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Leukocyte-associated immunoglobulin-like receptor-1 expressed in epithelial ovarian cancer cells and involved in cell proliferation and invasion

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ABSTRACT

Previous studies have shown that leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is expressed on most types of hamatopoietic cells and negatively regulate immune response, but the roles of LAIR-1 in tumor of the non-hematopoietic lineage have not been determined. Despite advances in therapy of epithelial ovarian cancer (EOC), many questions relating to EOC pathogenesis remain unanswered. The aim of this study was to investigate the clinical significance of LAIR-1 expression in EOC and explore the possible association between LAIR-1 and cancer. In this study, a tissue microarray containing 78 ovarian cancer cases was stained following a standard immunohistochemical protocol for LAIR-1 and the correlation of LAIR-1 expression with clinicopathologic features was assessed. LAIR-1 was detected to express in tumor cells of ovarian cancer tissues (73.1%) and EOC cell lines COC1 and HO8910, not in normal ovarian tissues. In addition, LAIR-1 expression correlates significantly with tumor grade ($p = 0.004$). Furthermore, down-regulation of LAIR-1 in HO8910 cells increased cell proliferation, colony formation and cell invasion. These data suggest that LAIR-1 has a relevant impact on EOC progression and may be helpful for a better understanding of molecular pathogenesis of cancer.

1. Introduction

Epithelial ovarian cancer (EOC) is a heterogeneous disease, comprising defined histological subtypes of serous, endometrioid, mucinous and clear cells cancer. The distinct histotypes differ with regard to their epidemiology, genetic changes, gene expression, tumor markers and responsiveness to therapy [1]. Despite advances in therapy, EOC is still the most lethal of the gynaecological cancers. The overall cure rate remains only approximately 30% due to late diagnosis and many questions relating to EOC pathogenesis are needed to be answered [1–4]. Therefore, a better understanding of molecular pathogenesis of ovarian cancer is crucial for advancing the diagnosis and treatment of this disease.

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1), also known as CD305, is a transmembrane glycoprotein carrying immunoreceptor tyrosine-based inhibition motifs (ITIM) in its cytoplasmic tail and is known to be expressed on almost all immune cells and $CD34^+$ hematopoietic progenitor cells. The extracellular matrix collagens are high affinity ligands for LAIR-1. Upon engagement of LAIR-1, the tyrosines within its ITIMs undergo phosphorylation to recruit phosphatases SHP-1, SHP-2 and C-terminal Csk, and negatively regulate the immune response and cell differentiation [5,6]. LAIR-1 has been reported to inhibit the proliferation of human primary leukemias and myeloid leukemia cell lines [7,8]. Recently, several studies demonstrated that the expression level of LAIR-1 in chronic lymphocytic leukamia (CLL) is related to the stage of disease [9,10]. LAIR-1 expression is lower in high risk CLL compared to that in intermediate-risk and low-risk

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CLL and is higher in early than in advanced CLL stages. Although these data imply that LAIR-1 may contribute to the abnormal proliferation of malignant hematopoietic cells, no information is available to date regarding the relationship between LAIR-1 expression and tumors of non-hematopoietic lineage. The aim of this study was thus to investigate the clinical significance of LAIR-1 expression in EOC and explore the possible association between LAIR-1 and cancer.

2. Materials and methods

2.1. Cell lines and culture conditions

Eight ovarian cancer (OVCA) cell lines (COC1, HO8910, OVCA433, OVCAR-3, HEY, CAOV3, A2780 and SKOV3) were obtained from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). COC1 and OVCAR-3 were maintained in the RPMI 1640 medium with 10% fetal bovine serum (FBS, Invitrogen), HO8910 in the DMEM/HG with 10% FBS, and other cell lines in the DMEM/F12 with 10% FBS.

2.2. Antibodies

Anti-LAIR1 antibody [lc12] (ab14826) was purchased from Abcam (Cambridge, MA). Purified Mouse Anti-Human LAIR-1 (clone DX26) and PE mouse Anti-Human LAIR-1 (clone DX26) and isotype-matched conjugated mAb were purchased from BD Pharmingen (San Diego, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody and CY3-conjugated goat anti-mouse antibody were purchased from Zhongshan Jinqiao Biotechnology Limited Company (Beijing, China).

2.3. Tissue microarrays and immunohistochemical staining

Immunohistochemical staining was performed on an Ovarian Cancer Tissue Microarray (No: BC11115, Xi'an Alena Biotechnology Ltd) using EliVision™ plus Kit (Maixin Biological Ltd, Fuzhou, China). The tissue microarray contains 78 ovarian carcinoma samples including 61 serous adenocarcinoma, 10 mucinous adenocarcinoma, 2 endometrioid adenocarcinoma and 5 clear cell carcinoma. And there are 10 lymph node tissues with metastatic carcinoma, 5 ovary tissue proximal to cancer and 5 normal ovary tissues included in the tissue microarray too. Following standard dewaxing, rehydration and antigen retrieval, a monoclonal mouse anti-LAIR1 antibody [lc12] (1:100) used as primary antibody was applied to tissue section over night at 4 °C. Antigen visualization was performed with diaminobenzidine (DAB-0031/1031kit, Maixin Biological, Fuzhou, China), followed by counterstaining with hematoxylin. A same-species IgG was used as isotype controls.

2.4. Immunohistochemical staining evaluation

All immunostained slides were evaluated by two independent pathologists blinded to all clinical and histopathological data. A semi-quantitative score based on staining intensity and percentage of positive cells was created by inspecting three different areas from each case. Intensity of staining was classified on a scale of 0–3 (0: no staining; 1: weak; 2: moderate; 3: strong). The percentage of positively stained cells was scored by cell count on a scale of 0–4: 0, ≤5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; 4, >75%. An immunoreactive score was derived by intensity score values plus the cell positivity score: <2, negative; 2–3, weak positive; 4–5, moderate positive; 6–7, strong positive.

2.5. Quantitative real time PCR (qRT-PCR)

Relative levels of expression of *lair-1* were detected using qRT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, USA) and reverse-transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Canada). qRT-PCR was performed using Thermo Scientific DyNAmo ColorFlash SYBR Green qPCR kit (F-416). The forward and reverse primers used for *lair-1* PCR amplification were 5′- TCTCCTCCTCCTGGTCCTCTTC-3′ and 5′-GCCTTGTCTGCTGTCCTCTCTA-3'. Housekeeping gene *GAPDH* was used as the internal control, the primers used for *GAPDH* were 5' -GTCTCCTCTGACTTCAACAGCG -3′ and 5′- ACCACCCTGTTGCTG-TAGCCAA -3'. The relative LAIR-1 mRNA expression levels were normalized against *GAPDH* using the comparative ΔΔCt method and relative fold change of gene was calculated by the equation $2^{-\Delta\Delta Ct}$.

2.6. Western blot analysis

Western blot analysis was performed to detect the expression of LAIR-1 in a panel of OVCA cell lines. Cells were lysed using RIPA lysis buffer (Beyotime, China). Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane, blocked with 5% skim milk. The membrane were probed with a monoclonal mouse anti-LAIR1 antibody [lc12] (1: 500) (ab14826) followed by HRP–conjugated goat anti-mouse antibody (1: 6000) incubation. Protein bands were detected by an ECL kit (Pierce, USA) according to the manufacturer's instructions. The immune blot of GAPDH was used as a loading control.

2.7. Flow cytometry (FCM) and confocal laser scanning microscopy (CLSM) analysis

To investigate cell location of LAIR-1 molecules, HO8910 cells and COC1 cells were stained with PE mouse Anti-Human LAIR-1 (clone DX26). After incubation for 20 min at 4 °C, cells were analyzed by FCM (EPICS XL, Beckman Coulter, USA) and CLSM (LEICA TCS SPE, Germany). To study the intracellular expression of LAIR-1 of HO8910 cells, cells were first fixed and permeabilized, followed by staining with purified mouse anti-Human LAIR-1 (1:100) (clone DX26) in a humidified chamber overnight at 4 °C. The secondary CY3-conjugated goat anti-mouse antibody (1:100) was then used and incubated for 1 h at room temperature with minimal exposure to light, cells were analyzed by CLSM. The cell nucleus was stained by Hoechst 33258 (Amresco, USA). Isotype controls were used in each experiment.

2.8. Knockdown of LAIR-1 by shRNA in HO8910 cells

The pGMLV-SC1-shRNA-LAIR-1 vector was constructed according to the manufacturer's instructions (pGMLV-SC1, a small hairpin RNA (shRNA)i Vector, Shanghai Genomeditch Co. Ltd). Four shRNA-LAIR-1 lentiviral vectors were generated to knockdown the expression of LAIR-1 in HO8910 cells (shRNA-LAIR-1). The four shRNA targeting sequences for LAIR-1 were as follows: 1: 5′-CCGTCGGACAACAGTCACAAT-3′; 2: 5′-GACCTGGCTGTTGATGTTCTA-3′; 3: 5′-GGAGAGTAGATCCACATACAA-3′; 4: 5′-GGCCTTATCGCTG-CATCTATT-3′. Four LAIR-1 shRNA double-stranded oligonucleotides were formed using following primers, 1F: 5′-gatccGCCGTCGGA-CAACAGTCACAATTTCAAGAGAATTGTGACTGTTGTCCGACGGTTTTT
Tg-3′, 1R: 5′-aattcAAAAAACCGTCGGACAACAGTCACAATTCTCTTGAA
ATTGTGACTGTTGTCCGACGGCg-3′; 2F: 5′-gatccGACCTGGCTGTT-GATGTTCTATTCAAGAGATAGAACATCAACAGCCAGGTCTTTTTTg-3′,
2R: 5′-aattcAAAAAAGACCTGGCTGTTGATGTTCTATCTCTTGAATAGA
ACATCAACAGCCAGGTCg-3′; 3F: 5′-gatccGGAGAGTAGATCCACATA-

CAATTCAAGAGATTGTATGTGGATCTACTCTCCTTTTTTg-3′, 3R: 5′-aattcAAAAAAGGAGAGTAGATCCACATACAATCTCTTGAATTGTATGTGGATCTACTCTCCg-3′ and 4F: 5′-gatccGGCCTTATCGCTGCATCTATTAGAGAACTTAATAGATGCAGCGATAAGGCCTTTTTTg-3′, 4R: 5′-aattcAAAAAAGGCCTTATCGCTGCATCTATTAAGTTCTCTAATAGATGCAGCGATAAGGCCg-3′) and cloned into lentiviral pGMLV-SC1 vectors. Nonsense scrambled oligonucleotide (TTCTCCGAACGTGTCACGT) was used as a control (Control shRNA). Recombinant lentiviruses were produced by transient transfection of 293T cells with LAIR-1-shRNA lentiviral vectors and used to infect HO8910 cells. Four stable HO8910 cell lines with LAIR-1 shRNA knockdown (LAIR-1 shRNA1-4) were obtained. The efficiency of LAIR-1 knockdown was confirmed by qRT-PCR and Western blotting.

2.9. Cells proliferation assay and apoptosis analysis

Cell Proliferation was determined using a CCK8-based cell proliferation assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. For analysis of induction of apoptosis, cells were stained with Annexin V/PI (BD Pharmingen) according to the product instructions. Cells were then analyzed for apoptosis by flow cytometry.

2.10. Colony formation assay

800 cells were suspended in 3 ml of DMEM medium, plated in a well of six-well plate, and then incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 14 days. Then, the colonies were fixed with paraformaldehyde, stained with Giemsa (ECM550, Chemicon) and counted.

2.11. Cell invasion assay

A BD Biocoat™ Matrigel™ Invasion Chamber (BD Biosciences Cat. No. 354480) was used for the invasion assay. The cells were resuspended in serum-free DMEM at 2×10^5 cells per ml. 500 μl cell suspensions were placed in the upper chambers, and 700 μl medium with 30% FBS was filled in the lower chambers. After 24 or 48 h, non-invased cells on the upper surface of the filters were removed by wiping with a cotton swab and cells on the lower surface of the membranes were stained with Giemsa dye. Three different fields of the stained cells were photographed and counted for each transwell filter.

2.12. Statistical analysis

Data were expressed as mean ± SD. Student's t test was used to compare the values between subgroups. Crosstabs and Pearson χ^2 test were used to test the correlation between the expression levels of LAIR-1 and clinical parameters. The statistical analyses were performed using the SPSS 16.0 software (SPSS). A P value of <0.05 was considered significant.

3. Results

3.1. Expression of LAIR-1 and their correlation with clinicopathologic variables in EOC

Immunohistochemical results showed that LAIR-1 was mainly expressed in the membrane and cytoplasm of carcinoma cells in 73.1% (57/78) EOC samples. In contrast, LAIR-1 expression was not detected in normal ovarian tissues ($n = 5$) and ovarian tissues proximal to cancer ($n = 5$). Besides, 17.9% (14/78) of tumor samples contained small numbers of LAIR-1-positive tumor infiltrating lymphocytes (TIL). Representative IHC staining images are shown in Fig. 1. The clinicopathologic analysis showed that the expression of LAIR-1 in tumor cells was significantly correlated with tumor grade (coefficients: 0.338, $P = 0.004$). A high expression level of LAIR-1 was associated with a poor differentiation, and a low expression level was associated with a well differentiation. Other clinical parameters including age, histologic subtypes and stages showed no significant correlation with LAIR-1 expressions. LAIR-1

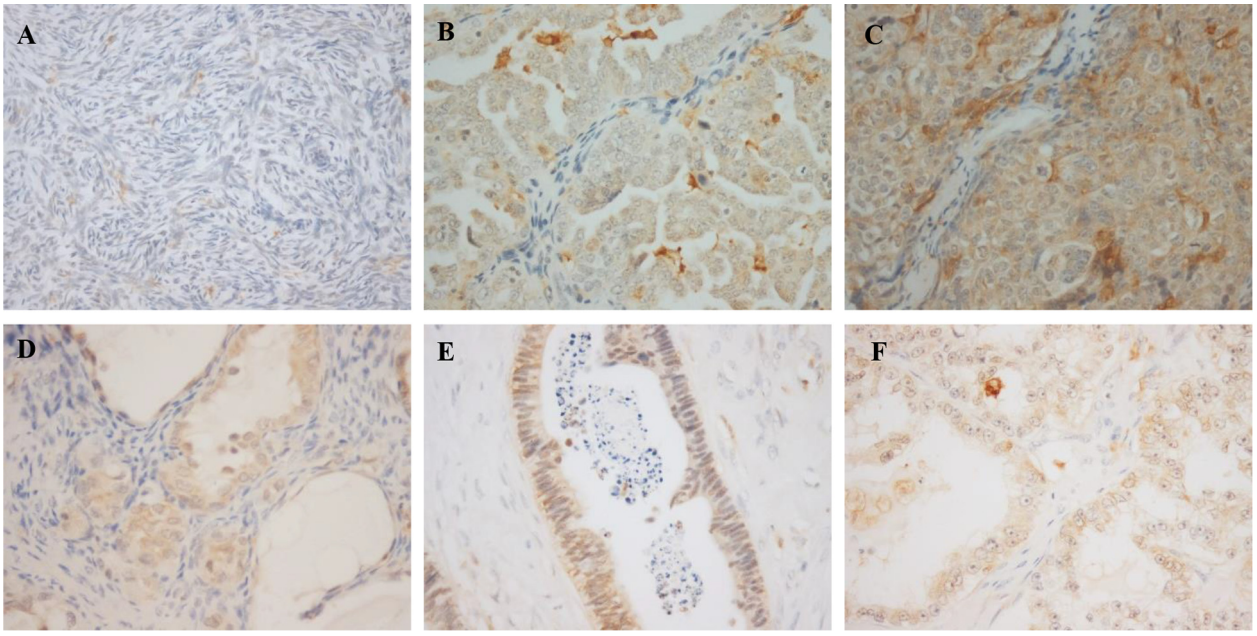

Fig. 1. Immunohistochemical analysis of LAIR-1 in ovarian tissue and ovarian carcinomas. Representative examples of immunohistochemical results: (A) LAIR-1 is not expressed in normal ovarian tissue; (B) LAIR-1 is weakly expressed in tumor cells of high-grade serous epithelial ovarian cancer (EOC); (C) LAIR-1 is strongly expressed in tumor cells of low-grade serous EOC; (D) LAIR-1 is moderately expressed in tumor cells of mucinous EOC; (E) LAIR-1 is weakly expressed in tumor cells of endometrial EOC; (F) LAIR-1 is weakly expressed in tumor cells of clear cell EOC. Magnification 400×.

Table 1
Correlation between LAIR-1 and clinicopathological characteristics in 78 EOC specimens.

Variables	Total	LAIR-1 expression						
		None	Weak	Moderate	Strong	Positive rate%	Correlation	*P*-value
Age (Year)								
< 50	40	11	21	3	5	72.5	−0.024	0.835
≥50	38	10	22	3	3	73.7		
Histologic type							−0.183	0.108
Serous	61	15	33	6	7	75.4		
Mucinous	10	5	5	0	0	50.0		
Clear cells	5	1	3	0	1	80.8		
Endometrioid	2	0	2	0	0	100.0		
FIGO stage							0.037	0.750
I	45	15	21	4	5	66.7		
II	17	1	12	1	3	94.1		
III	13	5	8	0	0	61.6		
IV	3	0	2	1	0	100.0		
Grade								
G1	18	10	7	1	0	44.4	0.338	**0.004**
G2	18	3	13	1	1	83.3		
G3	35	7	18	4	6	80.0		

P-values of $\chi 2$-tests are indicated; bold, statistically significant ($P < 0.05$).

expression and clinicopathologic parameters in 78 EOC specimens are summarized in Table 1.

3.2. Detection of LAIR-1 expression in human OVCA cell lines

Expression of LAIR-1 in eight OVCA cell lines (COC1, HO8910, HEY, OVCA433, OVCAR-3, CAOV3, A2780 and SKOV3) were analyzed by qRT-PCR and western blotting. qRT-PCR results showed that COC1 and HO8910 cells exhibited a higher LAIR-1 mRNA expression level compared with six other cell lines (Fig. 2A). Consistent with the results of qRT-PCR analysis, western blotting showed the different level of LAIR-1 protein in these cell lines, with remarkably higher level in COC1 and HO8910 cells (Fig. 1B). The cell location of LAIR-1 molecules was further investigated on COC1 and HO8910 cells using FCM and CLSM analysis. Interestingly, we found LAIR-1 proteins were localized in both the plasma membrane and cytoplasm of COC1 cells, while predominately localized in the cytoplasm of HO8910 cells (Fig. 2C and D). This finding was consistent with immunohistochemical data indicating that LAIR-1 resides within cytoplasmic compartments or on the plasma membrane of the tumor cells in EOC tissues of different subtypes.

3.3. Knockdown of LAIR-1 promotes the proliferation, colony formation and the invasive ability of HO8910 cells

To reveal the role of LAIR-1 in ovarian cancer cell biology, we knocked down *lair-1* using lentiviral-mediated shRNA constructs in the HO8910 cells based on their relatively high endogenous *lair-1*

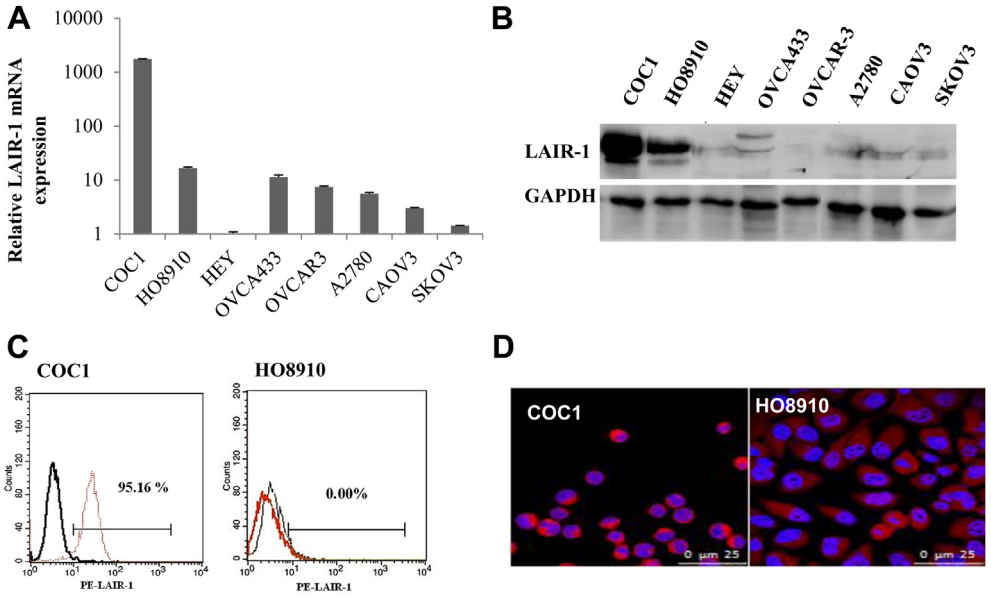

Fig. 2. Expression of LAIR-1 in OVCA cell lines. (A) qRT-PCR analysis showed the different expression level of LAIR-1 mRNA in eight OVCA cell lines. (B) Western blotting analysis revealed the different expression level of LAIR-1 protein in the eight OVCA cell lines, which confirmed that COC1 and HO8910 cell lines had higher expression of LAIR-1 compared with the other six OVCA cell lines. (C) Flow cytometry analysis of LAIR-1 expression on COC1 and HO8910. (D) Confocal microscopic analyses confirmed LAIR-1 was predominately localized in the cytoplasm of HO8910 cells, and the cell membrane and cytoplasm of COC1 cells. Representative COC1 cells were stained with the PE-conjugated anti-LAIR-1 mAb (red). Representative HO8910 cells were stained with purified mouse Anti-Human LAIR-1 (1:100) followed by the secondary CY3-conjugated goat anti-mouse antibody (Red). Cell nucleus was stained with Hoechst 33258 (blue). Scale bars indicate 25 μm. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

expression. Four stable HO8910 cells with *lair-1* knockdown (LAIR-1 shRNA1-4) were obtained. The efficiency of *lair-1* knockdown was confirmed by qRT–PCR (Fig. 3A) and western blotting (Fig. 3B). According to the knockdown efficiency of *lair-1*, the LAIR-1 shRNA4 was chosen to study the function in HO8910 cells.

We performed proliferation, soft agar colony formation and cell invasion assay on cell lines HO8910. Inhibition of LAIR-1 expression in HO8910 cells using LAIR-1 shRNA (Scramble shRNA as negative control) resulted in no significant difference in cell apoptosis (data not shown), but significantly promotes cell proliferation (Fig. 3C), the colony formation (Fig. 3D) and the invasive ability of HO8910 (Fig. 3E). Thus, endogenous LAIR-1 may play an essential role in ovarian cancer progression.

4. Discussion

Since its discovery, LAIR-1 has been reported to be mainly expressed in hamatopoietic cells and to function as an inhibitory receptor. Multiple transmembrane and extracellular matrix collagens are high affinity ligands for LAIR-1 [11,12]. In this study, the expression of LAIR-1 was observed in tumor cells of four histotypes of EOC and some OVCA cell lines. Together with the result that the expression of LAIR-1 correlates to the tumor grade of EOC suggests that LAIR-1 may facilitate cell adhesion to collagens in tumor microenvironment, and be involved in solid tumor progress or metastasis. Results from other reports have already proved that engagement of LAIR-1 could inhibit the proliferation of myeloid leukemia cell lines [7] and AML blasts [8], and that the lack of LAIR-1 expression in high-risk CLL results in the absence of a negative signal that regulates kinase activation and cell division [9]. Our results show that LAIR-1 could inhibit the proliferation, colony formation and cell invasion of ovarian cell line and indicate that LAIR-1 may play a negative regulatory role in tumor biology.

Previous studies have demonstrated that a higher level of LAIR-1 expression is associated with a less-differentiated phenotype in several types of hamatopoietic cells [11,13–15]. In the present study, we show that LAIR-1 is expressed in tumor cells of majority EOC samples of four distinct histology. Moreover, the expression level of LAIR-1 is positively correlated to tumor grade which indicates the higher the LAIR-1 expression, the poorer the tumor differentiation. These results suggest that LAIR-1 may be a potential biomarker for EOC diagnosis and prognosis.

Our previous studies showed that the soluble level of LAIR-1 in sera from tumor patients was significantly higher than that in healthy individuals and the expression of LAIR-1 on the $CD3^+CD4^+$ T cells or $CD3^+CD8^+$ T cells in the peripheral blood of cervical cancer and endometrial carcinoma patients was significant greater than that in patients with hysteromyom or precancerous lesion [16]. A recent study has reported that tumor-expressed collagens can bind to LAIR-1 and trigger immune inhibitory signaling to inhibit the function of NK cells, a possible mechanism to evade anti-cancer immune response [17]. Recently, high expression of LAIR-1 in tumor-associated DCs was also observed in mouse models of ovarian cancer which suggests that signaling through

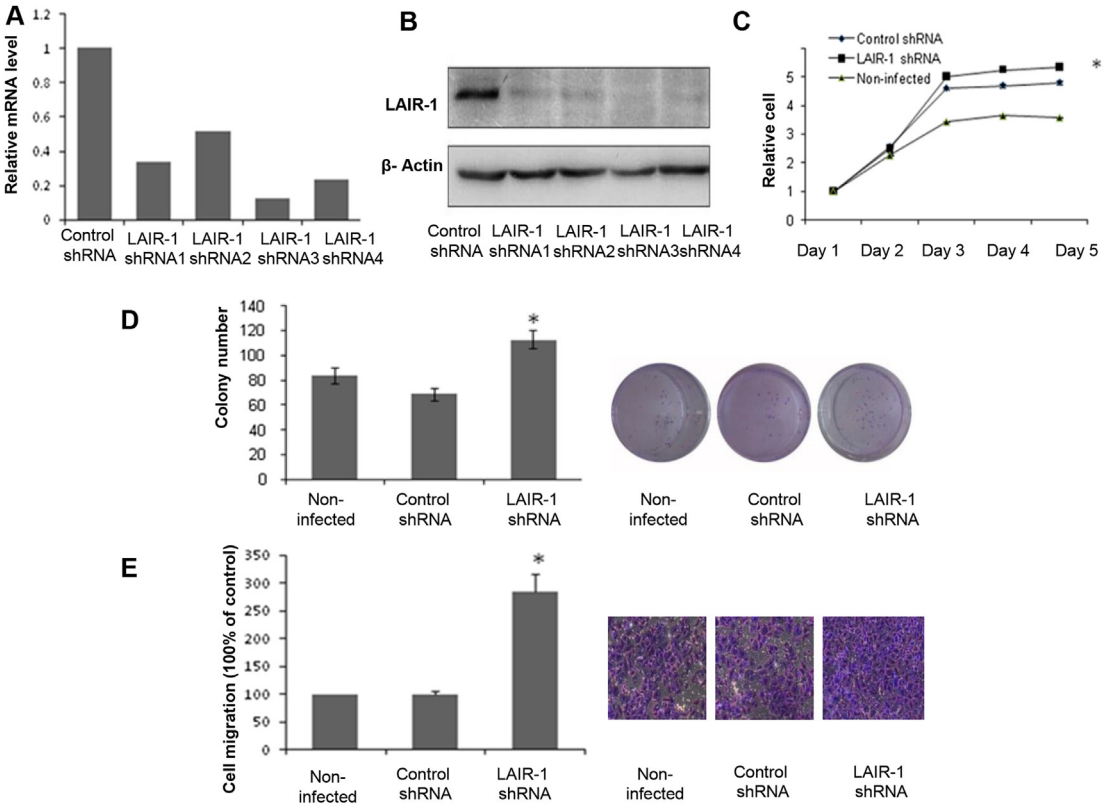

Fig. 3. The biological effects of LAIR-1 on HO8910 cells. (A) Efficiency of LAIR-1 knockdown level in OVCA cell line HO8910 by shRNA. Four shRNA constructs against LAIR-1 was introduced into HO8910 cells by lentiviral infection (LAIR-1 shRNA1-4) and the relative expression of LAIR-1 mRNA was measured by qRT–PCR. Scramble-shRNA was used as a control. Error bars represent mean ± SD of triplicate samples; (B) Western blot analysis confirms the qRT–PCR results and shows knockdown of LAIR-1 at the protein level; (C) CCK8 proliferation assay showed that proliferation of HO8910 were enhanced by LAIR-1 shRNA (*$P < 0.05$, LAIR-1 shRNA vs. control shRNA and non-infected cells (D) The colony formation assay revealed that LAIR-1 knock-down increased colony formation in HO8910 cells. The experiment was performed three times and reported as the mean ± SD. The asterisk indicates a significant difference between LAIR-1-shRNA HO8910 cells and negative control and non-infected HO8910 cells (E) The cell invasion assay showed that more cells of stable HO8910 cell line with LAIR-1 shRNA knockdown invaded to the detection fields compared with the control shRNA and non-infected HO8910 cells; *$P < 0.05$. Mean ± SD was from 3 independent experiments. Magnification × 100.

this molecule upon interaction with collagen produced by tumor cells might help define the poorly immunogenic status of these cells in the tumor microenvironment [18]. In this study, we also noticed that less than 20% of tumor sample contains small numbers of LAIR-1-positive lymphocytes. Thus, expression of LAIR-1 in both tumor cells and the immune cells indicates that the regulation role of LAIR-1 in tumor biology will be complicated and requires further studies.

In conclusion, we demonstrate for the first time that LAIR-1 is expressed in non-hematopoietic cancer cells. LAIR-1 was detected to express in EOC tissues and some cell lines, but not in normal epithelial ovarian cells. In addition, LAIR-1 expression correlates significantly with tumor grade. Down-regulation of LAIR-1 expression in EOC cell line by lentivirus-mediated shRNA increased cell proliferation, colony formation and cell invasion. Our findings suggest that LAIR-1 has a relevant impact on EOC progression and may be helpful for a better understanding of molecular pathogenesis of cancer. The mechanisms of LAIR-1 that influence the biological functions of tumor cells and the relationship between LAIR-1 expression and other cancer types deserve further investigation.

Conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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