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Interleukin-33 predicts poor prognosis and promotes ovarian cancer cell growth and metastasis through regulating ERK and JNK signaling pathways

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Abstract

Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer. Patients are often diagnosed with advanced stage EOC which has a high chance of cancer recurrence and metastasis and thus with a poor survival rate. It remains a huge challenge to understand the cellular and molecular mechanisms of the aggressive behaviour of EOC cells. In this study we investigated the role of an immunomodulatory cytokine IL-33 and its receptor ST2 in mediating the growth and metastasis of EOC, and the association of their expression with disease prognosis. Our data show that both IL-33 and ST2 were highly up-regulated in EOC tumour tissues compared with normal ovary and ovarian benign tumours, and the expression levels were further increased in tumour tissues at the metastatic site compared with the primary site. The expression levels of IL-33 and ST2 were also positively correlated with the expression of Ki-67, and negatively correlated with the survival time of EOC patients. Using EOC cell lines, we observed that cells knocked down of IL-33 gene by siRNA had reduced migratory and invasive potential, while full length human IL-33 (fl-hIL-33) promoted the invasive, migratory and proliferative capacity of EOC cells and this process could be inhibited by IL-33 decoy receptor sST2. Signaling pathway analysis suggested that IL-33 increased the phosphorylation of ERK and JNK which was blocked by sST2. Fl-hIL-33-induced increases in EOC cell migration, invasive potential and proliferation were specifically abrogated by treatment with the ERK inhibitor U0126 while JNK inhibitor SP600125 only disrupted IL-33-induced enhancement of EOC cell viability. Taken together, our data suggest that IL-33/ST2 axis closely associates with poor prognosis of EOC patient survival time, and it promotes ovarian cancer growth and metastasis through regulating ERK and JNK signaling pathways. Thus IL-33 and/or ST2 might be potential prognosis markers and therapeutic targets for EOC patients.

Key words: fl-hIL-33, ovarian cancer, metastasis, survival, invasion

Abbreviations:

EOC: Epithelial ovarian cancer (EOC)

IL-33: interleukin-33

fl-hIL-33: full length human IL-33

sST2: soluble ST2

DC: dendritic cells

NK: natural killer cells

Highlights:

- Increased IL-33/ST2 expression in EOC tumours, particularly EOC metastatic tumours
- Expression levels of IL-33/ST2 negatively correlate with patient survival time
- Fl-hIL-33 promotes the invasive, migratory and proliferative capacity of EOC cells
- Fl-hIL-33 promotes ECO growth and metastasis through ERK and JNK signaling pathways

1. Introduction

Ovarian cancers are a group of heterogeneous, rapidly progressing gynaecological malignancies with high rate of mortality in women. Epithelial ovarian cancer (EOC) is the most lethal ovarian cancer accounting for 80 to 90% of all cases, with serous ovarian carcinoma as the most common and most aggressive subtype of EOC (Bell, 2005; Kurian et al., 2005; Marquez et al., 2005). Despite recent advances in surgical resections and systemic chemotherapies, the prognosis of EOC remains poor and the 5-year survival rate is only approximately 30% after initial diagnosis (Armstrong, 2002; Jemal et al., 2009; McGuire et al., 1996). The main reason for the poor survival rate is that currently there are no effective screening methods for early symptoms of EOC which are often silent. 70% of patients are first diagnosed with advanced stage of the disease with metastasis, which remains the leading cause of cancer relapse and ultimately death from EOC (Goff et al., 2000; Heintz et al., 2006; Stirling et al., 2005). Thus there is an urgent need to investigate the molecular mechanisms associated with the aggressive growth and metastatic ability of ovarian cancer.

IL-33 is a member of the IL-1 cytokine family, originally described as a nuclear protein in high endothelial venules (Baekkevold et al., 2003). IL-33 is expressed in many organs and cells (Schimitz et al., 2005), it induces synthesis of various chemokines and cytokines such as IL-5 and IL-13 via binding to its heterodimeric receptor ST2 and IL-1R accessory protein (Liew, 2012). ST2 exists in two forms due to the alternative splicing: a transmembrane full-length form (ST2L) and a soluble, secreted form (sST2) acting as a decoy receptor (Oshikawa et al., 2002). ST2 is expressed by various immune cells including dendritic cells (DCs), Th2 cells, mast cells and natural killer (NK) cells (Lohning et al., 1998; Moritz et al., 1998; Xu et al., 1998), as well as tissue specific cells such as endothelial cells and decidual stromal cells (Hu et al.,

2014; Zeyda et al., 2013).

Since the discovery of IL-33 in 2005 (Schmitz et al., 2005), numerous studies have suggested that IL-33 plays diverse but important roles in immune mediated diseases such as infection, allergy and autoimmune diseases (Liew, 2012). Interestingly, a few recent studies in cancer indicate a potential role of IL-33 in modulating anti-tumour immunity and tumour growth. Elevated levels of IL-33 have been detected in the serum samples of gastric cancer (Sun et al., 2011), non-small cell lung cancer (Hu et al., 2013) and breast cancer patients (Liu et al., 2014), while the serum levels of sST2 were reported to be associated with worse prognostic factors in hepatocellular (Bergis et al., 2013) and breast cancer (Lu et al., 2014), suggesting IL-33/ST2 signaling pathway is likely to be involved in the development of a variety of cancers. However, the function of IL-33 in tumour progression is less clear. While transgenic expression of IL-33 in mice activated NK and CD8 T cells and inhibited tumour growth and metastasis in melanoma and lung carcinoma animal models (Gao k., et al., 2013; Gao X., et al., 2015), induced IL-33 expression enhanced the tumourigenic activity of rat glioma cells (Fang et al., 2014). In addition, the IL-33/ST2 axis promoted mouse mammary carcinoma growth and metastases by facilitating intratumoural accumulation of immunosuppressive cells (Jovanovic et al., 2011 and 2014). Thus despite convincing evidence suggesting a role for IL-33/ST2 in cancer growth and progression, its exact function and more importantly the underlying mechanisms in tumour development remain unclear.

In this study we focused on the role of IL-33/ST2 in the development of EOC and examined the underlying mechanism of its function. We evaluated the correlation of the expression levels of IL-33 and ST2 in dissected tumour tissues with the survival

time of these EOC patients. Furthermore, we investigated the function of IL-33/ST2 axis in EOC cell growth, migration and invasion and the important signaling pathways. To our knowledge, our study is the first to demonstrate that IL-33 and/or ST2 may be a potential prognosis markers for EOC patients, and IL-33/ST2 axis plays a key role in the control of EOC growth and metastasis.

2. Materials and methods

2.1. Patients

A total of 192 specimens were used for this study. 20 normal ovary tissues (hysterectomy specimens with non-ovarian disease), 20 benign ovarian tumours and 152 EOC samples (88 serous cystadenocarcinoma tumours, 16 mucinous cystadenocarcinoma tumours, 3 endometrioid carcinoma tumours, 5 clear cell carcinoma tumours, 2 undifferentiated carcinoma tumours, together with 38 metastatic lesions in the pelvic and peritoneal cavities) were obtained from the Department of Gynecology, the First Affiliated Hospital of China Medical University between 2004 and 2010. All patients were clinically staged according to the International Federation of Gynecology and Obstetrics (FIGO) system. The cases were selected based on the availability of the resection tissues, clinical follow-up data and no history of preoperative radiation or chemotherapy. Ages of the 114 patients ranged from 26 to 76 years (mean age, 53 years) and the average duration of follow-up was 45.2 months (range from 1 to 110 months). Two pathologists separately assessed the specimens to document primary tumour diagnosis as well as the presence of metastases in the pelvic and peritoneal cavity, tumours were pathologically graded according to the Silverberg grading system. The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of China Medical University in accordance with the Helsinki Declaration.

2.2 Cell lines

Human EOC cell lines HO8910, CAOV3 and SKOV3-DDP were obtained from Cell Resource Center, Chinese Academy of Medical Sciences, Beijing. CAOV3 was cultured in DMEM with high glucose supplement (Sigma), HO8910 and SKOV3-DDP were cultured in RPMI 1640 (GIBCO). Both media were supplemented with 2mM

L-glutamine, penicillin-streptomycin (100U/ml) and 10% FBS and cells were cultured at the standard culture conditions.

2.3 IL-33 siRNA transfection

EOC cell lines were transfected with IL-33 or scramble siRNAs according to the manufacturer's instruction (OriGene Technologies, USA). Briefly cells were plated in 6-well plates (3×10^5/well) and allowed to reach 50% confluence for transfection. 7.5μl of Lipofectamine 3000 (Life Technology) and 1μl of IL-33 or scramble siRNAs were separately pre-incubated in 125μl of Opti-MEM culture medium (Life Technology) for 5mins. The two solutions were then mixed and incubated at room temperature for 5mins. The mixture was then added to the cells pre-incubated in 1750μl of fresh culture medium. After 48h, the cells were harvested and transfection efficiency was evaluated using western blot analysis.

2.4 Cell viability and proliferation assay

Thiazolyl Blue Tetrazolium Bromide (MTT) assay is commonly used to measure cell metabolic activities, thus a composite readout of cell viability and proliferation. EOC cells in a 96-well plate (5×10^3/well) were starved of serum for 16h. Cells were then incubated in the absence or presence of 10ng/ml full length human IL-33 (fl-hIL-33, provided by Professor Mu, Peking University. Briefly, fl-Hil-33 was expressed in *Escherichia Coli* and purified by Ni-NTA affinity chromatogaraphy, endotoxin was removed and the levels were confirmed to be <0.1 unit/μg of protein. The primary structure of fl-hIL-33 was confirmed by sequencing). 200ng/ml sST2 (R&D Systems), 2μmol/L U0126 (Sigma) or 10μmol/L SP600125 (Sigma) for various time periods in different experiments. 20μl of MTT solution (5mg/ml in PBS, Sigma) was then added into each well and cells were incubated for a further 3h. The medium was removed

and 100µl/well of dimethyl sulfoxide (DMSO) (Sigma) was added, the plate was gently rotated for 10mins before the absorbance was measured at 570nm.

2.5 Wound healing assay

EOC cells were seeded into six-well plates (3×10^5/well). When cells reached 70-80% confluence, complete medium was replaced with medium containing 1% FBS and cultured for a further 16h. The cultures were then scratched using a 200µl pipette tip across the cell monolayer and cells were gently washed with warm PBS and maintained in fresh 1% FBS medium with or without rhIL-33, sST2, U0126 and SP600125 as required in different experiments. Cell migration was photographed at 0h and 48h. The area of the wound in each well was calculated using Image software.

2.6 Transwell invasion assay

EOC cell invasion was evaluated using a matrigel invasion chamber. Briefly, the cell inserts (Corning) were coated with 100µl of matrigel (BD BioScience) and then placed in a 24-well plate. Cells (4×10^5/ml) suspended in 100µl of medium containing 0.1% FBS with/without rhIL-33, sST2, U0126 or SP600125 were seeded in the top chambers. Dimethylsulfoxide (DMSO) was used to dissolve U0126 and SP600125 in our experiments, the final concentration of DMSO in the cell culture was 0.05% (v/v). The lower chambers were filled with 600µl of medium containing 10% FBS. After 48h incubation, the inserts were removed and washed in PBS before being fixed and stained with hematoxylin. The non-invading cells together with the matrigel were removed and the cells migrating to the lower surfaces were examined and counted at a magnification of ×200 in five predetermined fields. Each experiment was carried out in triplicate.

2.7 Immunohistochemistry

Formalin-fixed, paraffin-embedded EOC tissue sections (5μm) were dewaxed and rehydrated before an antigen retrieval step. Sections were then incubated with anti-human primary antibodies for IL-33 (Enzo Life Sciences), ST2 (Sigma-Aldrich), Ki-67 (Abcam) or matching IgG isotypes overnight. Slides were then stained with species specific biotinylated secondary antibodies (R&D Corporation), streptavidin–HRP and detected with substrate AEC (3-amino-9-ethylcarbazole) (Vector Laboratories). After that, slides were counterstained with hematoxylin. The staining intensity was scored independently by two pathologists with strong staining marked as 2+, weak staining marked as 1+ and minimal staining as 0 [29, 30]. Three sections from each sample were examined.

For EOC cell lines, cells were cultured on glass coverslips coated with poly-D-lysine (Sigma) with or without rhIL-33 in the culture medium for 48h. Cells were then fixed and incubated with anti-Ki-67 and other primary antibodies, and the subsequent staining steps were performed as described above.

2.8 Protein extraction and western blot

Total protein was harvested from cells and tissues, and protein concentrations were determined using Quick Start Bradford protein assay (Bio-Rad). Aliquots of samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were incubated with primary antibodies at 4 ºC overnight. Protein bands were detected by incubation with HRP-conjugated antibody (Pierce Biotechnology, USA) and visualized with Clarity Western ECL Substrate (Bio-Rad). Following antibodies used in the study were purchased from Cell Signaling Technology (Danvers, USA): β-actin, NF-κB, phospho-NF-κB p65 (Ser536), p38, phospho-p38

(Thr180/Tyr182), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p44/42MAPK/ERK, and phospho-p44/42MAPK/ERK (Thr202/Tyr204).

2.9 Statistical analysis

Results are expressed as mean+SEM and the data are representative of at least three separate experiments. The Wilcoxon–Mann–Whitney test was used to determine the statistical differences of cell proliferation, migration and invasion between two samples. The comparison between protein expression levels in different groups (Fig 1C and Tables 1 and 2) was assessed using Fisher's exact test. Overall survival curves were generated using Kaplan–Meier method and compared using a log-rank test. P value <0.05 was regarded as statistically significant.

3. Results

3.1 The expression levels of IL-33 and ST2 are associated with metastasis and proliferation of EOC

We first examined whether IL-33 and ST2 were expressed by normal ovary and ovarian tumour tissues using immunohistochemical staining, and the expression levels were then analyzed according to the established criteria for high-expression (staining 2+) and low-expression (staining 0 or 1+) (Gonzalez-Campora et al., 2011). Our data showed that IL-33 was undetectable in normal ovary tissues, and the expression levels were highly up-regulated in EOC samples with IL-33 positive staining localized to the cytoplasm as well as on the cell nucleus (Fig 1A). Similar to IL-33 expression, ST2 was not expressed by normal ovary tissues while high levels of ST2 positive staining were observed in EOC tissues with ST2 localized to the membrane and cytoplasm of cells (Fig 1B). Interestingly, when we compared the expression levels of IL-33 and ST2 in the tumours collected from the primary and metastatic sites, stronger staining of both IL-33 and ST2 was observed by the metastatic tumours, with additional nuclear staining of ST2 in the metastatic tumour cells but not primary tumour cells (Fig 1A and 1B).

To understand whether there is a correlation between the expression levels of IL-33 and ST2 and EOC metastasis, we screened the paraffin-embedded tissues of 20 normal ovary, 20 benign tumours and 114 EOC samples. Our results (Table 1) show that all normal ovary tissues had low expression of IL-33 or ST2, with 10%-20% of ovary benign tumours had high expression levels of the molecules. However in EOC samples, there was a significant upregulation of IL-33 expression with 59% of primary site and 76% of metastatic EOC tumours had high levels of expression. The statistical

difference was more significant for ST2 expression as 87% of metastatic tumours had high ST2 expression compared to 66% of primary site tumours. Furthermore there was a significant correlation between the expression levels of IL-33 and ST2 in EOC and tumour metastasis. Using western blotting, we further assessed the difference of IL-33 and ST2 expression in paired primary site and metastatic site EOC tumours. Samples were collected at operative resection with each case being confirmed by pathology examination. Our data show (Fig 1C) that out of twelve patients, eight had increased expression of IL-33 in metastatic tumours compared with the primary tumours while nine out of twelve metastatic tumours had increased ST2 expression, the differences were statistically significant for both IL-33 and ST2 using Fisher's test. Taken together our findings from both western blotting and immunohistochemical staining suggest that high levels of IL-33 and ST2 expression were closely correlated with EOC metastasis.

3.2 The up-regulation of IL-33 and ST2 in EOC is an indicator of tumour cell proliferation and poor prognosis

In addition to the tumour metastasis, we further analyzed whether there was a correlation between the expression levels of IL-33 and ST2 and patient age and other clinic pathological characteristics of EOC. Surprisingly, our data suggested that there was no significant correlation between the expression levels of IL-33 or ST2 and patient age, tumour histologic types, FIGO stages or differentiation (Table 2). However, IL-33 and ST2 expression levels were positively correlated with the expression of Ki-67 (Fig 2A, Table 2), further confirming a potential role of IL-33/ST2 signaling axis in EOC growth and metastasis. To understand the clinical implications of the up-regulation of IL-33 and ST2, we collected and analyzed the clinical follow up

data of EOC patients. Our Kaplan-Meier patient overall survival analysis curves showed that higher expression level of IL-33 was closely correlated with reduced survival time for EOC patients (Fig 2B), and the correlation was even more significant with the expression level of ST2 (Fig 2C). Thus the data here show that IL-33/ST2 axis plays an important role in ovarian cancer progression and are poor prognostic markers for patients.

3.3 Knock down of IL-33 gene reduces the migratory and invasive potential of EOC cells

The close correlation of IL-33/ST2 expression with EOC progression, particularly tumour metastasis suggests that IL-33/ST2 may be important in ovarian cancer cell growth, migration and invasion. We studied the role of IL-33 in EOC cell behaviour using cell lines. First we examined the expression of IL-33 and ST2 by EOC cell lines CAOV3, HO8910 and SKOV3-DDP. In agreement with our staining data of EOC patient tumour tissues, both IL-33 and ST2 were highly expressed by all three cell lines as confirmed by both immunohistochecmial staining (data not shown) and western blot (Fig 3A). Next, we disrupted IL-33 gene expression in the cell lines using siRNA, and tested the effect of endogenous IL-33 on EOC cell function. After transfection of IL-33 specific siRNA, the expression of IL-33 was dramatically decreased in all three cell lines as expected (Fig 3B) when compared with scramble control cells. Furthermore, cell viability, migration and invasion assays were performed to evaluate the role of IL-33 in EOC cell behaviour. While IL-33 knock-down had no effect on cell viability/proliferation in all three cell lines (data not shown), the cells transfected with IL-33 siRNA had significantly reduced their migration (Fig 4A) and invasion capacity (Fig 4B) as tested by the wound healing and transwell invasion assays.

3.4 Fl-hIL-33 increases the migratory, invasive and proliferative potential of EOC cells

To understand the function of IL-33/ST2 in EOC development, we investigated the effect of exogenous IL-33 on cell metabolic activity, invasion and migration using CAOV3 and HO8910 cell lines. Cells were first incubated with or without 200ng/ml sST2 for 3h before adding 10ng/ml of fl-hIL-33 in the culture for a further 48h. As shown in Figure 4, fl-hIL-33 not only increased the cell migration (Fig 5A) and invasion (Fig 5B), but also enhanced the cell viability/proliferation of both cell lines (Fig 5C). Furthermore, the staining of Ki-67 protein was also elevated in the cells treated with fl-hIL-33 (Fig 5D). While the administration of IL-33 decoy receptor sST2 was able to block the stimulatory effect of fl-hIL-33 on EOC cell migration, invasion and proliferation, sST2 also decreased IL-33-induced up-regulation of Ki-67 in EOC cells (Fig 5).

3.5 Fl-hIL-33 increases ovarian cancer migration, invasion and proliferation through ERK and JNK pathways

Numerous signaling pathways are involved in the aggressive behaviour of cancer cells, unraveling these signaling networks will provide important insights into cancer development. To understand the molecular mechanisms of IL-33/ST2 effect on ovarian cell growth and metastasis, we next investigated the changes in the signaling pathways of EOC cells after treatment with fl-hIL-33. We observed an obvious increase in the phosphorylation of JNK and ERK with a peak of phosphorylation occurring at 10mins after fl-hIL-33 stimulation, but no change was observed with NF-κB and p38 (Fig 6A). Phosphorylation of ERK and JNK was completely blocked

by sST2 (Fig 6B). We further demonstrated that U0126 (2μmol/l), an inhibitor of MAPK/ERK, significantly inhibited the phosphorylation of ERK in CAOV3 and HO8910 cells (Fig 7A). It also blocked the IL-33 mediated increase of cell migratory (Fig 7C) and invasive (Fig 7D) potential, together with cell viability/proliferation in CAOV3 cells (Fig 7E). When the JNK pathway inhibitor SP600125 (10μmol/l) was added to the cells, it inhibited JNK phosphorylation in CAOV3 and HO8910 cells as expected (Fig 7B). Surprisingly SP600125 had no effect on the IL-33-induced increase in cell migratory (Fig 7C) and invasive (Fig 7D) potential, and was only able to disrupt IL-33-induced enhancement of cell viability/proliferation (Fig 7E) of CAOV3 cells. DMSO was used to dissolve SP600125 and U0126 and our control experiments confirmed that low concentrations of DMSO used in the study had no effect on EOC cell behavior and the phosphorylation of JNK and ERK (Supplementary Fig 1). Collectively, our data demonstrate that the different roles of IL-33/ST2 in ovarian cancer cell growth, migration and invasion are mediated by complex signaling pathways.

4. Discussion

New evidence suggests that IL-33/ST2 axis is likely to play a role in cancer development and progression (Milovanovic et al., 2012), however its exact function in ovarian cancer growth and metastasis has not been defined. To our knowledge, our study is the first to demonstrate that the expression levels of both IL-33 and ST2 in EOC tumours are closely correlated with tumour metastasis and patient prognosis, and IL-33/ST2 axis contributes to EOC progression through mechanisms of promoting cancer cell growth and migration.

Several recent studies suggest that serum levels of IL-33 (Sun et al., 2011; Hu et al., 2013) and sST2 (Bergis et al., 2013; Lu et al., 2014) correlate with clinical prognosis of patients in various malignancies. In this study we have demonstrated that IL-33 and ST2 are highly elevated in EOC tumours compared with normal and benign tumour ovary tissues, and the levels are significantly increased in the tumours at the metastatic sites compared with the primary site tumours. These data suggest the IL-33/ST2 signaling pathway is very likely to be involved in the development of advanced EOC. Indeed, analysis of our clinical follow up records of a large cohort of EOC patients for nearly 10 years reveals that high levels of IL-33 and ST2 expression in EOC tissues are closely correlated with reduced survival rates of patients, indicating that IL-33 and ST2 are poor prognosis markers for EOC patients. Thus our data add to the current knowledge that IL-33/ST2 are potential prognostic markers for some malignant cancers.

Although emerging evidence strongly supports a role for IL-33/ST2 in cancer development, the precise underlying mechanism through which IL-33 facilitates cancer cell growth and metastasis is unclear. Our study suggests that IL-33, highly

elevated in EOC tumour tissues, is able to modulate the behaviour of cancer cells directly. It facilitates EOC progression and metastasis through promoting tumour cell invasion, migration and proliferation. While sST2 is able to block the function of IL-33 in enhancing EOC cell migration, invasion and cell viability, IL-33 gene disruption using siRNA reduces the cell migration and invasion but not the cell viability. The further up-regulation of IL-33 and ST2 by the metastatic EOC tumours indicates that IL-33 might act in an autocrine feedback mechanism to promote EOC progression and enhance the aggressive behaviour of EOC cells. Indeed as IL-33 is also regarded as an alarmin molecule and an important immunoregulatory cytokine (Pei et al., 2014), it might be argued that IL-33 is released by cancer cells into the tumour microenvironment where it binds to its receptor ST2 expressed on cancer cells and the infiltrating immune cells. As inflammation, immunity and cancer development are intimately related, it is highly likely that IL-33 might be one of the key molecules employed by cancer cells to promote cancer development through directly modifying cancer cell behaviour and indirectly modulating the infiltrating immune cells, thus linking the communications between these cells in an autocrine and/or paracrine feedback mechanism to promote cancer progression.

A few studies have examined the role of IL-33 in anti-tumour immune responses during cancer development (Gao et al., 2013; Jovanovic et al., 2011; Milovanovic et al., 2012) however with contradictory results. One study suggested that IL-33 acted as a crucial mediator in inflammation-associated pancreatic carcinogenesis by up-regulating secretion of the pro-inflammatory IL-6 and IL-8 cytokines (Schmieder et al., 2012). Transgenic IL-33 was shown to be able to activate NK and CD8 T cells and inhibited tumour growth and metastasis in melanoma and lung carcinoma animal models (Gao K, et al., 2013; Gao X, et al., 2015). Other reports showed that IL-33

enhanced type-2 immune responses (Barbour et al., 2014; Besnard et al., 2011; Kurowska-Stolarska et al., 2009; Jiang et al., 2012; Miller et al., 2008;) and thus suppressed activities of NK cells and accelerated cancer progression in tumour bearing animals (Jovanovic et al., 2014; Solinas et al., 2009). The reason for the discrepancy is not known, the data may reflect the different microenvironment in different cancer development and IL-33 may have specific anti-tumour immune responses in different cancer. Chronic inflammation underlies the progression of ovarian cancer (Lavoue et al., 2013), whether IL-33 influences the development of EOC through modulating the immune system is currently unknown and awaits further investigation.

Apart from the immune cells, cancer cells also have constant communications with other surrounding cells in the tumour microenvironment. Indeed, IL-33 promotes the invasiveness of head and neck squamous cell carcinoma through mediating the invasiveness of the key tumour-surrounding carcinoma-associated fibroblasts (Chen et al., 2013), suggesting IL-33 is one of the key mediators between cancer and stromal cells. As IL-33 also induces angiogenesis and vasopermeability (Choi et al., 2009), a study in squamous cell carcinoma of the tongue suggested that IL-33 influences the malignant potential of the tumour through the promotion of angiogenesis in the tumour microenvironment (Ishikawa et al., 2014). In our immunohistochemical staining data, we observed that IL-33 and ST2 were highly expressed by cancer cells and also the surrounding stromal cells, suggesting there might be a close interaction between the EOC and stromal cells.

IL-33 binds to ST2 and activates the downstream signaling pathways through NF-κB as well as MAPKs pathways (Liew, 2012), key signaling pathways for cancer cell

activities such as proliferation, migration and invasion. Current evidence indicates that gene- and cell-specific differences often exist in IL-33 activated signaling pathways (Milovanovic et al., 2012; Zhou et al., 2014). NF-κB is a key player in innate immunity and inflammation, and the IL-33 effect on cytokine production appeared to be mediated via NF-κB activation as shown in pancreatic carcinoma cells (Schmieder et al., 2012). Other studies show that P38 signaling pathway was associated with the pro-melanogenic activity of IL-33 in primary melonocytes (Zhou et al., 2014), while IL-33 enhanced proliferation and invasiveness of decidual stromal cells were mediated via both NF-κB and ERK1/2 signaling (Hu et al., 2014). To understand the molecular mechanisms underlying the function of IL-33 in EOC cell proliferation, migration and invasion, we examined the phosphorylation of multiple MAPKs, including ERK1/2, JNK and p38 as well as NF-κB in the EOC cell lines. Our results identified important roles for the ERK and JNK signaling pathways in IL-33 mediated aggressive behaviour of EOC cells as there was dramatic increase in the phosphorylation of ERK and JNK after fl-hIL-33 stimulation, and the increase was blocked by IL-33 decoy receptor sST2. However, neither NF-κB or p38 phosphorylation was detected, suggesting they were not involved in IL-33 mediated EOC cell metastasis and proliferation. We further demonstrated that the ERK pathway is specifically associated with IL-33-mediated increases in the invasive, migratory and proliferative potential of EOC cells, but the JNK pathway was only involved in IL-33-mediated increases in proliferation using ERK and JNK specific inhibitors. Therefore, specific function of fl-hIL-33 in different cells is likely to be regulated through distinct signaling pathways, while it increased EOC cell invasion and migration through the ERK pathways, but it promoted cell proliferation through both the ERK and JNK pathways.

In conclusion, we demonstrate that high levels of IL-33 and ST2 are expressed by EOC tumours, particularly those at the metastatic sites. We also show that the expression levels of both molecules are significantly correlated with poor prognosis of patient survival time. IL-33 facilitates ovarian cancer progression by promoting EOC cell proliferation, migration and through ERK and JNK specific pathways. The information obtained from our study provides an important insight into the mechanisms of metastases in EOC. Our data indicate that IL-33 and ST2 are potential prognostic markers as well as possible therapeutic targets for clinical intervention for EOC and other cancer patients.

Conflict of interests

The authors declare that they have no conflict of interest.

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Legends:

Fig 1. Expression levels of IL-33 and ST2 are up-regulated in EOC tumours compared with normal ovary tissues. (A) Representative immunohistochemical staining of IL-33 in normal ovary, EOC tumours at primary site and metastatic site at 100× and 400× original magnification. (B) Representative immunohistochemical staining of ST2 in normal ovary, EOC primary site and metastatic site. Arrows indicate tumour cells in the tissue. (C) Western blots of IL-33 and ST2 in patient primary site tumours (P) compared with metastases (M). Fisher's exact test was used to analyze the statistical difference for IL-33 and ST2 expression between primary and metastatic site EOC tumous, both $P<0.05$.

Fig 2. Correlation of IL-33/ST2 expression with patient survival time. (A) Expression of Ki-67 at EOC tumours at 100× and 400× original magnification. (B) Kaplan-Meier patient overall survival analysis curves for 114 EOC patients with high (2+) or low (0 or 1+) expression of IL-33. (C) Kaplan-Meier patient overall survival analysis curves for EOC patients with high (2+) or low (0 or 1+) expression of ST2. The follow up period was 45.2 months (range, from 1 to 110 months).

Fig 3. IL-33 and ST2 expression by EOC cell lines and knockdown of IL-33 gene by siRNA. (A) Western blot examined the expression of IL-33 and ST2 by CAOV3, HO8910 and SKOV3-DDP cell lines; (B) After transfection of IL-33 siRNA1, siRNA2 or scramble control siRNA, the expression of IL-33 was examined in all three cell lines by western blot.

Fig 4. Knock down of IL-33 gene reduces the invasive and migratory potential of EOC cell lines. Cell lines transfected with IL-33 specific siRNAs or scamble control siRNA were examined for cell migration (A) and invasive capacity (B) using wound healing and transwell invasion assays respectively. Data were expressed as mean+SEM, * P < 0.05. Results are representative of 3 different experiments.

Fig 5. Fl-hIL-33 promotes the invasive and migratory potential of EOC cells and increased cell proliferation. CAOV3 and HO8910 cells were treated with or without fl-hIL-33 (10ng/ml), in some wells cells were pre-incubated with sST2 (200ng/ml) for 3h before adding fl-hIL-33. The effect of IL-33 on cell migration (A), invasion (B) and cell viability (C) was tested using wound healing, transwell invasion and MTT assays respectively. (D) Immunohistochemical staining of the cells with Ki-67 antibody. Data in A, B, and C were expressed as mean+SEM with the control as "100%" in cell viability assay. * P < 0.05. Results are representative of 3 different experiments.

Fig 6. Fl-hIL-33 increases the phosphorylation of ERK and JNK and this was blocked by sST2. (A) CAOV3 and HO8910 cells were stimulated with fl-hIL-33 (10ng/ml) for the indicated times (0, 10, 30, 60, 120mins) and then harvested for western blot assay of P-NF-κB, NF-κB, P-JNK, JNK, P-P38, P38, P-ERK1/2, ERK1/2 and β-actin. (B) CAOV3 and HO8910 cells were pretreated with 200ng/ml sST2 for 3h before adding rhIL-33 (10ng/ml) for 10mins. Cells were then harvested and examined for the expression of phosphorylation of MAPKs JNK and ERK.

Fig 7. The effect of fl-hIL-33 on EOC cell invasion, migration and proliferation was

specifically mediated by the ERK and the JNK pathways. CAOV3 and HO8910 cells were pre-treated with 2µmol/ml U0126 (inhibitor of ERK) and 10µmol/ml SP600125 (inhibitor of JNK) for 30mins before being incubated with rh-IL-33 (10ng/ml) for 10mins. Phosphorylation of ERK (A) and JNK (B) was evaluated by western blot. (C-E) CAOV3 cells were pre-treated with U0126 or SP600125 before adding rhIL-33, and then used to study the migratory (C), invasive (D) potential and cell viability (E). Data are expressed as mean+SEM, with the control as “100%” in cell viability assay. * $P < 0.05$. Results are representative of 3 different experiments.

Supplementary Fig 1. The effect of low level of DMSO on EOC cell behaviour and phosphorylation. CAOV3 cells were treated with or without 0.05% DMSO in the culture and its effects on cell invasion (A), migration (B) and cell viability/proliferation (C) were tested using the methods described in the manuscript. (D) CAOV3 and HO8910 cells were cultured with or without 0.05% DMSO, and then cells were harvested and examined for the expression of phosphorylation of JNK and ERK by western blot.

Table 1. IL-33 and ST2 expression in normal ovary, benign ovarian tumour and EOC tissues

Groups	**n**	No. of Patients (%) IL-33 protein expression		No. of Patients (%) ST2 protein expression	
		0 or 1+	2+	0 or 1+	2+
Normal ovary	20	20 (100)	0 (0)	20 (100)	0 (0)
Benign tumours	20	18 (90)	2 (10)	16 (80)	4 (20)
EOC					
Primary	114	47 (41)	67 (59)*	39 (34)	75 (66)*
metastasis	38	9 (24)	29 (76)$^{\%}$	5 (13)	33 (87)$^{\#}$

* Expression levels of IL-33 or ST2 were compared between normal ovary and ovarian benign tumours, $P < 0.01$; $^{\%}$ comparison of expression levels of IL-33 between primary site and metastatic site tumours, $P < 0.05$; $^{\#}$ comparison of expression levels of ST2 between primary site and metastatic site EOC, $P < 0.01$. P values were calculated by Fisher's exact test.

Table 2. Correlation of IL-33 and ST2 expression with clinicopathological features of EOC

Characteristic	**n**	No. of Patients (%) IL-33 expression			No. of Patients (%) ST2 expression		
		0 or 1+	2+	P Value	0 or 1+	2+	P Value
Age (years)				0.774			0.881
>60	51	22(43)	29(57)		17(33)	34(67)	
≤60	63	25(40)	38(60)		22(35)	41(65)	
Histologic type				0.155			0.187
Serous carcinoma	88	34(39)	54(61)		28(32)	60(68)	
Mucinous carcinoma and others	26	13(50)	13(50)		11(42)	15(58)	
FIGO disease stage				0.117			0.107
I-II	28	14(50)	14(50)		12(43)	16(57)	
III-IV	86	33(38)	53(62)		27(31)	59(69)	
Differentiation				0.112			0.098
Well and moderate	70	32(46)	38(54)		27(39)	43(59)	
Poor	44	15(34)	29(66)		12(27)	32(73)	
Ki-67 expression				0.006			0.002
0 or 1+	47	25(53)	22(47)		22(47)	25(53)	
2+	67	22(33)	45(67)		17(25)	50(75)	

P values were calculated by Fisher’s exact test.

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