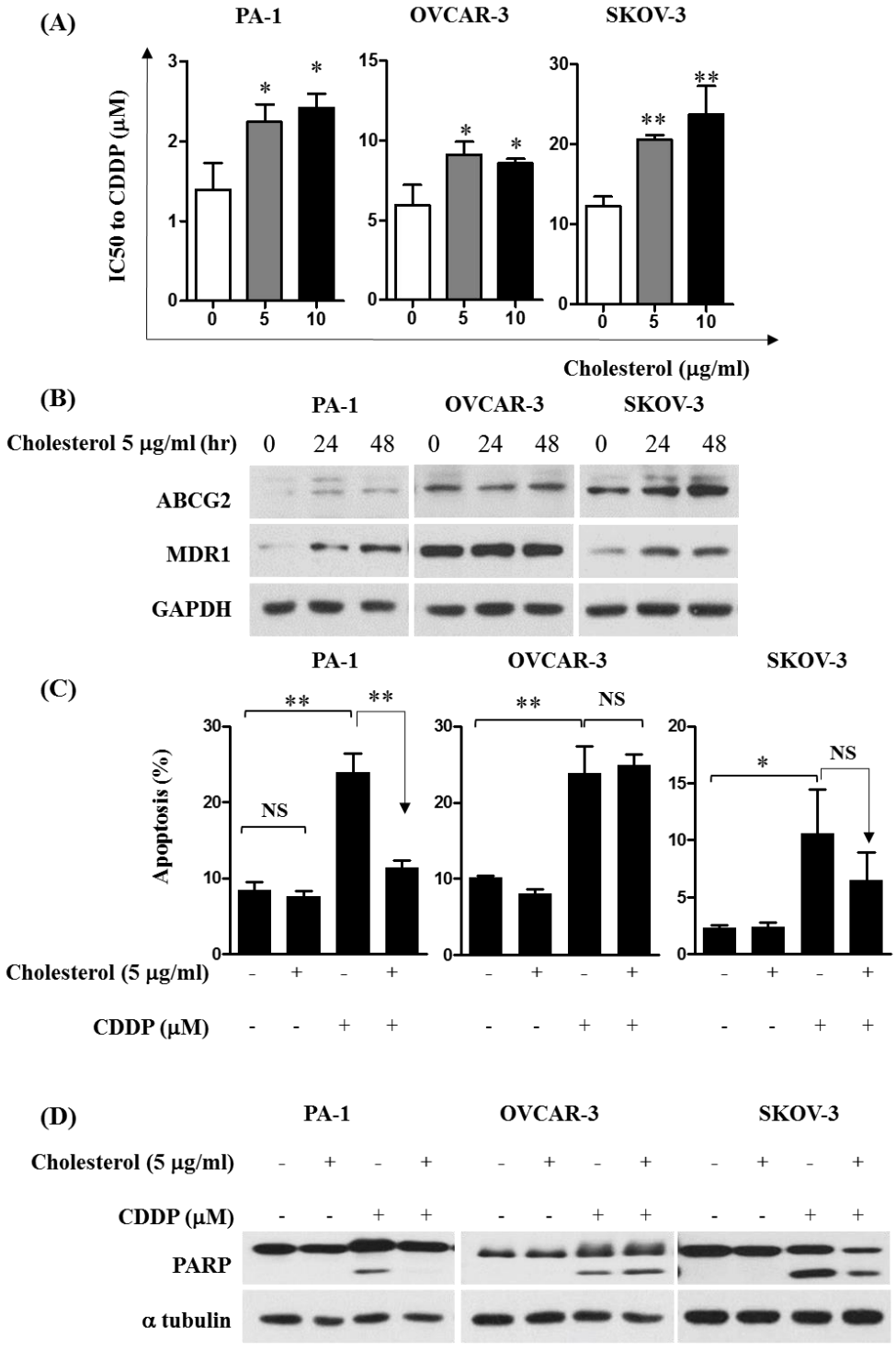

(A)
PA-1
OVCAR-3
SKOV-3
IC50 to CDDP (μM)
Cholesterol (μg/ml)
(B)
PA-1
OVCAR-3
SKOV-3
Cholesterol 5 μg/ml (hr)
0 24 48 0 24 48 0 24 48
ABCG2
MDR1
GAPDH
(C)
PA-1
OVCAR-3
SKOV-3
Apoptosis (%)
NS
Cholesterol (5 μg/ml)
CDDP (μM)
(D)
PA-1
OVCAR-3
SKOV-3
Cholesterol (5 μg/ml)
CDDP (μM)
PARP
α tubulin

Fig. 3. Cholesterol reduce ovarian cancer cell response to CDDP

(A) Effect of cholesterol pre-treatment on ovarian cancer cell IC50 to CDDP. Ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3 were pre-treated with an increasing cholesterol concentration from 0 to 10 μg/ml for 24 and were treated with an increasing CDDP concentration for 48 hr. IC50 to CDDP were calculated. * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

(B) Effect of cholesterol on ABC transporter protein expression. Ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3 were treated with cholesterol 5 μg/ml for 24 to 48 hr. ABC transporter protein expression were assessed by Western blotting.

(C) Suppression of CDDP-induced apoptosis by cholesterol pre-treatment. Ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3 were pre-treated with cholesterol 5 μg/ml for 24 hr and then treated with CDDP for 24 hr. Apoptotic cell death was analyzed by Annexin V/PI staining. * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

(D) Reduction of CDDP-induced PARP cleavage by cholesterol pre-treatment. Ovarian cancer cell lines, PA-1, OVCAR-3 and SKOV-3 were trated with cholesterol 5 μg/ml for 24 hr and were treated with CDDP for 24 hr. PAPR protein cleavage were assessed by Western blotting.

3.3.4 Cholesterol increase ABC transporters through sterol sensor

Cholesterol is an essential components of mammalian cell membranes, which generates a semipermeable barrier between cellular comparments and modulates the functions of membrane proteins [17]. Excess cellular cholesterol is toxic, in this sense, the circulating cholesterol levels are tightly regulated [18]. The liver x receptor (LXR) family, provide a feed-forward reglatory system for the elimination of excess cholesterol, positively regulating the expression of genes encoding lipid transport proteins [19]. Moreover, previous report suggest cholesterol as an eseential modulator of the ABCG2 and MDR1 protein transport functions [20]. Here, we evaluated whether cholesterol treatment affect membrane expression of ABC transporters. PA-1 cells were treated with cholesterol 5μg/ml for 24 hr, membrane expression of ABCG2 and MDR1 were analyzed by Western blot. As shown in Fig. 4A, membrane expression of both ABCG2 and MDR1 were significantly increased by cholesterol treatment. In Parallel with these events, the sterol sensor, LXRα/β regulating the homeostasis of cholesterol, expression were increased in nucleus fraction (Fig. 4B).

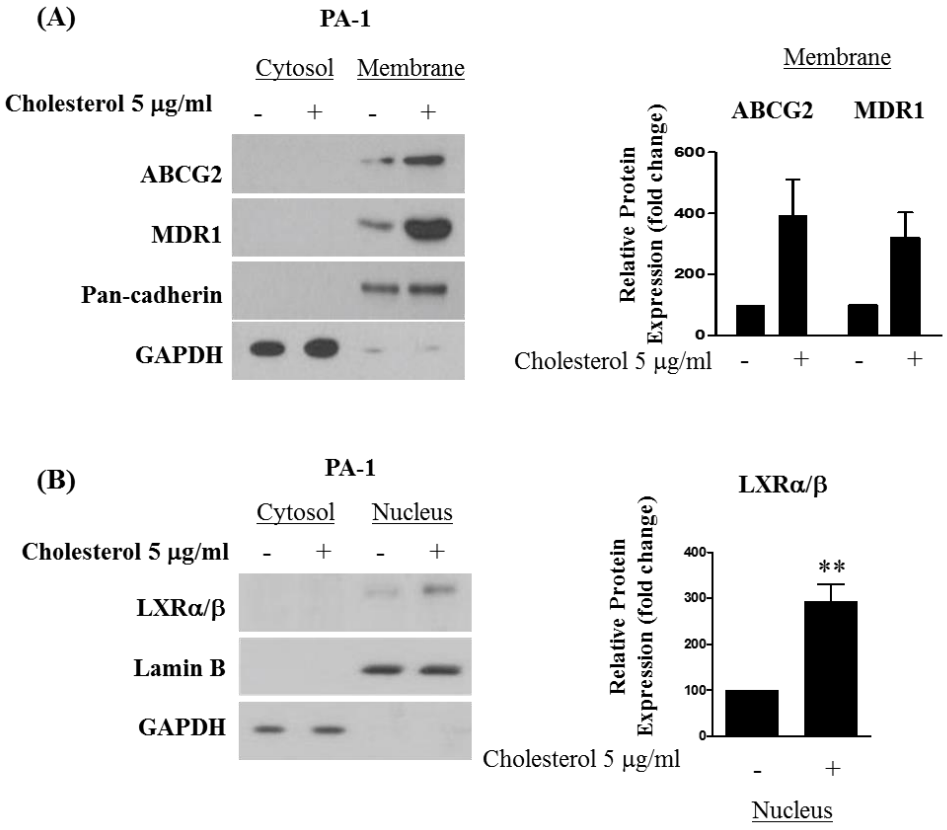

(A)
PA-1
Cytosol
Membrane
Cholesterol 5 μg/ml
- + - +
ABCG2
MDR1
Pan-cadherin
GAPDH
Membrane
ABCG2
MDR1
Relative Protein Expression (fold change)
600
400
200
0
Cholesterol 5 μg/ml - + - +
(B)
PA-1
Cytosol
Nucleus
Cholesterol 5 μg/ml
- + - +
LXRα/β
Lamin B
GAPDH
LXRα/β
Relative Protein Expression (fold change)
400
300
200
100
0
**
Cholesterol 5 μg/ml
- +
Nucleus

Fig. 4. Cholesterol increase membrane expression of ABC transporter proteins

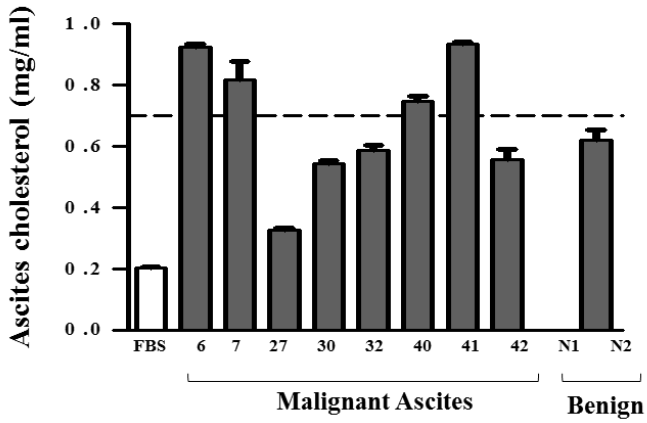
(A) Effect of cholesterol treatment on membrne expression of ABC transporter proteins. Ovarian cancer cell line, PA-1 was treated with cholesterol 5 μg/ml for 24 hr and cytosol and membrane protein were fractionated and protein expression were assessed by Western blotting.

(B) Effect of cholesterol treatment on liver x receptor expression. Ovarian cancer cell lines, PA-1, was treated with cholesterol 5 μg/ml for 24 hr and cytosol and nucler protein were fractionated and protein expression were assessed by Western blotting. ** represent $P < 0.01$.

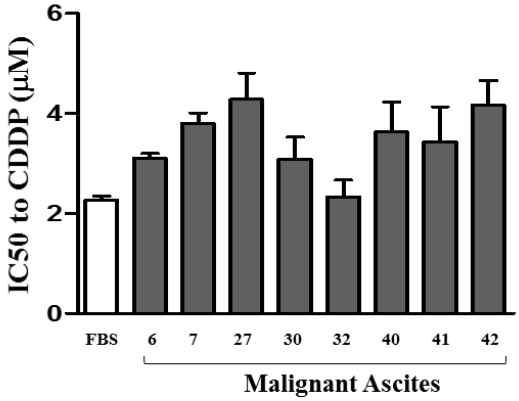
3.3.5 Ascites cholesterol increase chemoresistance of ovarian cancer cells

In previous report, cholesterol is enriched in ascites and ascites cholesterol have high diagnostic efficiency in the diagnosis of malignant ascites [7, 8]. Therefore, we postulated that cholesterol in ascites modulate response to CDDP, causing therapy failure in ovarian cancer patients with ascites. Eight ascites were collected from ovarian cancer patients and the ascites cholesterol levels were determined. As shown in Fig. 5A, cholesterol is elevated in ascites. Associated with these findings, ascites pre-treatment increased IC50 to CDDP at 48 hr in PA-1 cells (Fig. 5B). Moreover, ascites treatment increased both ABCG2 and MDR1 protein expression and LXRα/β expression (Fig. 5C). Taken together, these results indicated that cholesterol in ascites acted as an ABC transporters regulator via LXRα/β, thereby reducing response to CDDP in ovarian cancer cells.

(A)


Ascites cholesterol (mg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
FBS
6
7
27
30
32
40
41
42
N1
N2
Malignant Ascites
Benign

(B)


IC50 to CDDP (μM)
6
4
2
0
FBS
6
7
27
30
32
40
41
42
Malignant Ascites

(C)

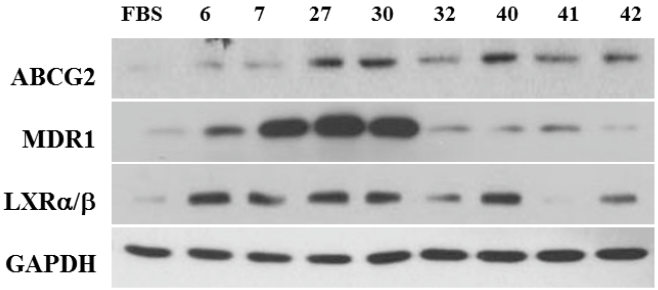

FBS
6
7
27
30
32
40
41
42
ABCG2
MDR1
LXRα/β
GAPDH

Fig. 5. Ascites reduce ovarian cancer cell response to CDDP

(A) Cholesterol level in ascites. The level of cholesterol in ascites were quantitated using cholesterol quantitation kit. FBS were used as control.

(B) Effect of ascites on ovarian cancer cell IC50 to CDDP. Ovarian cancer cell line, PA-1 were pre-treated with 10% ascites for 24 hr and were treated with an increasing CDDP concentration for 48 hr. IC50 to CDDP were calculated.

(C) Effect of ascites on ABC transporter protein and LXRα/β expression. Ovarian cancer cell lines, PA-1 were treated with 10% ascites for 24 hr. ABC transporter protein and LXRα/β were assessed by Western blotting.

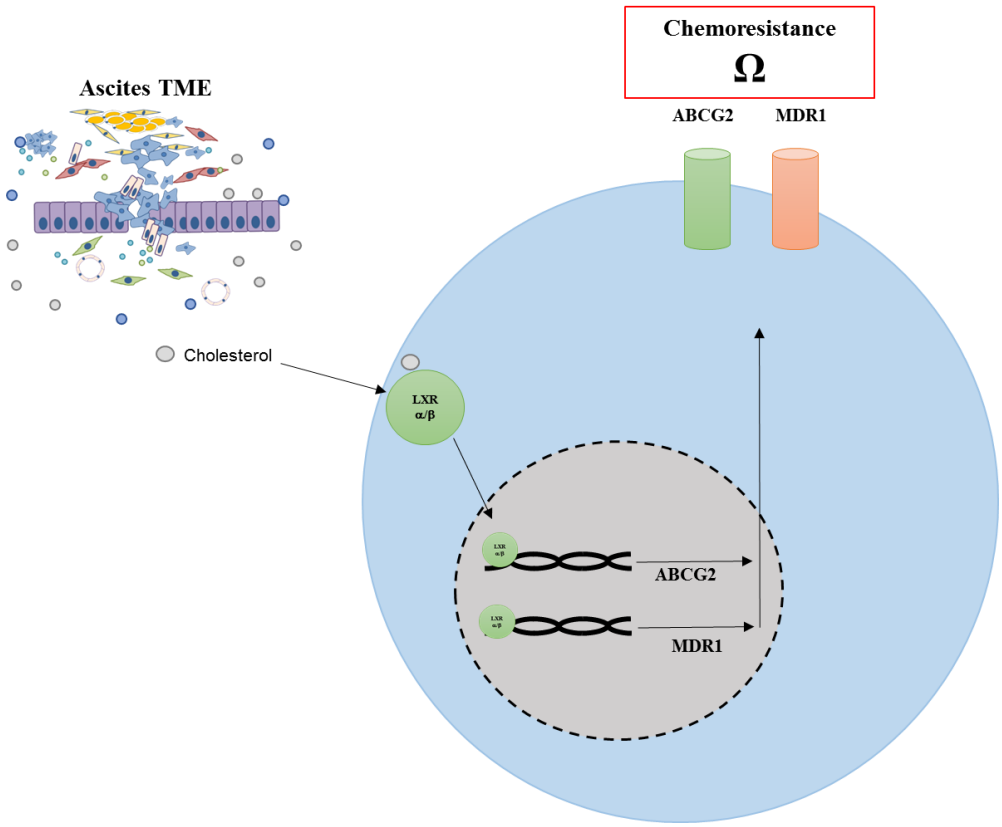


Fig. 6. Cholesterol in ascites tumor microenvironment promote chemoresistance via LXRα/β mediated ABCG2 and MDR1 expression in ovarian cancer cells

3.4 Discussion

We provide evidences that cholesterol acts as a key modulator of chemoresistance in ovarian cancer. In this study, we first demonstrated that cholesterol pre-treatment reduced CDDP response in ovarian cancer cells via increased expression of ABC transporters, ABCG2 and MDR1 (Fig. 3A and 3B), which are frequently reported drug resistant proteins in cancer [13]. Cholesterol pre-treatment increased expression of ABCG2 and MDR1 protein expression on cell membrane (Fig. 4A). Further, cholesterol modulate CDDP response were associated with LXRα/β expression in ovarian cancer cells (Fig. 4B).

Cholesterol, is a steroidal lipid which comprises around one third of the plasma membrane lipid content and is an essential component of membrane in animal cells [21, 22]. High levels of cholesterol can cause cytotoxicity, therefore is tightly regulated. Multiple mechanisms including regulated cholesterol uptake, synthesis and metabolism as well as efflux operate to maintain the correct intracellular cholesterol concentration [22]. Nevertheless, this regulation is dysregulated in cancer, potentially leading to multiple effect on tumor cell growth, apoptosis and sensitivity or resistance to chemotherapy [23]. Moreover, cancer cell membranes were found to be rich in cholesterol and cholesterol have been linked to reduced sensitivity to platinum-based chemotherapy in lung adenocarcinoma [10]. Recent studies in ovarian cancer also suggested that modulation of cholesterol synthesis via statins have a meaningful reduction in ovarian cancer risk [14-16, 24]. Additionally, intracellular cholesterol levels are tightly regulated under homeostasis by positively regulating the expression

of lipid transport proteins via LXR family of transcription factors [19]. Notably, these findings are consistent with our observation that high cholesterol treatment reduce chemosensitivity to CDDP via modulation of ABCG2 and MDR1 protein expression and increased nucleus expression of LXRα/β in ovarian cancer cells (Fig. 3 and Fig. 4).

Presence of ascites have been associated with reduced survival in ovarian cancer patients [25]. Recently, the link between ascites and chemoresistance in ovarian cancer were demonstrated in murine ovarian cancer cells [26] and elevated cholesterol in ascites were reported in recent metabolomics [8]. To date, no study has investigated a direct effect of cholesterol on ovarian cancer chemoresistance via ABCG2 and MDR1 protein expression, although each is identified as a potential risk factor in predicting poor prognosis for ovarian cancer patients. Our study is novel in suggesting that cholesterol alone is capable of driving the expression of these ABC transporters and confer chemoresistance in ovarian cancer cells. Specifically, we show that ascites is enriched with cholesterol, and these cholesterols are capable of inducing ABC transporter protein expression and thereby reducing sensitivity to CDDP in ovarian cancer cells (Fig. 5 and Fig. 6). Collectively, our studies underscore the importance of future studies investigating efficacy of modulating cholesterol levels in ovarian cancer patients with ascites, thus increasing chemotherapy efficacy.

3.5 References

1. Tapia G and Diaz-Padilla I. (2013). Molecular Mechanisms of Platinum Resistance in Ovarian Cancer.

2. Kipps E, Tan DS and Kaye SB. Meeting the challenge of ascites in ovarian cancer: new avenues for therapy and research. Nat Rev Cancer. 2013; 13(4):273-282.

3. Meads MB, Gatenby RA and Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. Nat Rev Cancer. 2009; 9(9):665-674.

4. Kim S, Kim B and Song YS. Ascites modulates cancer cell behavior, contributing to tumor heterogeneity in ovarian cancer. Cancer Sci. 2016; 107(9):1173-1178.

5. Ayhan A, Gultekin M, Taskiran C, Dursun P, Firat P, Bozdag G, Celik NY and Yuce K. Ascites and epithelial ovarian cancers: a reappraisal with respect to different aspects. Int J Gynecol Cancer. 2007; 17(1):68-75.

6. Lane D, Matte I, Rancourt C and Piche A. Prognostic significance of IL-6 and IL-8 ascites levels in ovarian cancer patients. BMC Cancer. 2011; 11:210.

7. Rana SV, Babu SG and Kocchar R. Usefulness of ascitic fluid cholesterol as a marker for malignant ascites. Med Sci Monit. 2005; 11(3):CR136-142.

8. Shender VO, Pavlyukov MS, Ziganshin RH, Arapidi GP, Kovalchuk SI, Anikanov NA, Altukhov IA, Alexeev DG, Butenko IO, Shavarda AL, Khomyakova EB, Evtushenko E, Ashrafyan LA, Antonova IB, Kuznetcov IN, Gorbachev AY, et al. Proteome-metabolome profiling of ovarian cancer ascites reveals novel

components involved in intercellular communication. Mol Cell Proteomics. 2014; 13(12):3558-3571.

9. Telbisz A, Muller M, Ozvegy-Laczka C, Homolya L, Szente L, Varadi A and Sarkadi B. Membrane cholesterol selectively modulates the activity of the human ABCG2 multidrug transporter. Biochim Biophys Acta. 2007; 1768(11):2698-2713.

10. Wu Y, Si R, Tang H, He Z, Zhu H, Wang L, Fan Y, Xia S, He Z and Wang Q. Cholesterol reduces the sensitivity to platinum-based chemotherapy via upregulating ABCG2 in lung adenocarcinoma. Biochem Biophys Res Commun. 2015; 457(4):614-620.

11. Kim SH, Kim SH, Kim YB, Jeon YT, Lee SC and Song YS. Genistein inhibits cell growth by modulating various mitogen-activated protein kinases and AKT in cervical cancer cells. Annals of the New York Academy of Sciences. 2009; 1171:495-500.

12. Gottesman MM, Fojo T and Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002; 2(1):48-58.

13. Fletcher JI, Haber M, Henderson MJ and Norris MD. ABC transporters in cancer: more than just drug efflux pumps. Nat Rev Cancer. 2010; 10(2):147-156.

14. Friedman GD, Flick ED, Udaltsova N, Chan J, Quesenberry CP, Jr. and Habel LA. Screening statins for possible carcinogenic risk: up to 9 years of follow-up of 361,859 recipients. Pharmacoepidemiol Drug Saf. 2008; 17(1):27-36.

15. Liu Y, Qin A, Li T, Qin X and Li S. Effect of statin on risk of gynecologic cancers: a meta-analysis of observational studies and randomized controlled trials. Gynecol Oncol. 2014; 133(3):647-655.

16. Stine JE, Guo H, Sheng X, Han X, Schointuch MN, Gilliam TP, Gehrig PA, Zhou C and Bae-Jump VL. The HMG-CoA reductase inhibitor, simvastatin, exhibits anti-metastatic and anti-tumorigenic effects in ovarian cancer. Oncotarget. 2016; 7(1):946-960.

17. Ikonen E. Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol. 2008; 9(2):125-138.

18. Cruz PM, Mo H, McConathy WJ, Sabnis N and Lacko AG. The role of cholesterol metabolism and cholesterol transport in carcinogenesis: a review of scientific findings, relevant to future cancer therapeutics. Front Pharmacol. 2013; 4:119.

19. Spann NJ and Glass CK. Sterols and oxysterols in immune cell function. Nat Immunol. 2013; 14(9):893-900.

20. Hegedus C, Telbisz A, Hegedus T, Sarkadi B and Ozvegy-Laczka C. Lipid regulation of the ABCB1 and ABCG2 multidrug transporters. Adv Cancer Res. 2015; 125:97-137.

21. Simons K and Vaz WL. Model systems, lipid rafts, and cell membranes. Annu Rev Biophys Biomol Struct. 2004; 33:269-295.

22. Simons K and Ikonen E. How cells handle cholesterol. Science. 2000; 290(5497):1721-1726.

23. Pelton K, Freeman MR and Solomon KR. Cholesterol and prostate cancer. Curr Opin Pharmacol. 2012; 12(6):751-759.

24. Yu O, Boudreau DM, Buist DS and Miglioretti DL. Statin use and female reproductive organ cancer risk in a large population-based setting. Cancer Causes Control. 2009; 20(5):609-616.

25. Huang H, Li YJ, Lan CY, Huang QD, Feng YL, Huang YW and Liu JH. Clinical significance of ascites in epithelial ovarian cancer. Neoplasma. 2013; 60(5):546-552.

26. Mo L, Pospichalova V, Huang Z, Murphy SK, Payne S, Wang F, Kennedy M, Cianciolo GJ, Bryja V, Pizzo SV and Bachelder RE. Ascites Increases Expression/Function of Multidrug Resistance Proteins in Ovarian Cancer Cells. PLoS One. 2015; 10(7):e0131579.

Chapter 4. Conclusion

4.1 Conclusion

Ascites, which is a readily accessible source of primary cancer cells and cancer-associated secreted bio-active factors, are underutilized. Although presence of ascites correlates with a poor prognosis in ovarian cancer patients, its association with chemoresistance is poorly understood. Further studies are needed to highlight both genetic and responsive heterogeneity and identify chemoresistance mechanisms in ovarian cancer. The contents of ascites may reflect the molecular signatures of the underlying disease, and ascites potentially harbor both diagnostic and prognostic factors, which can be used in biomarker discovery studies. The current stage of the ascites research field provides a foundation for future experiments examining larger numbers of patient samples for validating suggested markers, presenting significant technical challenges. A more detailed understanding of the relative contribution of ascites-regulated molecules on subsets of ovarian cancer cells will increase the understanding of ovarian cancer biology and will result in improved treatment. In conclusion, we summarized the functional roles of ascites in the progression of EOC and provide a new perspective regarding tumor heterogeneity.

4.2 Therapeutic implication of targeting tumor microenvironment

Ascites is one of the valuable resources highly attractive for biomarker discovery studies. Because it is easy to obtain abundant tumor-derived soluble factors as a promising biologic source. As opposed to serum, ascites being a proximal fluid might reflect events in early stage of ovarian cancer progression. Moreover, the concentration of cancer associated soluble factors is usually much higher in ascites than in serum [1]. Thus, the malignant ascites could be a promising biospecimen for investigating diagnostic, therapeutic, as well as prognostic markers.

4.2.1 Utility of ascites as a diagnostic factor.

The presence of ascites is not limited to malignant ovarian cancer, but also ascites is often present in patients with benign ovarian epithelial tumors. The clinical management of ascites associated with malignant tumors is quite different from those of benign lesions. However, it remains difficult in the clinic to differentiate benign and malignant ascites, particularly in the early diagnosis of malignant ascites [2]. Currently, cytological detection of ascites has become a gold standard for confirming malignant ascites [3]. This detection shows high specificity, but its sensitivity is low, which can easily result in misdiagnoses and repeated tests after multiple ascites collections, leading to delays in providing optimal therapeutic options and the increased discomfort from abdominocentesis [2]. Our group previously suggested

that a smaller amount of ascites may have good prognosis for the overall survival of patients with ovarian cancer [4]. Moreover, its components share profiles of possible, diagnostic, and prognostic factors. A number of soluble factors are present in abundance in ovarian cancer patient-derived ascites, but few have been validated for their biomarker potential; these are shown in Table 1

Several laboratory indexes have been reported, such as VEGF, matrix metalloproteinase, DNA heteroploid, and human leukocyte antigen system-G, which have some value in diagnosing malignant ascites; however, their applications are limited under clinical settings because of their complicated inspection techniques, including their difficult operation and high cost [5-8]. These reported tumor markers have some diagnostic sensitivity and specificity for diagnosing malignant ascites, but the diagnostic value of each index differs greatly in malignant ascites induced by different causes because of the complex etiology of malignant ascites [9]. It is thought that no identified tumor markers show high sensitivity and specificity in malignant ascites induced by all causes. Combined detection of tumor markers in the serum and ascites may improve their diagnostic value. Moreover, a recent study by Zhu et al. explored the values of tumor markers in the serum and ascites for identifying and diagnosing malignant ascites by analyzing the clinical data of patients diagnosed with ascites [2]; their findings suggested that compared to a single index, combined detection of tumor markers in the serum and ascites will significantly improve diagnostic sensitivity and specificity[2]. However, tumor markers that accurately identify malignant ovarian tumors are required for optimal patient management.

4.2.2 Personalized therapy

Personalized therapies have been added to the treatment strategy of malignant ascites. Several lines of targeted drugs have improved progression-free survival in some patients with ovarian cancer. The presence of ascites in women with advanced ovarian cancer may predict the population of women more likely to derive a long-term benefit from bevacizumab, an anti-angiogenic therapy [10]. Intra-peritoneal infusion of catumaxomab, a bispecific monoclonal antibody (anti-EpCAM and anti-CD3) activates immune cells, despite the prevailing immunosuppressive environment of malignant ascites [11]. The relative excess of CD8+ T cells in ascites is reportedly associated with significantly improved overall survival of ovarian cancer patients [12]. Moreover, integrated analysis for the soluble components in ascites may provide a powerful platform for discovering indicators of pathological processes or pharmacologic responses to a therapeutic intervention, leading to development of precision medicine [13, 14]. These reports highlight the potential use of ascites constituents in diagnostic and prognostic marker screening in ovarian cancer, and an increased understanding of ascites.

4.3 References

1. Giuntoli RL, 2nd, Webb TJ, Zoso A, Rogers O, Diaz-Montes TP, Bristow RE and Oelke M. Ovarian cancer-associated ascites demonstrates altered immune environment: implications for antitumor immunity. Anticancer Res. 2009; 29(8):2875-2884.
2. Zhu FL, Ling AS, Wei Q, Ma J and Lu G. Tumor markers in serum and ascites in the diagnosis of benign and malignant ascites. Asian Pac J Cancer Prev. 2015; 16(2):719-722.
3. Liu F, Kong X, Dou Q, Ye J, Xu D, Shang H, Xu K and Song Y. Evaluation of tumor markers for the differential diagnosis of benign and malignant ascites. Ann Hepatol. 2014; 13(3):357-363.
4. Kim HS, Kim JW, Chung HH, Park NH, Song YS and Kang SB. Disease confined within the ovary and smaller amount of ascites are good prognostic factors for survival of patients with squamous cell carcinoma arising from mature cystic teratoma of the ovary: a case series in Korea and review of the published reports. J Obstet Gynaecol Res. 2009; 35(1):99-105.
5. Chen Y, Liang B, Zhao YJ, Wang SC, Fan YB and Wu GP. Transcription expression and clinical significance of vascular endothelial growth factor mRNA and endostatin mRNA in pleural effusions of patients with lung cancer. Diagn Cytopathol. 2012; 40(4):287-291.
6. Cheng D, Liang B and Kong H. Clinical significance of vascular endothelial growth factor and endostatin levels in the differential diagnosis of malignant and benign ascites. Med Oncol. 2012; 29(2):1397-1402.
7. Wu KL, Chen HW, Lin SH and Chen JS. Intractable ascites, changes in serum vascular endothelial growth factor levels and thalidomide treatment in POEMS syndrome. Am J Med Sci. 2014; 347(6):510-512.
8. Zhang Y, Yu LK and Xia N. Evaluation of serum and pleural levels of endostatin and vascular epithelial growth factor in lung cancer patients with pleural effusion. Asian Pac J Trop Med. 2012; 5(3):239-242.

9. Wang XF, Wu YH, Wang MS and Wang YS. CEA, AFP, CA125, CA153 and CA199 in malignant pleural effusions predict the cause. Asian Pac J Cancer Prev. 2014; 15(1):363-368.

10. Ferriss JS, Java JJ, Bookman MA, Fleming GF, Monk BJ, Walker JL, Homesley HD, Fowler J, Greer BE, Boente MP and Burger RA. Ascites predicts treatment benefit of bevacizumab in front-line therapy of advanced epithelial ovarian, fallopian tube and peritoneal cancers: An NRG Oncology/GOG study. Gynecol Oncol. 2015; 139(1):17-22.

11. Fossati M, Buzzonetti A, Monego G, Catzola V, Scambia G, Fattorossi A and Battaglia A. Immunological changes in the ascites of cancer patients after intraperitoneal administration of the bispecific antibody catumaxomab (anti-EpCAMxanti-CD3). Gynecol Oncol. 2015; 138(2):343-351.

12. Markowska J, Lacki JK, Jaroszewski J and Wiktorowicz K. The usefulness of CD4/CD8 ratio evaluation in monitoring of ovarian cancer patients. Eur J Gynaecol Oncol. 1995; 16(1):54-58.

13. Seeber A, Martowicz A, Spizzo G, Buratti T, Obrist P, Fong D, Gastl G and Untergasser G. Soluble EpCAM levels in ascites correlate with positive cytology and neutralize catumaxomab activity in vitro. BMC Cancer. 2015; 15:372.

14. Liu C and Yang F. Akt2/ZEB2 may be a biomarker for exfoliant cells in ascitic fluid in advanced grades of serous ovarian carcinoma. Tumour Biol. 2015.

국문 초록

난소 암은 여성 암 중 가장 치명적인 암 종으로, 질병 자체가 증상이 없어 대부분의 환자가 말기에 진단되고, 빈번한 재발과 항암제 저항성 때문에 낮은 생존율을 보이는 심각한 질환이다.

다른 암 종과 마찬가지로 난소 암에 있어서도 종양 미세환경에 대한 연구가 점차 증가하고 있는 추세이다. 많은 암 중 특히 난소 암에서 복강 내에 과도하게 축적 되는 체액 성 물질인 복수가 차는 현상이 가장 빈번하게 일어나고, 재발성 난소 암 환자의 경우 대부분이 복수가 차고 그에 의한 복강 내 압박 증상을 통해 식욕 부진, 구역질, 복강 내 출혈, 복통 등의 여러 증상을 동반하게 되어 환자의 삶의 질에 영향을 미치게 된다. 복수를 이루고 있는 성분은 단순 체액 뿐만 아니라 여러 종류의 세포들과 비 세포 적인 구성요소들이 확인 되어 있으며, 난소 암에 있어 복수의 존재 여부는 전이 및 불량한 예후와 관련 있는 것으로 보고 된바 있다. 하지만, 많은 연구에도 불구하고 현재 복수에 역할에 대한 깊은 이해가 부족하고 관련 약제 개발이 더뎌지고 있는 실정이다. 난소 암의 치료 방법을 개선하기 위해서는 복수의 생물학적 특성이 암 전이 및 항암제 저항성에 대한 근본적인 원인 이해가 필요하다.

본 연구에서는 난소 암 환자 유래 복수가 염증 성 종양미세환경을

형성 함으로써, 암세포의 전이를 촉진 하는 것을 확인하였다. 염증 성 cytokine중 IL-6가 기존 보고보다 높은 레벨로 복수내 존재하는 것을 확인하였고, 중요성을 확인하기위해, neutralizing antibody를 사용하였다. 복수내 존재하는 IL-6는 정상 난소 표피세포주에는 아무런 영향을 미치지 못하였고, JAK2-STAT3 signaling를 매개로 하여 암세포의 전이를 유도함을 확인하였다. 더 나아가서, 복수가 모든 난소 암 세포주의 전이를 유도하지 못하는 것을 확인하였다. 흥미롭게도 복수내 존재하는 IL-6는 IL-6R를 세포막에 발현하는 세포주를 선택적으로 JAK2-STAT3 signaling을 활성화 시켰고, 이에 따른 암 세포 전이가 증가하는 것을 확인하였다. 이러한 연구결과를 종합해볼 때, 난소 암 환자 유래 복수는 염증 성 종양미세환경을 형성함으로써, IL-6R를 세포막에 발현하고 있는 난소 암 세포의 선택적 이동을 증가시킴을 알 수 있다. 비세포 적인 복수 구성요소가 암 세포의 전이에 미치는 분자적 기전을 이해 함으로써, 향후 난소 암 치료제로서 큰 효과를 기대해 볼 수 있을 것이라 사료된다.

항암제 저항성을 극복하기 위한 시도로 암세포의 미세 환경을 조절하려는 노력이 이루어 지고 있다. 난소암환자에 있어서도 복수의 존재 유무가 항암제 반응성과 깊은 연관성이 있다고 제안되고 있다. 최근 연구에서는 콜레스테롤이 항암제 저항성과 상관관계가 있다고 보고 되고 있으며, 난소 암 환자 유래 복수 내에도 콜레스테롤이 높게 존재한다고

보고 된다 있다. 하지만 관련 분자적 기전에 대한 연구는 미미한 실정이다. 본 연구에서는 복수 내 존재하는 콜레스테롤이 난소 암의 항암제 저항성과 연관성이 있는 ATP-binding cassette transporters (ABC transporters)의 활성 및 세포막 이동을 증가 시킴으로써 항암제 저항성을 유도함을 확인하였다. 콜레스테롤 레벨 조절이 ABC transporters의 발현을 감소시킴을 확인하였다. 이 현상과 더불어 난소 암의 표준 치료제인 cisplatin에 대한 저항성을 증가시키는 것을 확인하였다. 과도한 세포내 콜레스테롤 축적을 막기위해, 유출 시스템인 liver x receptor (LXR)의 활성을 유도함으로써, ABC transporter의 발현에 기여함을 확인하였다. 연구 결과를 종합하여 볼 때, 복수내 높은 레벨로 존재하는 IL-6 조절이 암세포의 전이를 억제 할 수 있는 치료법으로 제안 될 수 있고, 콜레스테롤 레벨 조절이 항암제 저항성을 극복 할 수 있는 대안이 될 것이라 기대된다.