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Kindlin-2 inhibits serous epithelial ovarian cancer peritoneal dissemination and predicts patient outcomes

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ABSTRACT

Kindlin-2 has been known to promote most cancer progression through regulation of multiple signaling pathways. However, a novel tumor suppressive role of Kindlin-2 was identified in serous epithelial ovarian cancer progression, which sharply contrasts to the tumor promoting roles for Kindlin-2 in most other cancers. While we demonstrated that Kindlin-2 was highly expressed in control tissues, a drastic low expression of Kindlin-2 was found in the tumor tissues of serous epithelial ovarian cancer, especially in the high-grade serous epithelial ovarian cancer. Importantly, Kindlin-2 inhibited serous epithelial ovarian cancer cell peritoneal dissemination in a mouse model. For clinical relevance, low Kindlin-2 expression correlated with higher tumor grade and older patients. Intriguingly, decreased Kindlin-2 expression predicts poor overall and progression-free survivals in serous epithelial ovarian cancer patients. Mechanistically, Kindlin-2 induced a mesenchymal to epithelial transition in serous epithelial ovarian cancer cells, at least in part, by up-regulation of estrogen receptor α which was recruited to the promoter of E-cadherin and thereby enhanced the transcription of E-cadherin. Collectively, we concluded that inadequate Kindlin-2 is an independent risk factor for serous epithelial ovarian cancer patients.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic cancer and currently ranks the fifth in cancer-related deaths among women [1]. Most of patient deaths from EOC are caused by serous subtype which is notorious for their late clinical stage and the poor outcome [2,3]. Thus, understanding the progression of serous EOC and discovering novel molecular prognostic indicators as well as new therapeutic targets remain a major challenge.

Unlike most other cancers, serous EOC rarely metastasizes through the vasculature [4], but rather disseminates predominantly within the serosal cavities, forming multiple solid nodules on the surfaces of the abdominal cavity or organs, as well as accumulating of peritoneal effusion fluid (ascites) [5]. The initial step of intraperitoneal dissemination begins with serous EOC detaching as single cells or clusters from the primary site, during which epithelial-to-mesenchymal transition (EMT) takes place [6]. Down-regulation of E-cadherin is one of the critical events for EMT and has been demonstrated in the floating EOC cells in ascites [7]. Moreover, EOC cells with low E-cadherin expression level are more invasive and the absence of E-cadherin expression in EOC predicts poor patient survival [3].

Kindlin-2 (FERMT2), a member of Kindlin protein family, has been indicated as a co-activator for integrin activation and an essential element of bidirectional integrin signaling [8,9]. We have found that Kindlin-2 was not only involved in some non-neoplastic lesions such as renal fibrosis [10], but also took various oncogenic roles in promoting tumor invasion by enhancing Wnt signaling [11] or silencing of the microRNA200 family [12]. However, Shi and Wu [13] demonstrated that Kindlin-2 might have a suppressive role by inhibiting mesenchymal cancer cell invasion. The

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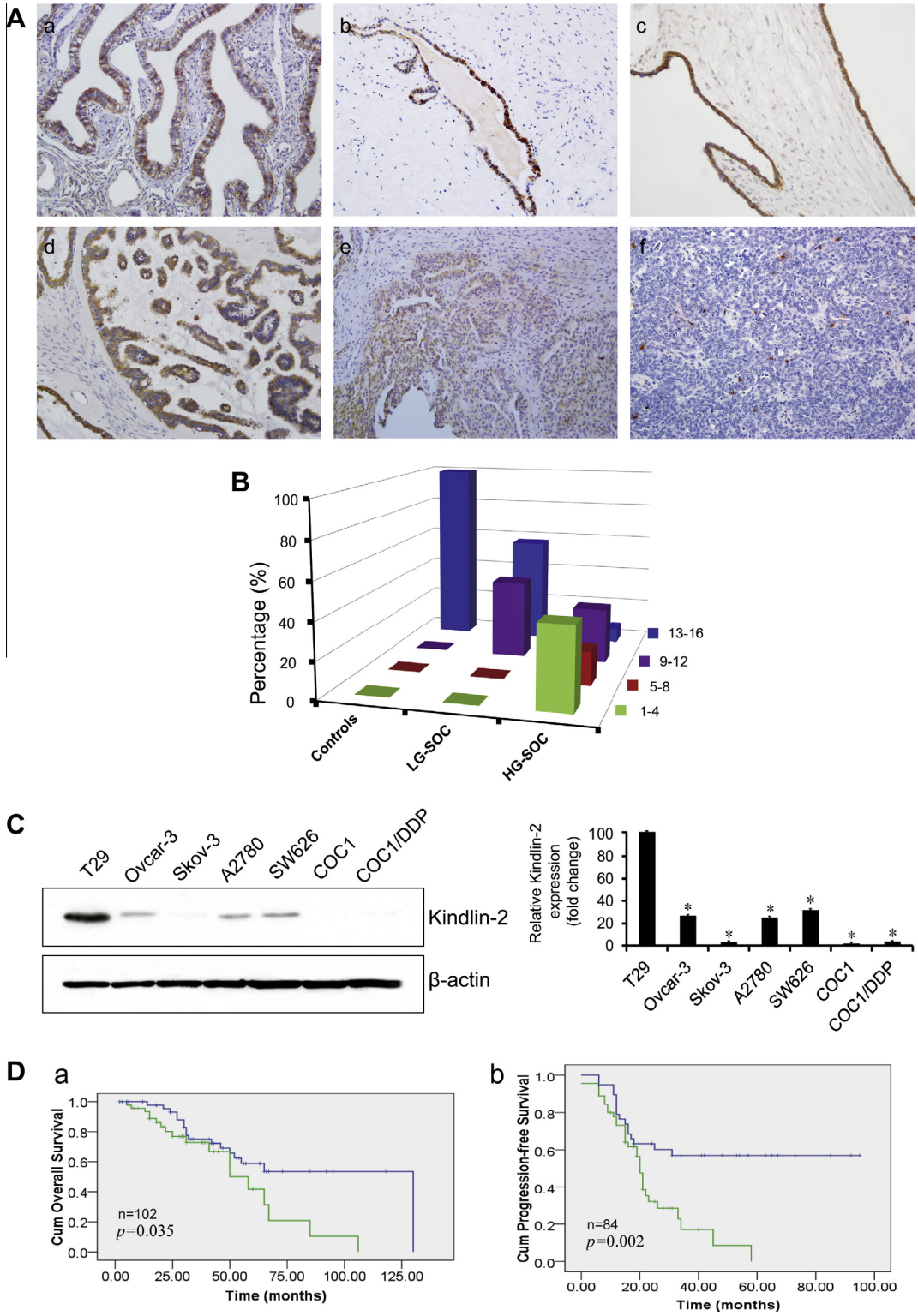

Fig. 1. Decreased expression of Kindlin-2 is correlated with the progression of serous EOC and poorer survival of patients. (A) Immunohistochemical staining of Kindlin-2 in (a) normal epithelium of fallopian tube; (b) inclusion cyst; (c) benign serous cystadenoma; (d) borderline serous cystadenoma; (e) low-grade serous EOC; (f) high-grade serous EOC (magnification 200×). (B) Kindlin-2 expression in control tissues and serous EOCs. Controls: including normal fallopian tube epithelia, inclusion cysts, benign serous cystadenomas and borderline serous cystadenomas. LG-SOC: Low-grade serous ovarian cancer; HG-SOC: High-grade serous ovarian cancer. (C) Western blot assay was performed in immortalized ovarian surface epithelial cell line (HOSE T29) and EOC cell lines $^{*}p < 0.05$. (D) Kaplan–Meier analysis showed (a) OS and (b) PFS for serous EOC patients with two different expression levels of Kindlin-2.

above contradictory results from different studies evoked our curiosity about the exact functions of Kindlin-2 in serous EOC, which represents a subtype of refractory EOC. In the present study, we investigated the alteration of Kindlin-2 protein expression level during the progression of serous EOC and analyzed their related clinicopathologic significances. Furthermore, we established the

relationship between Kindlin-2 and the serous EOC patient prognosis as well as the underlying mechanisms that support the clinical relevance.

2. Materials and methods

2.1. Tumor tissues

All tissues were obtained from the Department of Pathology, Peking University Health Science Center, detailed information showed in Supplementary Materials and methods. 113 serous EOC samples were graded according to M.D. Anderson Cancer (MDACC) grading system [14]. Given that low-grade serous EOC probably derived from ovarian surface epithelium (OSE)/inclusion cysts, while high-grade serous EOC arise from the fallopian tube [15,16], our study included both inclusion cysts and fallopian tube epithelia as normal controls. The use of these clinical samples was approved by the Institutional Ethics Committee of Peking University Health Science Center. All cases were used for immunohistochemistry.

2.2. Immunohistochemistry

Immunohistochemistry was performed as described previously [17]. In brief, formalin-fixed, paraffin-embedded, 4 μm thick tissue sections were stained with rabbit anti-Kindlin-2 and Ki-67 antibodies by EnVision + Dual Link System (K4061; Dako). Microwave antigen recovery, using citrate buffer (pH 6.0) was done. Diaminobenzidine (DAB) was used as chromogen and hematoxylin as counterstain. Negative control reactions used TBS instead of the specific primary antibody, and no positive staining was observed. Immunoreactivity was assessed by intensity and percentage of epithelium stained. Staining intensity was scored as 1 (negative); 2 (weak), 3 (moderate), and 4 (strong). The percentage of positive staining was rated as 1 (<25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). A composite "histoscore" was given as a product of average staining intensity (1–4) and the average percentage of positive cells (1–4), with a maximum of 16.

2.3. Cell culture and establishment of stable cell lines

The information of cell lines showed in Table S1. For the generation of stable cell lines, Skov-3 cells were transfected with pCMV-3 × FLAG or pCMV-3 × FLAG-Kindlin-2 plasmid using Lipofectamine 2000. Clones were selected by 800 μg/ml of G418 (Sigma) for 2 weeks. Mixed clones (6–10 independent clones) were maintained in medium containing 200 μg/ml of G418.

2.4. Western blot

Western blot was performed as described previously [12]. The antibodies used were shown in Table S2.

2.5. In vitro migration and invasion assays

In vitro migration and invasion assays were performed as described previously [12].

2.6. In vivo studies

5×10^6 Kindlin-2 or Flag transfected Skov-3 cells were inoculated subcutaneously (s.c.) (seven mice per group) or intraperitoneally (i.p.) (seven mice per group) into BALB/c female nude mice (Center of Experimental Animals, Peking University, Beijing, China). Mice with s.c. or i.p inoculation were killed at 6 weeks after cells injection. The mice were maintained according to the Guidelines of Animal Experiments by Peking University. The Ethics Committee of Peking University Health Science Center has approved this study (Permit No: LA2011-73). The procedures followed were in accordance with the ethical standard of the Helsinki Declaration of 1975, as revised in 1983.

2.7. RNA interference (RNAi)

Sequences of RNA interference (RNAi) oligonucleotides were as follows:

Control siRNA, 5′-UUCUCCGAACGUGUCACGU-3′; Kindlin-2 siRNA, 5′-AAGCUGGUGGAGAAACUCG-3′; ERα, 5′-UCAUCG CAUU CCUUGCAAA-3′. All RNAi oligonucleotides were purchased from Shanghai GenePharma Company (Shanghai, China). These RNAi oligonucleotides were transfected into cells by using a Lipofectamine 2000 kit (Invitrogen) for 72 h according to the manufacturer's instructions.

2.8. Chromatin immunoprecipitation (ChIP) assay

A chromatin immunoprecipitation (ChIP) assay was performed as described previously [12]. The primers for E-cadherin promoter were as follow: forward primer, 5′-CCCCATCTCCAAAACGAACAA-3′ and reverse primer, 5′-CCGGTGGCTCACTAAGACCTG-3′.

2.9. Real-time PCR (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using the SuperScript kit (Invitrogen). The primer sequences were as follows: ERα, forward primer, 5′-GCATTCTAC AGGCCAAATTCAGATAA-3′ and reverse primer, 5′-GCCATACTTCCC TTGTCATTGGT-3′; E-cadherin, forward primer, 5′-CGGCTGATACTG ACCCCACA-3′ and reverse primer, 5′-CGCTTCCTTCATAGTCAAACAC GA-3′; GAPDH, forward primer, 5′-GGCAAAGTGGAGATTGTTGC-3′ and reverse primer, 5′-AATTTGCCGTGAGTGGAGTC-3′.

2.10. Statistical analysis

The data were analyzed with the software package SPSS 16.0. Mann–Whitney test and Chi-Square test were used to analyze the association between Kindlin-2 expression level and

Table 1
Univariate and multivariate analysis of PFS.

	Univariate analysis			Multivariate analysis		
	HR	(95% CI)	*p*	HR	(95% CI)	*p*
Chemosensitivity: yes *vs.* no	7.401	(3.772, 14.525)	$p < 0.0001$	11.432	(4.085, 31.985)	$p < 0.0001$
High Kindlin2 *vs.* low Kindlin2	2.294	(1.249, 4.211)	$p = 0.007$	3.699	(1.396, 9.807)	$p = 0.009$
Ascites: yes *vs.* no	2.230	(1.210, 4.110)	$p = 0.01$	NS		
Omental metastasis: yes *vs.* no	2.491	(1.152, 5.383)	$p = 0.020$	NS		

NS, Not significant.

clinicopathologic parameters. Kaplan–Meier curves were plotted to assess the effects of Kindlin-2 expression level on overall survival (OS) and progression-free survival (PFS). Survival curves were compared using the log-rank test. Cox proportional hazard models were used to assess the prognostic significance of Kindlin-2 expression. Unpaired t test was used for comparison of data between two groups. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Decreased expression of Kindlin-2 protein is correlated with the tumor differentiation and poor prognosis of serous EOC patients

To uncover the possible involvement of Kindlin-2 in serous EOC progression, we examined the mRNA and protein expression profiles of Kindlin-2 in serous EOC. Oncomine database analysis

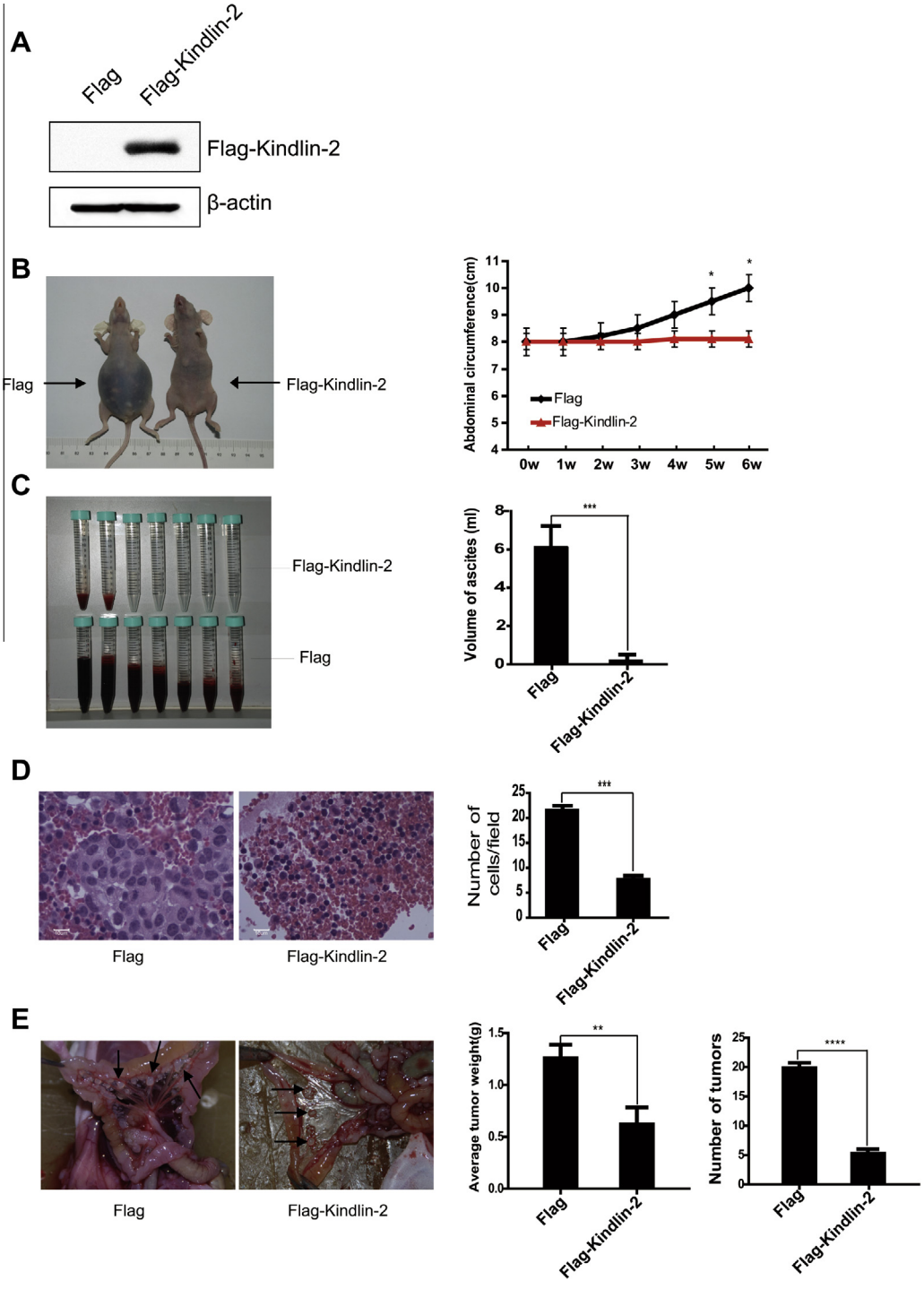

Fig. 2. Kindlin-2 blocks serous EOC cell peritoneal dissemination and production of bloody ascites in nude mice. (A) Overexpression of Flag-Kindlin-2 or Flag Skov-3 stable cells was detected by Western blot analysis. (B) The two stable cell lines were i.p. inoculated into nude mice. Abdominal circumference of nude mice displayed at indicated time $^{*}p < 0.05$. (C) Ascites of mice inoculated i.p. with the two stable cell lines were collected in centrifuge tubes and quantified $^{***}p < 0.0005$. (D) Cancer cells in ascites were obtained by centrifuge to make cell paraffin block, and then hematoxylin and eosin (H.E.) staining was performed and tumor cells in ascites were quantified $^{***}p < 0.0005$. (E) Representative views of the abdominal cavity of mice inoculated i.p. with two stable cells were photographed (left). Arrows indicated tumor nodules. The weight (middle) and the numbers of tumors (right) disseminated on the mesentery of nude mice were quantified $^{**}p < 0.005$, $^{****}p < 0.0001$, respectively.

indicated that Kindlin-2 mRNA expression is lower in serous EOC than that in normal ovary (Fig. S1). In a patient cohort study, strong immunostaining signals (score:13–16) of Kindlin-2 could be seen in all control samples, including 32 fallopian tube epithelia, 17 inclusion cysts, 17 benign serous cystadenomas, and 15 borderline serous tumors. While decreased expression of Kindlin-2 protein (score $\leqslant$ 12) were shown in nearly half (11/26, 42.3%) of low-grade serous EOCs (LG-SOC) and most (80/87, 92%) of high-grade serous EOCs (HG-SOC) ($p = 0.000$, Fig. 1Aa–f and B). Similar results were also demonstrated in EOC cell lines (Fig. 1C). These data suggested that Kindlin-2 expression reciprocally correlated with the tumor grade in serous EOC.

We grouped Kindlin-2 protein expression levels as high Kindlin-2 and low Kindlin-2, and then examined the correlation between Kindlin-2 expression levels and clinicopathological parameters in 113 serous EOC patients. The expression level of Kindlin-2 in older ($\geqslant$50 years) patients was found significantly lower than that in the younger (<50 years) ones ($p < 0.05$, Table S3), and it seemed that patients with reduced Kindlin-2 protein were inclined to suffer in omental metastasis and ascites production, unfortunately, this difference did not reach statistic significance ($p = 0.077$, $p = 0.078$, respectively, Table S3) probably due to the limited sample size and follow-up period. The patients with lower Kindlin-2 expression levels were significantly associated with shorter OS (for median OS, 56 months vs. 100 months) ($p < 0.05$, Fig. 1D-a) and PFS (for median PFS, 22 months vs. 90 months) ($p < 0.05$, Fig. 1D-b). Multivariate analysis further showed that decreased Kindlin-2 expression level was one of the independent risk factor for PFS ($p < 0.05$, Table 1). Taken together, our findings indicated that decreased Kindlin-2 expression level is correlated with the tumor differentiation and poor prognosis of serous EOC patients.

3.2. Kindlin-2 blocks serous EOC cell peritoneal dissemination in nude mice

To answer why the expression level of Kindlin-2 could predict serous EOC patient outcomes, we established serous EOC peritoneal metastatic mouse model. 5×10^6 Kindlin-2 overexpressed Skov-3 stable cells or control cells (Fig. 2A) were inoculated intraperitoneally (i.p.) into the nude mice separately. In contrast with the control mice, mice injected with Kindlin-2-overexpressed cells showed significantly decreased abdominal circumference ($p < 0.05$, Fig. 2B), less ascites production ($p < 0.0005$, Fig. 2C), reduced cancer cell emergence in ascites sediment ($p < 0.0005$, Fig. 2D), lower intraperitoneal dissemination potential (Fig. 2E-left), lower total i.p. tumor weight ($p < 0.005$, Fig. 2E-middle), and less tumor nodules ($p < 0.0001$, Fig. 2E-right). All these *in vivo* results indicated that Kindlin-2 could negatively regulate the peritoneal dissemination and ascites production potentials of serous EOC cell lines.

3.3. Kindlin-2 inhibits serous EOC cell migration, invasion and MMPs expression

Overexpression of Kindlin-2 protein in serous EOC cells not only made them changing from an elongated and spindle-like (mesenchymal phenotype) morphology to a cobblestone-like morphology (epithelial phenotype) (Fig. 3A), but also significantly downregulated their migration and invasion potentials (Fig. 3B) as well as MMPs expression (Fig. 3C), and knockdown of Kindlin-2 protein upregulated their migration and invasion potentials (Fig. 3D) and MMPs expression (Fig. 3E). In addition, both *in vivo* and *in vitro* experiments showed that Kindlin-2 had no effect on the proliferation of serous EOC cells (Fig. S2).

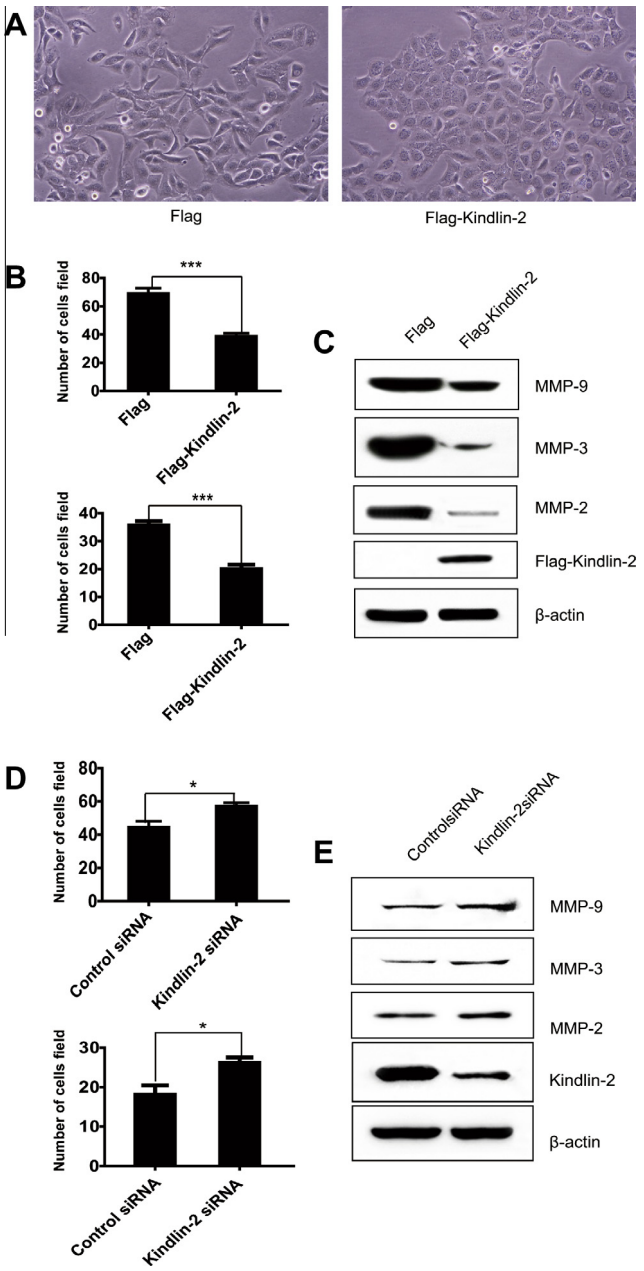

Fig. 3. Kindlin-2 impedes serous EOC cells migration, invasion and reduces MMPs expression. (A) Phase contrast microscopy image of the two stable cell lines. (B) *In vitro* migration (upper) and invasion assays (lower) were performed in Kindlin-2 overexpressed stable cells and control cells, $^{***}p < 0.0005$. (C) Western blot analysis on MMPs expression in the two stable cell lines. (D) Knockdown of Kindlin-2 with specific Kindlin-2 siRNA, and then migration (upper) and invasion assays (lower) were performed $^{*}p < 0.05$. (E) Western blot analysis on MMPs expression in Kindlin-2 knocked-down cells and the control cells.

3.4. Kindlin-2 upregulates E-cadherin at least in part via ERα recruitment to E-cadherin promoter

Overexpression of Kindlin-2 in serous EOC cells apparently upregualted E-cadherin and ERα (epithelial markers) while greatly downregualted Vimentin and Twist (mesenchymal markers) (Fig. 4A); whereas knockdown of Kindlin-2 downregulated E-cadherin and ERα while upregulated Vimentin and Twist (Fig. 4B). These data suggested that Kindlin-2 is an important regulator of mesenchymal to epithelial transition (MET). Importantly, chromatin immunoprecipitation (ChIP) assay identified that more

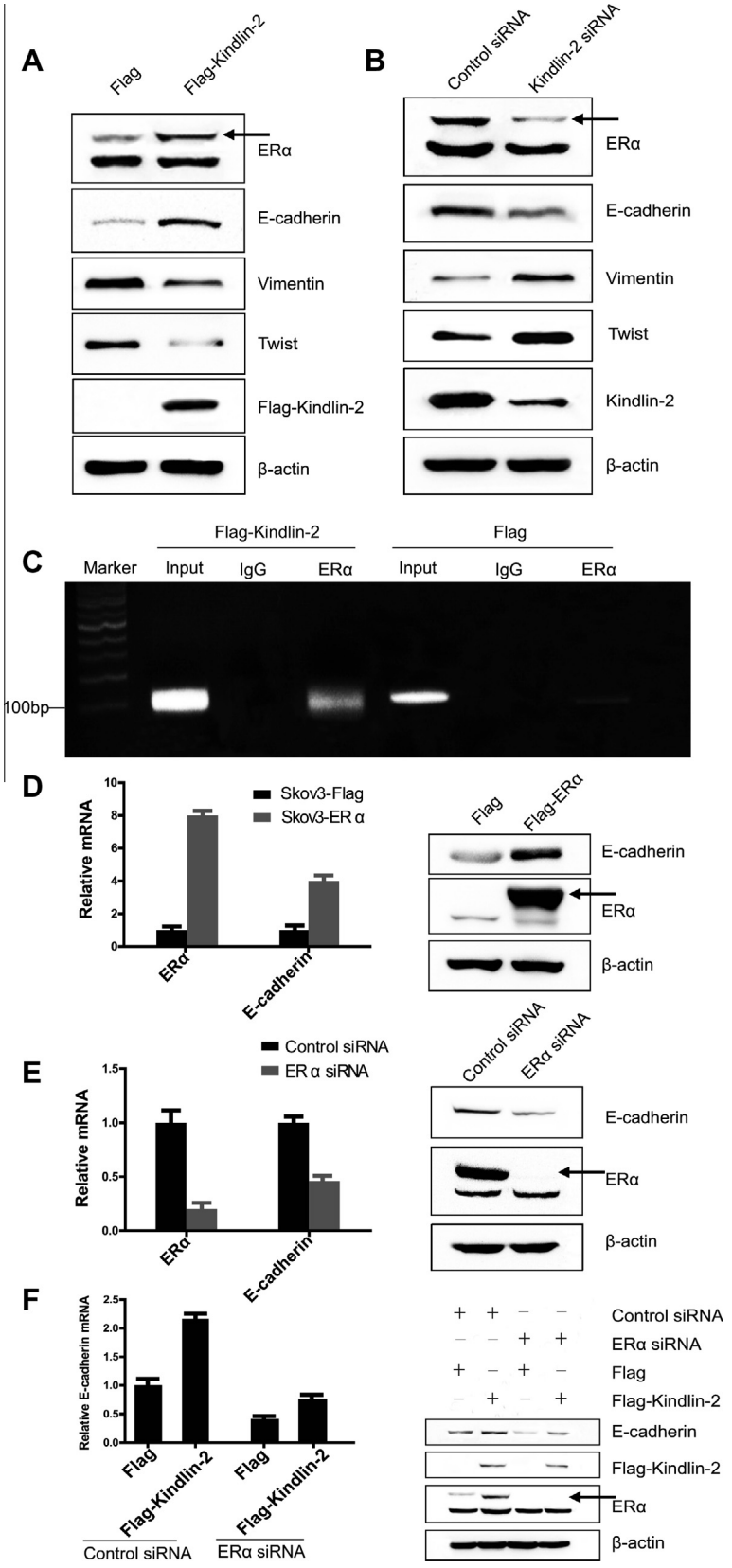

Fig. 4. Upregulation of E-cadherin by Kindlin-2 depends on ERα recruitment at the E-cadherin promoter. Western blot analysis was performed to detect EMT markers in (A) Kindlin-2 overexpressed stable cells and (B) Kindlin-2 knocked-down cells using indicated antibodies. (C) ChIP assay was performed with an anti-ERα antibody in Kindlin-2-overexpressed stable cells and control cells. Input DNA was used to normalize the results. (D) Exogenous transfection Skov-3 with ERα, and then RT-qPCR assay and Western blot analysis were performed to detect E-cadherin expression. (E) Knockdown of ERα using specific ERα siRNA in Kindlin-2-overexpressed stable cells, and then RT-qPCR assay and Western blot analysis were performed. (F) The ovarian cancer cell Skov-3 was transfected with or without ERα siRNA, and then transfected again with or without Flag-Kindlin-2, the level of E-cadherin was detected by RT-qPCR assay (left) and Western blot (right).

ERα can be recruited to the promoter of E-cadherin in Kindlin-2-overexpressed stable cells than that in control cells (Fig. 4C), uncovering a new mechanism of ERα regulation on the epithelial marker E-cadherin in serous EOC cells. Interestingly, overexpression of ERα alone could promote E-cadherin expression at both mRNA and protein levels (Fig. 4D) in serous EOC cell, which suggested that ERα recruited to the promoter of E-cadherin promoted the transcription of E-cadherin in serous EOC cells. Knockdown of ERα led to downregulation of E-cadherin in Kindlin-2-overexpressed cells at both mRNA and protein levels (Fig. 4E). Further, to clarify whether ERα was involved in the regulation of Kindlin-2 on E-cadherin, ERα was knocked down and then Kindlin-2 was overexpressed in serous EOC cells. As shown in Fig. 4F, the upregulation of E-cadherin by Kindlin-2 was weakened compared with control in ERα knockdown cells (Fig. 4F), indicating that ERα contributed to the regulation of Kindlin-2 on E-cadherin, however, other molecules also participate in the regulation of E-cadherin. Taken together, our results suggested that Kindlin-2 upregulated E-cadherin, at least in part, via ERα and thereby induced an MET phenotype.

4. Discussion

Unlike the previous findings that Kindlin-2 promotes tumor progression [12,17], in this study we found that Kindlin-2 is a protective factor for serous EOC patients by showing that lower expression of Kindlin-2 correlated with poor OS as well as PFS in serous EOC patient. These important conclusions make Kindlin-2 a prognostic marker for clinic monitoring of the therapeutic outcomes for serous EOC patients. The findings that Kindlin-2 inhibits serous EOC cell migration and invasion especially peritoneal dissemination raise the possibility that targeting Kindlin-2 blocks serous EOC cell dissemination to the peritoneal cavity, a most lethal step for serous EOC progression. Therefore, Kindlin-2 is of particular value for serous EOC patient prognosis and could be a therapeutic target for inhibition of serous EOC cell peritoneal dissemination.

EMT has been known to be an important step during serous EOC progression [6]. The epithelial marker E-cadherin was markedly downregulated in ascites cells that are more invasive [18]. However, malignant ascites cells with upregulated E-cadherin and reduced Vimentin were less tumorigenic [19]. Interestingly, our findings indicated that Kindlin-2 upregulated E-cadherin and downregulated Vimentin and Twist in serous EOC cells, a mechanism that inhibits EMT occurrence. We also found that Kindlin-2 up-regulated ERα. ERα has been considered as another epithelial marker in breast cancer cells and was proved to be a ligand-independent activator of E-cadherin [20]. We found that ERα contributed to Kindlin-2 upregulation of E-cadherin, a mechanism that was not known before and is important for maintenance of epithelial integrity in serous EOC cells. Re-expression of ERα in breast cancer cells led to their invasive phenotype reversion [21], yet absence of ERα mechanistically linked to E-cadherin suppression and EMT [22]. In ovarian cancer patients, decreased ERα expression was associated with shorter OS and PFS [23,24]. Therefore, the mutual regulation between Kindlin-2 and ERα might determine the prognosis in serous EOC patients, which deserves further investigation.

Till now, Kindlin-2 has been known to play multiple roles in different cancers. Kindlin-2 could promote breast cancer invasion via an epigenetic silencing of the microRNA200 gene family [12]. Kindlin-2 was required for malignant mesothelioma cell adhesion and migration [17], and it was also positively related to tumor invasion, lymph node metastasis, and patient outcome in gastric cancer [25]. However, along with our data, Kindlin-2 had shown suppressive roles in some cancers. For example, it has been shown to inhibit mesenchymal cancer cell invasion [13], and it was significantly downregulated in malignant uterine leiomyosarcoma tissue compared with benign uterine leiomyoma tissue [26]. These paradoxical findings suggested that, as mentioned above, Kindlin-2 is probably a versatile molecule that may function through different pathways in a cellular context dependent manner.

Taken together, our results showed that Kindlin-2 is an important regulator for serous EOC progression. Kindlin-2 might be a potential candidate suppressor, an independent prognostic factor and a therapeutic target for serous EOC patients.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc. 2014.02.087.

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