Cell-Free 59 kDa Immunoreactive Integrin-Linked Kinase: A Novel Marker for Ovarian Carcinoma

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ABSTRACT

Purpose: **We reported that the expression of integrin-linked kinase (ILK) is up-regulated in ovarian carcinomas and that ovarian cancer cells have high expression of ILK. In this study, we have examined the expression of cell-free 59 kDa immunoreactive (ir)ILK in the serum and peritoneal fluid (PTF) of patients with ovarian cancer and evaluated its potential as a serum biomarker for early-stage screening and for monitoring clinical status of patients after chemotherapy treatment.**

Experimental Design: **Thirty-six serum specimens, including normal ($n = 6$), benign ($n = 6$), borderline ($n = 4$), grade 1 ($n = 5$), grade 2 ($n = 5$), and grade 3 ($n = 10$), were evaluated for the expression of irILK by Western blotting. The expression of irILK was evaluated in PTF ($n = 10$) and peritoneal washings from women with benign ovarian cysts ($n = 4$). In addition, tissue-conditioned medium obtained from the cultures of primary ovarian tumors ($n = 9$) was examined for the presence of irILK. Finally, the potential of serum irILK as a biomarker for ovarian cancer screening was evaluated by comparison with cancer antigen 125 (CA 125) concentrations in cancer patients before and after chemotherapy.**

Results: **irILK expression was present in normal serum and in serum of patients with benign ovarian tumors. irILK expression was 6–9-fold higher in the serum of patients with grade 1, grade 2, and grade 3 ovarian cancer than in the serum of healthy volunteers and patients with benign ovarian tumors ($P < 0.01$). Enhanced expression of irILK in the serum of ovarian cancer patients correlated with the concentration of CA 125. High expression of irILK was present in all 10 PTF tested. Tissue-conditioned medium prepared from malignant ovarian tumors had 4-fold more irILK expression than conditioned medium obtained from borderline and benign tumors ($P < 0.01$). irILK expression in serum of cancer patients was reduced to basal normal levels after six cycles of Taxol/carboplatin and was consistent with the change of CA 125 levels before and after chemotherapy.**

Conclusions: **These data suggest that irILK is an ovarian tumor-associated antigen and implicates its potential not only as a biomarker for early-stage screening but also as a marker for monitoring the clinical condition of patients after treatment.**

INTRODUCTION

Epithelial ovarian cancer is a major cause of cancer morbidity and mortality in women. The identification of serum biomarkers suitable for early-stage detection and diagnosis holds great promise to improve the clinical outcome of such patients. The 5-year survival for women diagnosed with early-stage ovarian cancer is 90%, whereas it is only 40% for women diagnosed at late stage (stage 3 or 4). Despite considerable effort directed at early-stage screening, no cost effective screening test has been developed (1), and 75% of women still present with disseminated disease at diagnosis (2).

Cancer antigen 125 (CA 125) is the best-characterized serum marker for advanced stage ovarian cancer (3). CA 125 is elevated in >90% of patients with stage 3 and 4 ovarian cancer, whereas only 50% of patients with stage 1 disease show elevated levels. Hence, CA 125 has insufficient sensitivity to be used as a population-based screening marker for early detection and diagnosis. Hence, there is a critical need for new serological tumor markers that individually or in combination with other markers may deliver the required sensitivity for both early detection and in treatment monitoring.

Invasion and subsequent dissemination of cancer cells require loss of adhesion between tumor cells and extracellular matrix. These processes are mediated by transmembrane receptors called integrins (4). Integrins are intimately associated with the cytoskeleton through the cytoplasmic domain of the β subunit (5, 6). Integrin-linked kinase (ILK) is a serine-threonine kinase and has been demonstrated to associate with the cytoplasmic domains of integrins β1 and β3 (7, 8). ILK is widely expressed in a broad range of human tissues and cells of all lineages (9), and it has been implicated in the cellular control of cell-extracellular matrix interactions and cell proliferation (10, 11).

The present study demonstrates the presence of cell-free immunoreactive (ir)ILK in the serum of ovarian cancer patients. To our knowledge, this is the first study that demonstrates irILK in the biological fluids of the cancer patients. Compared with serum from healthy volunteers and women with benign tumors, irILK expression was significantly elevated in the serum of patients with ovarian cancer. irILK was also detected in the peritoneal fluid (PTF) of cancer patients. We also demonstrate that serum irILK expression was decreased by six cycles of chemotherapeutic treatment and was complementary to the change of CA 125 values before and after treatment. Our data suggest that ILK may have potential clinical value as a candi-

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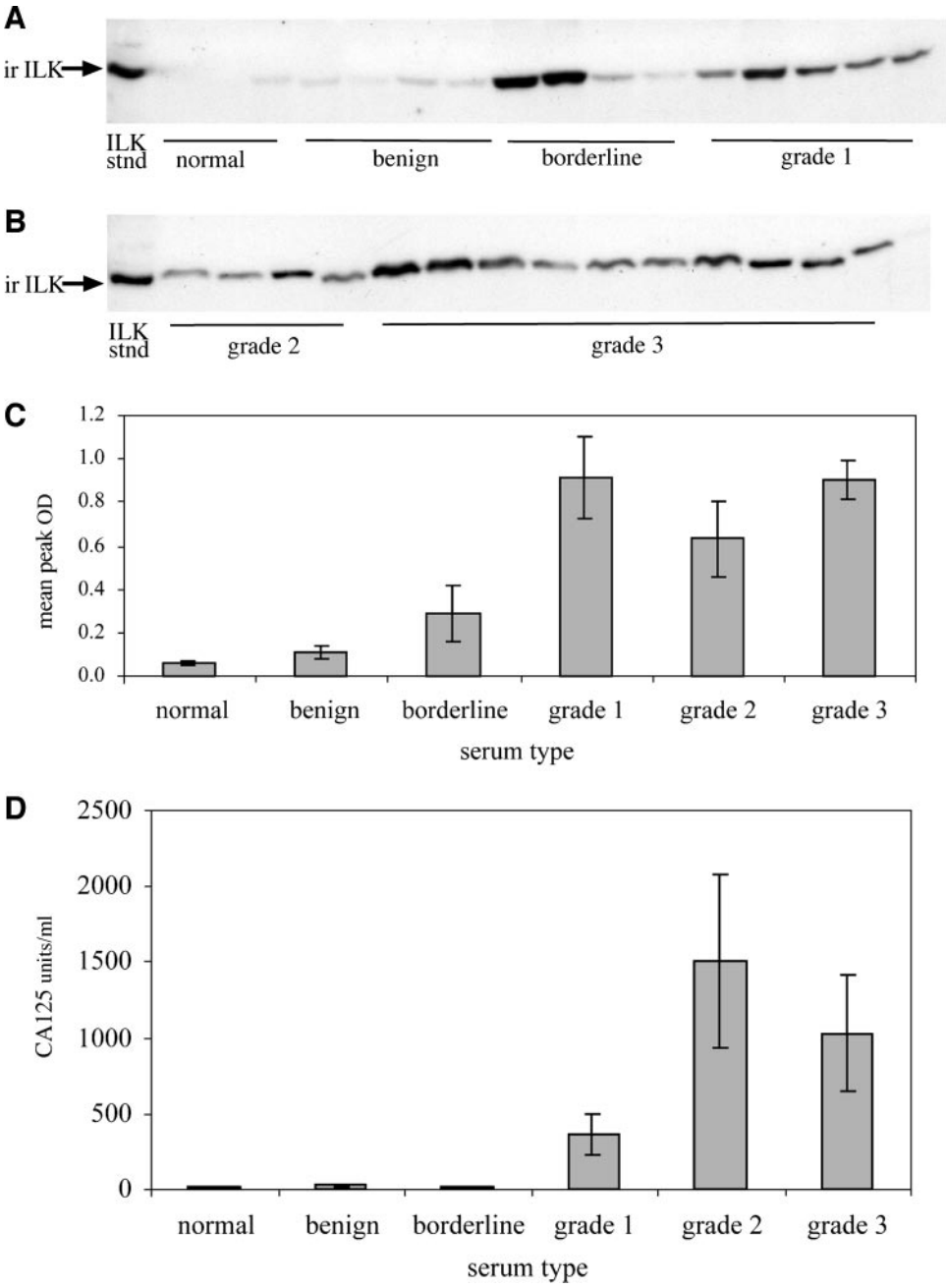

Fig. 1 Expression of immunoreactive integrin-linked kinase (irILK) in human serum. *A* and *B*, serum samples containing equal amounts of protein (50 μg) were electrophoresed on 10% SDS-PAGE gels under nonreducing conditions and transferred to nitrocellulose membranes. Membranes were probed with anti-ILK (Upstate Biotechnology, Lake Placid, NY) followed by peroxidase-labeled secondary antibody and visualized by the enhanced chemiluminescence. The number of samples in each group was normal ($n = 6$), benign ($n = 6$), borderline ($n = 4$), grade 1 ($n = 5$), grade 2 ($n = 5$), and grade 3 ($n = 10$). The results are representative of one experiment repeated three times. *C*, quantification of irILK expression was performed by densitometry and expressed as mean peak optical density (OD) ± SE of the number of samples described in each group. Results are representative of one experiment. The experiment was repeated three times. *D*, the quantitative determination of cancer antigen 125 (CA 125) levels in matching serum samples (*A* and *B*) was determined by using ACS: 180 OV Automated Chemiluminescence Systems. Results are represented as mean ± SE of the number of samples described in each group.

date marker for screening and in monitoring response to therapy in ovarian cancer.

MATERIALS AND METHODS

The study was approved by the Royal Women's Hospital, Melbourne, Research and Human Ethics Committee (Human Ethics Committee 02/29 and 02/30). Human blood was collected from healthy volunteers ($n = 6$) and patients ($n = 30$) presenting at the Oncology/Dysplasia Unit, Royal Women's Hospital, Melbourne, after the provision of a participant information statement and with informed consent. The mean age of healthy volunteers participating in the study was 47 years while that of women presenting with ovarian cancer was 62 years. All 4 patients with borderline tumors had stage 1 disease. Of the 5 patients with grade 1 cancer, 3 had stage 1, 1 had stage 2, and 1 had stage 3 disease. All patients with grade 2 and grade 3 ovarian cancer had stage 3 disease.

Ovarian cancer patients with serous, mucinous, endometrioid, and clear cell carcinoma subtypes were included in the study. Of the 6 patients in benign group, 4 were diagnosed with serous benign tumor and the other 2 had mucinous benign tumors. In the borderline group, 3 patients had serous and 1 had

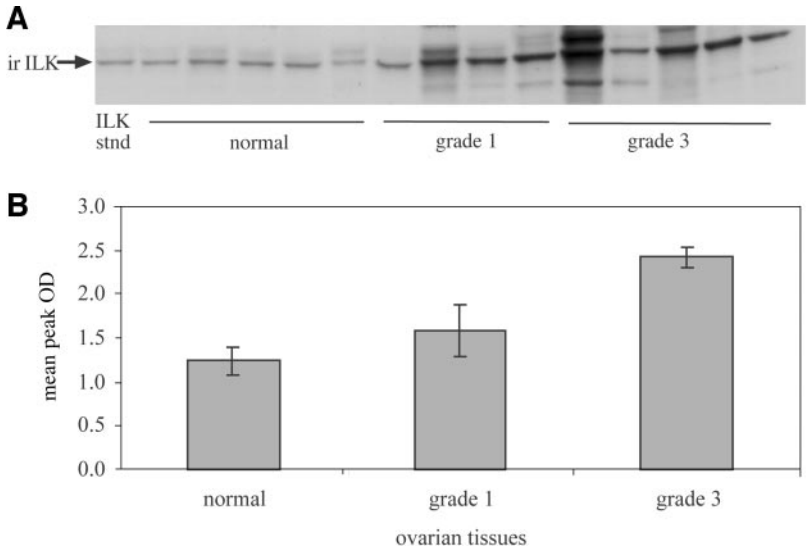

Fig. 2 Expression of immunoreactive integrin-linked kinase (irILK) in ovarian tumors. *A,* frozen ovarian tissue specimen was homogenized and centrifuged, and Western blotting was performed on the supernatant as described in "Materials and Methods." Seven μg of protein were loaded for each tissue homogenate. The results are representative of one experiment repeated three times. *B,* quantification of irILK expression in tissues was performed by densitometry and expressed as mean peak optical density (OD) ± SE of the number of samples described in each group. Results are representative of one experiment repeated three times.

mucinous tumor subtype. Of the patients in grade 1, 1 was diagnosed with serous, 2 with mucinous, and the other 2 with endometrioid subtypes of tumor. Grade 2 patients constituted of 3 serous and 2 endometrioid subtypes, whereas 6 serous, 3 endometrioid, and 1 clear cell carcinoma patients were included in grade 3. Of the 10 PTF specimens included in the study, 7 belonged to women with serous cyst adenocarcinoma, and 3 specimens were from patients with endometrioid carcinoma. All these women, except 1 with endometrioid carcinoma, were diagnosed as stage 3. The 1 patient with endometrioid carcinoma was staged as 1b. Peritoneal washings from women with benign ovarian cysts was collected from women with serous and mucinous benign cysts of the ovary (2 in each group).

Whole blood (10 ml) was collected by venipuncture into plain collection tubes for serum (blood was allowed to clot at room temperature for 30 min). Samples were centrifuged at 2000 × *g* for 10 min, after which, serum was collected. An aliquot (100 μl) was removed for the determination of total protein. Serum was stored at −80°C until analyzed.

Resected tissues not required for clinical analysis were obtained from patients who presented for surgery at the Royal Women's Hospital, Melbourne, after the provision of a participant information statement and only with informed consent. Normal ovaries, needed for control comparisons, were removed from patients undergoing surgery as a result of suspicious ultrasound images, palpable abdominal masses, and family history. The pathology diagnosis and tumor grade was determined by two staff pathologists in the Department of Pathology, Royal Women's Hospital, Melbourne. The histological classification of the tumors was performed by the method described by Silverberg (12). Of the five grade 1 tumors tissues, two were of

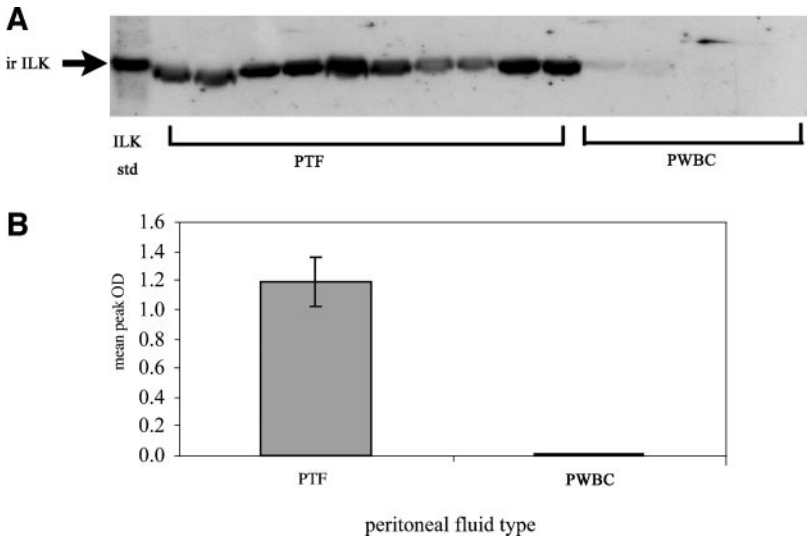

Fig. 3 Expression of immunoreactive integrin-linked kinase (irILK) in peritoneal fluid (PTF) and peritoneal washings from women with benign ovarian cysts (PWBC) of ovarian cancer patients. *A,* PTF ($n = 10$) and PWBC ($n = 4$) samples containing equal amounts of protein (50 μg) were analyzed by Western blotting as described in "Materials and Methods." The results are representative of one experiment repeated three times. *B,* quantification of irILK expression in PTF and BCF was performed by densitometry and expressed as mean peak optical density (OD) ± SE of the number of samples described in each group. Results are representative of one experiment repeated three times.

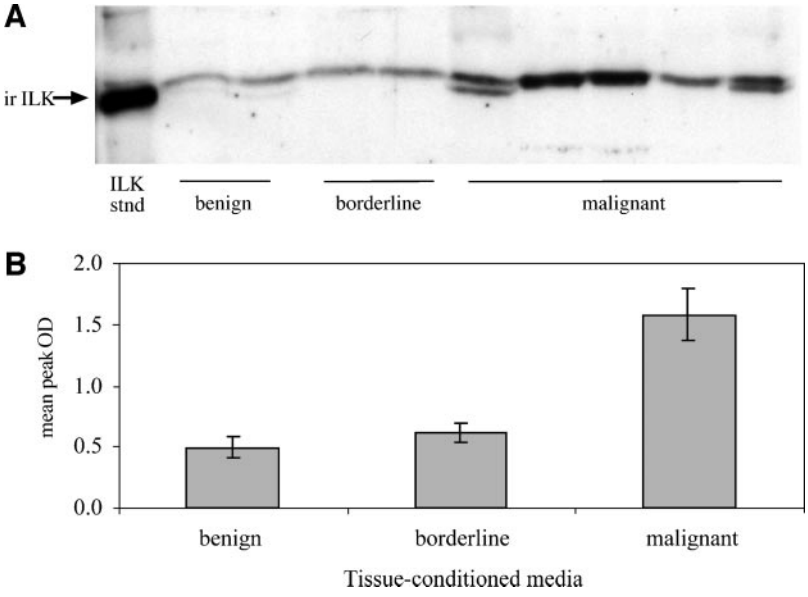

Fig. 4 Immunoreactive integrin-linked kinase (irILK) expression in tissue-conditioned medium. *A,* tissue-conditioned medium was prepared as described in "Materials and Methods." Samples were analyzed by Western blotting using ILK antibody. The results are representative of one experiment repeated three times. *B,* quantification of irILK expression in tissue-conditioned medium was performed by densitometry and expressed as mean peak optical density (OD) ± SE of the number of samples described in each group. Results are representative of one experiment repeated three times.

mucinous, and three were of serous origin. Five serous and three endometrioid tumor tissues constituted the grade 3 group.

Protein Assay. Total protein content was determined using a commercial protein assay kit with BSA standards according to the manufacturer's instruction (Pierce, Rockford, IL).

Western Blotting. Serum samples containing equal amounts of protein were electrophoresed on 10% SDS-PAGE gels under nonreducing conditions. The expression of irILK in the serum, PTF, and peritoneal washings from women with benign ovarian cysts was determined as described previously (13). Frozen ovarian tissue specimen were cut into small pieces (0.1 mg) and homogenized in Tris-HCl buffer [10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotonin (pH 7.0)] by repeated uniform strokes (approximately six). Samples were centrifuged at 10,000 × *g* for 20 min. The supernatant was recovered, and relative protein concentration was determined. At this stage, the samples can be stored at −20°C and are stable for months. Western blotting as described above determined the ILK expression of the tissue samples with equal protein loads.

Preparation of Tissue-Conditioned Medium. A fresh ovarian tissue specimen was cut into small pieces (0.1 mg wet weight) and incubated in 3 ml of serum-free RPMI 1640 supplemented with 2 mM glutamine (JRH Biosciences, Melbourne, Australia) and 100 μg/ml of penicillin/streptomycin (JRH Biosciences) for 48 h in humidified 5% CO_2 incubator. The tissue-conditioned medium was collected by centrifugation and concentrated 20–30-fold by using Biomax Ultrafree Centrifugal Filter Unit (Millipore, Bedford, MA) with a 10-kDa pore diameter cutoff. Samples with equal protein load were analyzed by Western blotting to determine the expression of irILK in the tissue-conditioned medium of primary cultures.

Determination of CA 125 Value. CA 125 values were determined by using ACS:180 OV Automated Chemiluminescence Systems (Bayer, Fernwald, Germany).

Chemotherapeutic Treatment. Patients diagnosed with ovarian cancer were treated with combination therapy consisting of carboplatin (AUC 5)/Taxol (175 mg/m^{2}body weight) after surgery. The combination drugs were given to patients every 3 weeks by i.v. infusion. Each patient underwent six cycles of chemotherapy, and CA 125 values were determined before and after each cycle.

Statistical Methods. Student's *t* test was used for statistical analyses. Statistical significance was indicated by $P < 0.05$. Data are presented as means ± SE. To determine variation of irILK expression in the serum of normal, benign, and four groups of ovarian cancer patients (borderline, grade 1, grade 2, and grade 3), Friedman nonparametric two-way analysis and Kruskal-Wallis one-way analysis were performed.

RESULTS

The mean serum irILK expression for grades 1, 2, and 3 disease was 6–9-fold higher than in control subjects ($P < 0.01$; Fig. 1*A–C*). Notably women with borderline tumors had 2-fold higher irILK expression than the control group. To determine variation of irILK expression in the serum of normal, benign, and four groups of ovarian cancer patients (borderline, grade 1, grade 2, and grade 3), Friedman nonparametric two-way analysis and Kruskal-Wallis one-way analysis were undertaken. The results indicate that there is a significant difference in the irILK expression among the groups ($\chi^2 = 14$, $df = 5$, $P = 0.016$). The average association between variables was determined by Kendall's coefficient of concordance = 0.700. Similar results were obtained with Kruskal-Wallis test ($\chi^2 = 16.06$, $df = 5$, $P = 0.007$).

Enhanced serum expression of irILK in the patients with ovarian cancer correlated with elevated serum CA 125 concentrations (Fig. 1*D*). Enhanced expression of ILK was also observed in ovarian tumor tissues, but it was not so pronounced as in the serum (Fig. 2, *A* and *B*). Compared with normal ovaries,

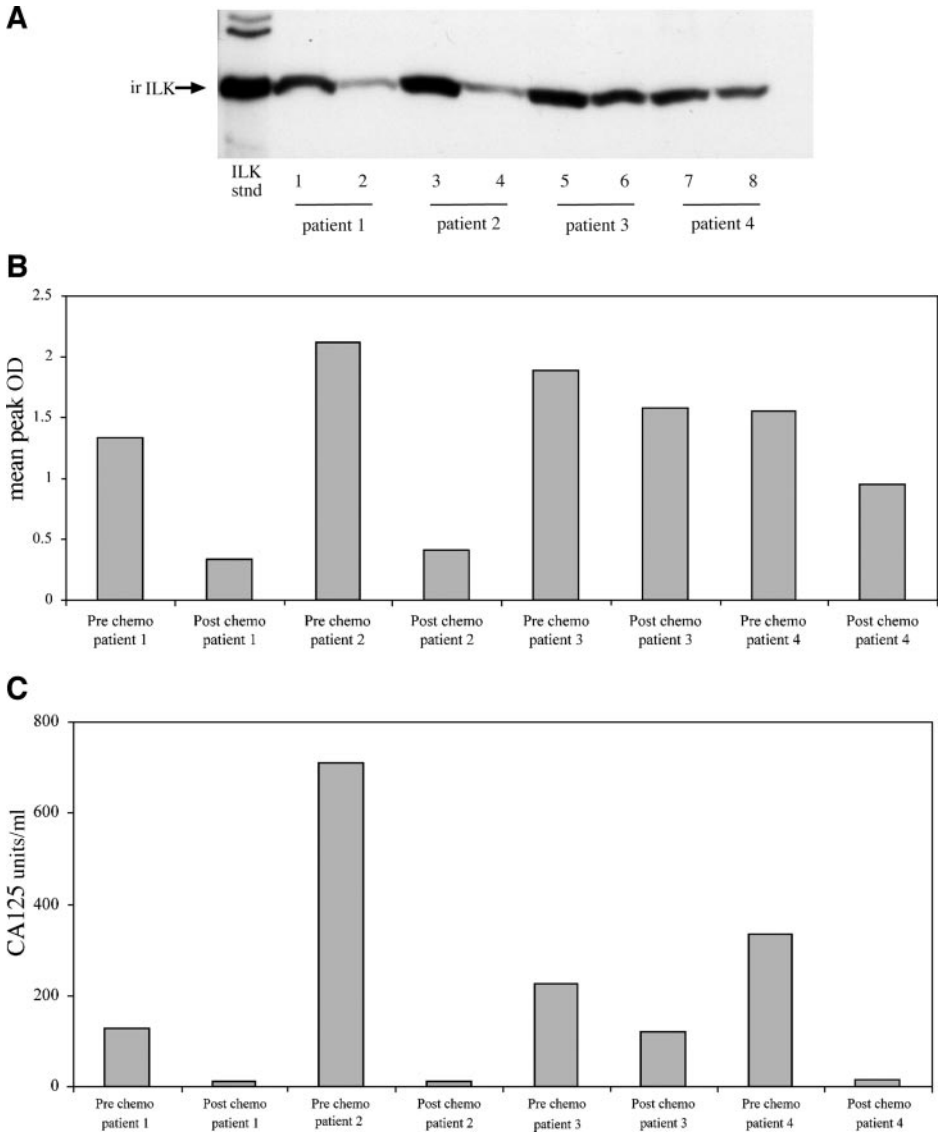

Fig. 5 Effect of chemotherapy on the expression of immunoreactive integrin-linked kinase (irILK) in the serum of ovarian cancer patients. *A*, matching serum samples from patients with grade 1 ovarian cancer before and after six cycles of carboplatin/Taxol were resolved by Western blotting. *Lanes 1*, *3*, and *7* are serum samples from patients diagnosed with grade 1 ovarian cancer before the surgery. *Lanes 2*, *4*, and *8* are the matching serum samples from the same patients after surgery and six cycles of chemotherapy. *Lanes 5* and *6* are matching serum samples from a patient diagnosed with grade 1 ovarian cancer before and after surgery and two cycles post chemotherapy. *B*, quantification of irILK expression in the serum samples was performed by densitometry and expressed as peak optical density (OD) ± SE of the number of samples described above. *C*, the quantitative determination of cancer antigen 125 (CA 125) levels in the matching serum samples of patients with grade 1 ovarian cancer.

the expression of ILK was enhanced by 1.3-fold in grade 1 and 1.6-fold ($P > 0.01$) in grade 3 ovarian tumor tissues (Fig. 2, *A* and *B*).

As peritoneum constitutes the tumor host microenvironment and is the first site to accumulate proteins produced by the tumor, we determined the expression of irILK in the PTF of the cancer patients. Significant expression of irILK was present in the PTF of patients with grade 3 ovarian cancer ($n = 10$), whereas some basal expression can be detected in two of the peritoneal washings from women with benign ovarian cysts samples whereas the other two were negative for irILK expression (Fig. 3, *A* and *B*; $P < 0.01$). The basal expression of irILK in the two peritoneal washings from women with benign ovarian cysts may result due to the contaminating blood in the specimen. No irILK expression was detected in benign cystic fluid obtained from women with benign cysts of the ovaries (data not shown). To determine whether ovarian tumors contribute to the presence of irILK in the peritoneum and subsequently in the serum of cancer patients, the expression of irILK was determined in the tissue-conditioned medium (concentrated 20–25-fold) prepared from primary ovarian tumors obtained from benign, borderline, and grade 3 cancer patients. Conditioned medium from benign and borderline tumors showed expression of irILK, but it was 8-fold lower than the expression determined in the tissue-conditioned medium of malignant tumors (Fig. 4, *A* and *B*; $P < 0.01$).

To determine whether chemotherapeutic treatment had any affect on the expression of irILK in the serum of ovarian cancer patients, matching samples from patients were tested for ILK expression before and after six cycles of chemotherapy. The expression of irILK was reduced after six cycles of chemotherapeutic treatment and paralleled reduction of serum concentrations of CA 125 after treatment (Fig. 5, *A* and *B*). Suppression of irILK expression after two cycles of chemotherapy was also

noted, but it was not as pronounced as after six cycles. Reduction in CA 125 concentration after chemotherapy in a four-serum specimen was greater than that compared with irILK expression, which may have been the result of a limited number of samples used in the study. Overall, the data obtained provide evidence of the potential role of irILK as a biomarker for not only early-stage screening but also depicts its usage in clinically monitoring patients after chemotherapy.

DISCUSSION

In this study, we report the identification of circulating p59 irILK in the serum of ovarian cancer patients. In addition, we show that the expression of soluble irILK is increased significantly in the serum of patients with low-grade ovarian tumors and is sustained in high-grade tumors. irILK is also observed in PTF and tissue-conditioned medium obtained from primary malignant ovarian tumors and is expressed at 4-fold lower levels in tissue-conditioned medium obtained from benign and borderline tumors. These results are consistent with the overexpression of ILK in ovarian tumor tissues that we have recently demonstrated (13) and are consistent with the hypothesis that circulating irILK is the product of overexpressed cellular ovarian tumors that is shed in the peritoneum and absorbed in the circulation.

The ILK signaling cascade is involved in tumor growth, tumorigenesis, and in the development of a metastatic phenotype (14). These events suppress apoptosis and promote and sustain cell survival by inhibiting apoptotic proteins such as Bad, caspase-9, and cell cycle transition by blocking proteolysis of cyclin D1 (15). ILK functions are, in part, mediated through a kinase activity that phosphorylates the downstream protein kinase B/Akt (16) and glycogen synthase kinase-3β (17). Recent studies in ILK null mice (18) and in Drosophila (19) have shown that the phosphorylation of protein kinase B/Akt and glycogen synthase kinase-3β is independent of ILK kinase activity. Kinase dead ILK mutants have been reported to interfere with paxillin binding rather than kinase activity itself, indicating that the kinase activity is not essential for ILK functions in cells (20). Hence, cells require threshold level of ILK expression to demonstrate ILK-mediated functions.

Results from our present study demonstrate that overexpression of ILK in ovarian tumor cells may result in its excretion in the tumor microenvironment. The role of this soluble ILK (if any) still needs to be determined. It is interesting to note that serum irILK expression distinguishes benign from borderline tumors better than CA 125 concentrations. However, CA 125 concentrations correlate better with ovarian tumor grade than irILK expression. Moreover, there is a correlation between the CA 125 and irILK in monitoring the effects of chemotherapy. The fact that serum irILK expression behaves in a similar fashion as serum CA 125 concentrations after two and six cycles of chemotherapy suggest that irILK may complement CA 125 in the follow-up of patients with ovarian cancer.

In conclusion, the data obtained in this study suggests that irILK may be a potential clinical biomarker for the monitoring and detection of ovarian cancer. Future studies to quantify changes in the circulatory concentration of irILK and its association with disease progression are required to fully assess the clinical utility of this biomarker.

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