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High histone H3K18 lactylation level is correlated with poor prognosis in epithelial ovarian cancer

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Protein lactylation has a poor prognosis in malignant tumors, but its impact on the prognosis of epithelial ovarian cancer (EOC) remains unknown. We analyzed 112 patients with EOC. Immunohistochemical staining was used to detect the level of pan lactylation (Pan Kla) and histone H3K18 lactylation (H3K18la) in the EOC tissues and normal ovarian tissues. The result showed that the protein lactylation level in EOC was higher than in normal tissues. Then, we analyzed the relationship between overall survival (OS), progression-free survival (PFS) of EOC, and lactylation. The result showed that patients with high histone H3K18la levels had poorer OS (p=0.028) and PFS (p<0.001). Multivariate Cox regression analysis of PFS showed histone H3K18la was an independent risk factor (p=0.001). In addition, we found that both histone H3K18la and Pan Kla in the cytoplasm were associated with platinum recurrence time (p=0.002/p=0.003). The results also indicated that the H3K18la level was related to a tumor stage (p=0.037). Furthermore, we explored the effects of lactylation on the metastasis of ovarian cancer. The results indicated a significant increase in migration in the promoter group compared to the negative control group and inhibitor group. In conclusion, high histone H3K18la level is associated with poor prognosis in EOC. Protein lactylation may have a significant impact on EOC and could potentially be used as a target for EOC therapy in the future.

Key words: histone lactylation; epithelial ovarian cancer; survival; clinical features

Ovarian cancer is the third most common gynecologic malignancy worldwide and the leading cause of death from gynecological cancer [1]. In 2020, 313,959 women were diagnosed with ovarian cancer, and 207,252 died [2]. The most prevalent type of ovarian cancer is epithelial ovarian cancer (EOC). Due to the lack of specific symptoms and effective early diagnostic methods, approximately 75% of patients are diagnosed at an advanced stage, resulting in a 5-year relative survival rate of 29% for those with advanced disease, compared to up to 92% for those with early-stage disease [1]. At present, the main treatment approach for EOC in clinical practice involves surgery, followed by chemotherapy and targeted therapies such as anti-angiogenic drugs and poly-adenosine diphosphate-ribose polymerase (PARP) inhibitors [3]. However, most patients with EOC experience relapse or develop platinum resistance at an advanced stage. Over the past few decades, the 5-year survival rate for EOC in developed countries has remained at 47% [1], while in China, it has hovered around 40% with an overall poor prognosis. Consequently, researchers have been investigating various factors that influence the prognosis of EOC, including tumor recurrence [4], stage [5], metastasis [6], and platinum resistance [7]. Therefore, it is crucial to explore additional factors that affect the prognosis of EOC to provide novel insights and treatment strategies for targeted therapy.

The Warburg effect [8] is a unique metabolic feature of tumors, characterized by the preference for glycolysis to generate energy even in the presence of oxygen, resulting in the accumulation of large amounts of lactate. As a byproduct of glycolysis, lactate has been found to have metabolic functions, such as providing energy to tumor cells [8], inhibiting the cytotoxic function of immune cells [9], and regulating natural immune signaling pathways [10]. Researchers have continuously explored the effects of lactate on tumors, and in 2019, Professor Yingming Zhao's team discovered a novel post-translational modification of proteins known as lactylation [11]. They observed that lactate, accumulated during metabolism, can serve as a precursor for lactylation modification of histone lysine, participating in the homeostatic regulation of M1 macrophages involved in bacterial infection, as well as regulating inflammation, cancer, and other diseases. Protein lactylation modifications represent an important mechanism through which lactate exerts its functions, marking the first time that the metabolite lactate has been linked to the regulation of gene expression [12, 13]. Studies have demonstrated that histone lactylation promotes the conversion of M1-type macrophages to tumor-associated M2-type macrophages by activating M2-like gene expression; histone lactylation also regulates the expression of certain metabolic enzyme genes, affecting metabolic reprogramming in non-small cell lung cancer [14]. Furthermore, lactylation levels were found to be higher in ocular melanoma tumor samples compared to normal samples, and inhibition of histone lactylation effectively suppressed tumor progression [15]. Additionally, the level of histone lactylation is correlated with poor prognosis in colon cancer patients [16]. These findings suggest that histone lactylation modification may be a negative prognostic factor for patients with tumors. However, to date, lactylation modification has not been studied in EOC. Therefore, it would be interesting to investigate whether it affects the prognosis of patients with EOC.

Patients and methods

Patients and surgical specimens. This study included 112 cases of pathologically confirmed ovarian cancer tissues from Sun Yat-sen University Cancer Hospital from 2005 to 2015, and 3 cases of normal ovarian tissues from the Department of Gynecology of Guangdong Provincial People's Hospital, which were derived from ovarian tissues of patients with cervical carcinoma, patients with endometrial carcinoma, and patients with benign tumors of the ovary. The trial was approved by the Ethics Committee of Sun Yat-sen University Cancer Hospital and the Ethics Committee of Guangdong Provincial People's Hospital (Ethics approval No. S2024-678-02; SZR2021-037). The platinum-free interval is the time interval from the cessation of platinum chemotherapy to tumor recurrence or progression. Depending on the length of the platinum-free interval, a platinum-free interval of ≥ 6 months was defined as a platinum-sensitive recurrence and <6 months as a platinum-resistant recurrence.

Cell lines and cell cultures. The A2780 and OVCAR3 human ovarian cancer cell lines were kindly provided by Professor Zheng Min (Department of Gynecology, Sun Yat-Sen University Cancer Center, Guangzhou, Guangdong, China). The A2780 cell line was cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco), and the OVCAR3 cell line was cultured in RPMI-1640 medium with 10% FBS.

Immunohistochemical staining. Immunohistochemical methods were used to detect the levels of protein Pan lactylation (Pan Kla) and histone H3K18 lactylation (H3K18la) in 112 ovarian cancer tissues. Simply, paraffin-embedded sections were baked in a 65 °C oven for 2 h, dewaxed with xylene, rehydrated with concentration gradient ethanol, and the specimens inhibited endogenous peroxidase activity with 0.3% hydrogen peroxide, followed by antigen retrieval (EDTA9.0) with high pressure, and incubated overnight with pan lactylation polyclonal antibody (PTM-1401RM) and histone H3K18 lactylation monoclonal antibody (PTM-1406RM) at 4 °C. After washing, the sections were incubated with an anti-rabbit secondary antibody with the sections at room temperature for 1 h. After washing, the sections were stained with DAB colorant, 10% Meyer's hematoxylin, dehydrated, and fixed by sealing the sections with gum. Two researchers assessed the extent of immunostaining in each formalin-fixed paraffin-embedded section. Scoring was done according to cancer positivity and staining intensity. Tissues were categorized into 4 grades based on staining intensity: 0 indicates no staining, 1 indicates weak staining (light yellow), 2 indicates moderate staining (yellowish brown), and 3 indicates strong staining (brown), and cancer cell positivity was defined as the percentage of the tissue sections that were positive for different levels of staining, and the score for each slice was the multiplication of the two, giving an *H*-score range of 0–300. The optimal threshold for the level of histone H3K18la was defined as a staining score ≥175 indicates a high level of histone H3K18la, and a staining score <175 indicates a low level of histone H3K18la; the optimal critical value for the level of Pan Kla was defined as follows: in the cytoplasm, a staining score ≥125 indicates high level of Pan Kla, and <125 indicates low level of Pan Kla, and in the nucleus, a staining score ≥25 indicates high level of Pan Kla and <25 indicates a low level of Pan Kla [17].

Wound healing assay. First, the cells were digested and plated in a 6-well plate (about 5×10^5 cells/well). Overnight, the cells grew to 80–90%. The next day, scratching the cells vertically with Sterile gunhead and washing them with PBS for 3 times. Then, the serum-free medium was added to continue the culture. The cells were observed and pictures were taken at 0 h, 12 h, and 24 h respectively, and then the healing rate according to the scratch area was calculated. All these cells were divided into four groups: negative control

group, inhibitor group, promotor group, and both inhibitor and promotor group. The inhibitor used was oxamate (10 mM in the A2780 cell line, 20 mM in the OVCAR3 cell line, purchased from Jingjie PTM BioLab (Hangzhou) Co., Inc.) which inhibits glycolysis to reduce lactate. The promotor used was L-lactate (50 mM both in A2780 and OVCAR3 cell lines, purchased from Jingjie PTM BioLab (Hangzhou) Co., Inc.).

Transwell assay. A 24-well Transwell system (Corning) and polycarbonate filters (8-μm pores, Corning) were used in the Transwell assay. The cells were starved for 12–24 h in a serum-free medium. Then the cells were digested with trypsin (Gibco) and washed with PBS (Gibco) 2 times to further remove the influence of serum. The cells were resuspended in a serum-free medium and the cell density was adjusted to 5×10^5 cells/ml. Two hundred μl of cell suspension were placed in the upper compartment, and 600 μl of medium containing 10% FBS was added to the lower chamber. After 48 h of incubation, the cells in the Transwell system were fixed with 4% paraformaldehyde for 20 min, and stained with 0.25% crystal violet for 15 min. Finally, photographed under an inverted microscope. All the groups are the same as in the wound healing test.

Statistical analysis. All statistical analysis was performed using the SPSS 26.0 statistical package, and graphs were plotted using GraphPad Prism 8.0 and Adobe Illustrator. The relationships between lactylation modification levels and clinicopathological features were analyzed using the Chi-square test. In addition, the correlation between lactylation level and clinicopathological features was calculated by the Pearson correlation coefficient. The relationship between survival of EOC and lactylation level was analyzed by Kaplan-Meier (KM) curve univariate analysis, and differences were calculated by log-rank test. Cox's proportional risk regression model was applied to the multivariate analyses. A p-value of <0.05 was considered statistically significant in all analyses.

Results

Differences in the level of histone lactylation modification between normal ovarian tissue and EOC tissue. To investigate the potential difference in histone lactylation modification levels between normal ovarian tissues and EOC tissues, we performed immunohistochemical staining using pan lactylation antibody and H3K18 lactylation (H3K18la) antibody. Three normal ovarian tissue samples and 112 EOC

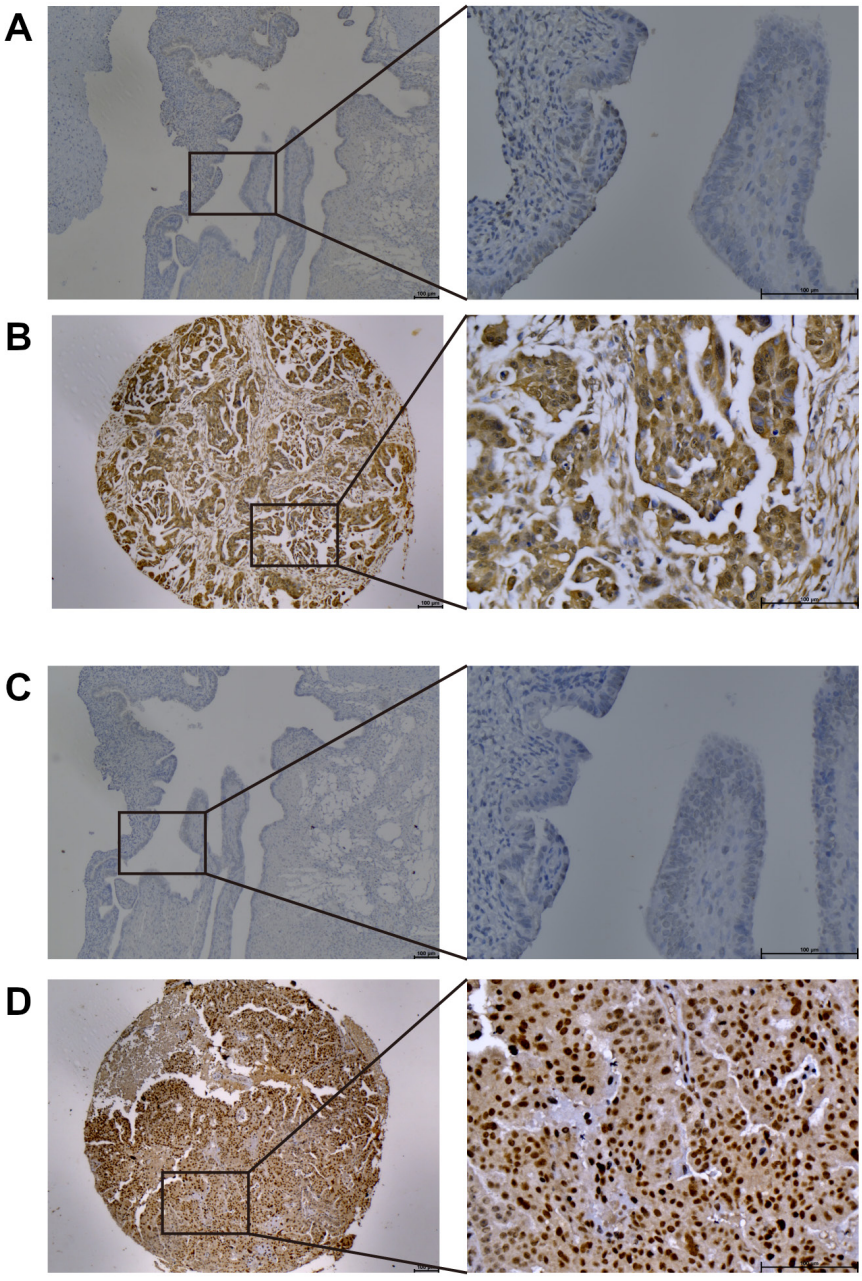

Figure 1. Lactylation level in normal ovarian tissue and EOC. A) Pan Kla in normal ovarian cancer; B) Pan Kla in EOC; C) H3K18la in normal ovarian tissue; D) H3K18la in EOC (scale bar: 100 μm).

tissue samples were analyzed. Our findings revealed that the majority of normal ovarian tissues exhibited either no staining or light staining when stained with either the pan lactylation antibody or the H3K18la antibody. In contrast, among the 112 EOC tissues, the staining results varied. For the pan lactylation antibody: in the cytoplasm, the staining fraction was ≥125 in 54 cases and <125 in 58 cases; in the nucleus, the staining fraction was ≥25 in 74 cases and <25 in 38 cases. Similarly, for the H3K18la antibody, 54 cases displayed staining scores ≥175, whereas 58 cases exhibited staining scores <175 (Figures 1A–1D). These findings demonstrated a discernible difference in histone lactylation modification levels between normal ovarian tissues and EOC tissues.

Table 1. Clinical characteristics and lactylation level in EOC.

Characteristics	Group	Number of cases	Pan Kla				H3K18-lactylation	
			Cytoplasm		Nucleus			
			Low	High	Low	High	Low	High
Age (years)	<50	53	29	24	14	39	31	22
	≥50	59	29	30	24	35	27	32
FIGO Stage	I	19	8	11	9	10	16	3
	II	16	6	10	3	13	5	11
	III	65	38	27	21	44	32	33
	IV	12	6	6	5	7	5	7
Grade	Low	20	9	11	7	13	13	7
	High	92	49	43	31	61	45	47
Lymph node metastasis	Yes	36	17	19	11	25	21	15
	No	76	41	35	27	49	37	39
Distant metastasis	Yes	17	8	9	7	10	8	9
	No	95	50	45	31	64	50	45
Platinum recurrence	Yes	51	34	17	18	33	18	33
	No	61	24	37	20	41	40	21
Platinum recurrence time	≥6 m	47	33	14	17	30	18	29
	<6 m	4	1	3	1	3	0	4
	No Rec	61	24	37	20	41	40	21
CA125 (U/ml)	<882	57	29	28	20	37	29	28
	≥ 882	55	29	26	18	37	29	26

Table 2. Pan Kla in association with clinical characteristics variables using the Chi-square test.

Characteristics	Group	Number of cases	Pan Kla in the cytoplasm		χ^2	p-value
			Low	High		
Age (years)	<50	53	29	24	0.35	0.556
	≥50	59	29	30		
FIGO stage	I	19	8	11	3.20	0.362
	II	16	6	10		
	III	65	38	27		
	IV	12	6	6		
Grade	Low	20	9	11	0.45	0.503
	High	92	49	43		
Lymph node metastasis	Yes	36	17	19	0.44	0.506
	No	76	41	35		
Distant metastasis	Yes	17	8	9	0.18	0.672
	No	95	50	45		
Platinum recurrence time	≥6 m	47	33	14	11.32	**0.003**
	<6 m	4	1	3		
	No Rec	61	24	37		
CA125 (U/ml)	<882	57	29	28	0.04	0.845
	≥882	55	29	26		

Notes: Pearson's Chi-square test and Fisher's exact test were used for analysis; p-values in bold indicate significance ($p<0.05$)

Correlation of H3K18la level with overall survival (OS) and progression-free survival (PFS) in EOC patients. To further investigate the potential impact of lactylation modification levels on the survival time of patients with EOC, we conducted a follow-up study on 112 patients with EOC. Simultaneously, immunohistochemical staining was performed on 112 ovarian cancer tissue samples and 3 normal ovarian tissue samples using two kinds of antibodies: a pan lactylation antibody and an H3K18la antibody. The follow-up data and experimental results were then combined for survival analysis, and the data are presented in Table 1. Furthermore, the results of KM survival analysis suggested that EOC patients with high levels of histone H3K18la modification had poorer OS and PFS compared to those with low levels, showing a statistically significant difference (Figures 2A, 2D). However, there was no significant difference in the OS of EOC patients regardless of the levels of lactylation in the cytoplasm or the nucleus (Figures 2B, 2C). Notably, higher levels of lactylation in the cytoplasm were associated with longer PFS (Figure 2E). Therefore, we speculate that protein lactylation in the cytoplasm and H3K18la may play different roles in EOC. The latter might potentially impact the occurrence and development of tumors by affecting the expression of oncogenes or tumor suppressor genes.

Correlation of protein lactylation level with recurrence time of EOC patients. In addition, we also analyzed the correlation between the clinical characteristics of these 112 EOC patients (Table 1)

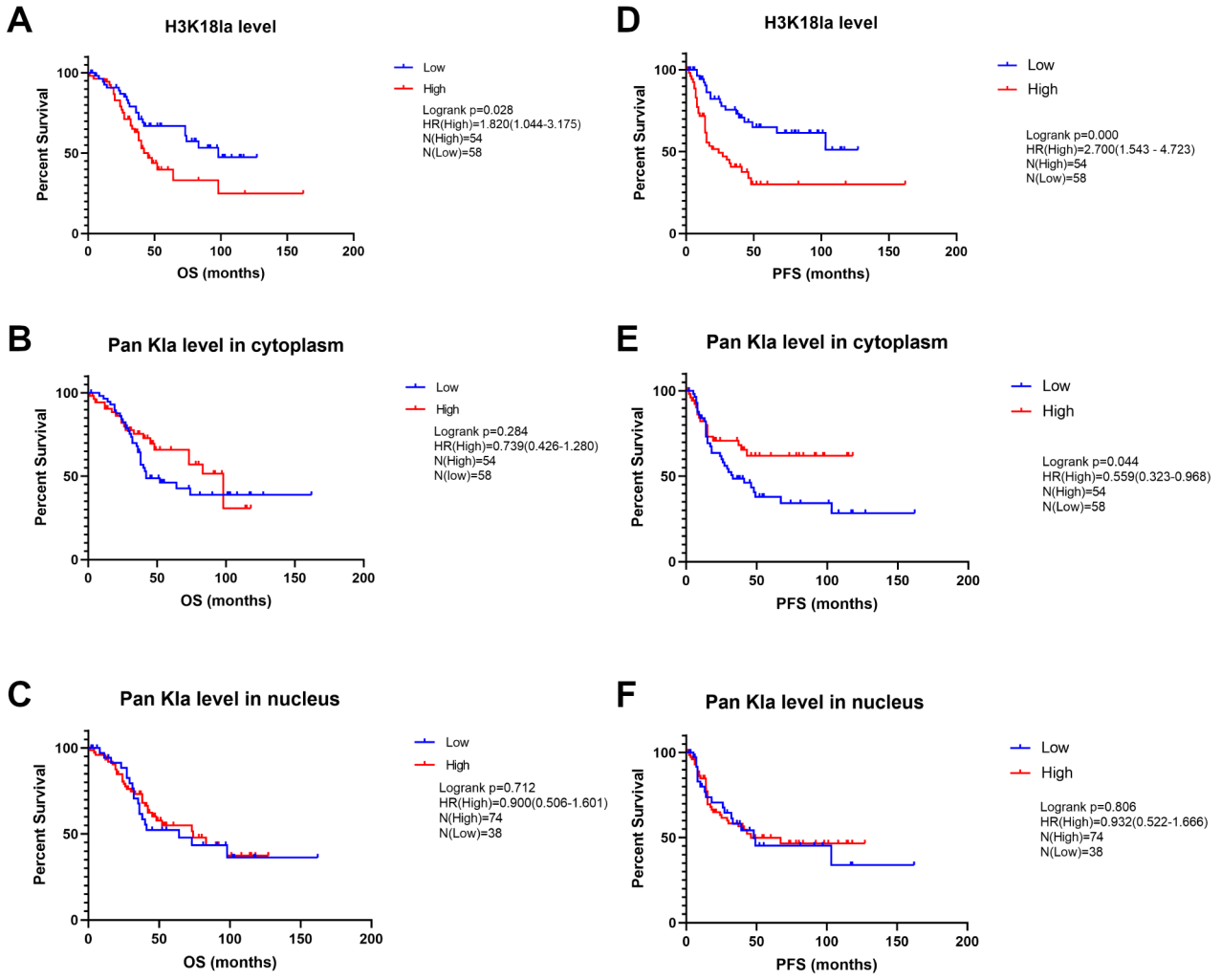

Figure 2. Lactylation in different sites, Kaplan-Meier curves of OS and PFS in EOC patients. A) Lactylation in H3K18, Kaplan-Meier curves of OS; B) Lactylation in the cytoplasm, Kaplan-Meier curves of OS; C) Lactylation in the nucleus, Kaplan-Meier curves of OS; D) Lactylation in H3K18, Kaplan-Meier curves of PFS; E) Lactylation in the cytoplasm, Kaplan-Meier curves of PFS; F) Lactylation in the nucleus, Kaplan-Meier curves of PFS.

and the level of protein lactylation modification. We found that the protein lactylation level correlated with the recurrence time of patients with EOC. As we all know, about 75% of ovarian cancer patients might experience a relapse. Based on the recurrence time, it was generally defined as within or beyond 6 months. Platinum-sensitive recurrence was defined as those that occurred after 6 months, and platinum-resistant recurrence was defined as those that occurred within 6 months. Among these 112 patients, up until the follow-up, there were a total of 51 cases of recurrence, with 4 cases experiencing recurrence within 6 months, 47 cases experiencing recurrence after 6 months, and 61 cases without any recurrence. In patients with recurrence, there were 17 cases with a high level of Pan Kla in the cytoplasm and 34 cases with a low level. Conversely, in patients without recurrence, there were 37 cases with a high level of Pan Kla in the cytoplasm and 24 cases with a low level. In patients with recurrence within 6 months, there were 3 cases with a high level of Pan Kla in the cytoplasm and 1 case with a low level. On the other hand, in patients with recurrence after 6 months, there were 14 cases with a high level of Pan Kla in the cytoplasm and 33 cases with a low level. All these cases were analyzed using a Chi-square test, which revealed that the level of Pan Kla in the cytoplasm correlated significantly with the recurrence time of patients with EOC (Table 2). However, this didn't occur in Pan Kla in the nucleus (Table 3). We observed a correlation between the level of histone H3K18la and the recurrence time of these patients (Table 4). Among patients with recurrence within 6 months, there were 4 cases with a high level of histone H3K18la and 0 cases with a low level. For patients with recurrence after 6 months, there were 29 cases with a high level of histone H3K18la and 18

Table 3. Pan Kla in association with clinical characteristics variables using the Chi-square test.

Characteristics	Group	Number of cases	Pan Kla in the nucleus		χ^2	p-value
			Low	High		
Age (years)	<50	53	14	39	2.53	0.111
	≥50	59	24	35		
FIGO stage	I	19	9	10	3.57	0.312
	II	16	3	13		
	III	65	21	44		
	IV	12	5	7		
Grade	Low	20	7	13	0.01	0.911
	High	92	31	61		
Lymph node metastasis	Yes	36	11	25	0.27	0.604
	No	76	27	49		
Distant metastasis	Yes	17	7	10	0.47	0.493
	No	95	31	64		
Platinum recurrence time	≥6 m	47	17	30	0.87	0.280
	<6 m	4	1	3		
	No Rec	61	20	41		
CA125 (U/ml)	<882	57	20	37	0.07	0.792
	≥882	55	18	37		

Notes: Pearson's Chi-square test and Fisher's exact test were used for analysis; p-values in bold indicate significance ($p<0.05$)

Table 4. H3K18-lactylation in association with clinical characteristics variables using the Chi-square test.

Characteristics	Group	Number of cases	H3K18-lactylation		χ^2	p-value
			Low	High		
Age (years)	<50	53	31	22	1.81	0.178
	≥50	59	27	32		
FIGO stage	I	19	16	3	12.28	**0.006**
	II	16	5	11		
	III	65	32	33		
	IV	12	5	7		
Grade	Low	20	13	7	1.70	0.192
	High	92	45	47		
Lymph node metastasis	Yes	36	21	15	0.91	0.340
	No	76	37	39		
Distant metastasis	Yes	17	8	9	0.18	0.672
	No	95	50	45		
Platinum recurrence time	≥6 m	47	18	29	12.37	**0.002**
	<6 m	4	0	4		
	No Rec	61	40	21		
CA125 (U/ml)	<882	57	29	28	0.04	0.845
	≥882	55	29	26		

Notes: Pearson's Chi-square test and Fisher's exact test were used for analysis; p-values in bold indicate significance ($p<0.05$)

cases with a low level. In patients without recurrence, there were 21 cases with a high level of histone H3K18la and 40 cases with a low level. Although the lactylation level in these two sites was associated with the recurrence time of patients with EOC, the lactylation level in the cytoplasm was higher in patients with platinum-resistant recurrence compared to those with platinum-sensitive recurrence, while the level of H3K18la is lower in patients with platinum-resistant recurrence (Figures 3A, 3B). It further indicated the distinct roles of lactylation in different parts of the protein.

Correlation of histone H3K18la level with pathological stage in EOC patients. The pathological stage has been considered to be crucial for predicting the prognosis of patients with EOC. Therefore, we analyzed the relationship between lactylation levels and the pathological stage of EOC patients. As shown in Table 4, there were three cases with a high lactylation level of histone H3K18 and 16 cases with a low level in stage I patients. Additionally, there were 11/5, 33/32, and 7/5 cases with high/low lactylation levels in patients with stage II, stage III, and stage IV, respectively. We conducted a Chi-square analysis for correlation analysis, and the results showed that the lactylation level of histone H3K18 was correlated with the pathological stage of EOC patients, and it was statistically significant for the prognosis of patients with EOC. Moreover, Figures 4A and 4B visually demonstrate that the histone H3K18la level in stage I is significantly lower compared to stages II, III, and IV, with statistical significance.

Pathologic stage, pathologic grade, recurrence, lymph node metastasis, distant metastasis, and CA125 level as factors affecting the prognosis of EOC patients. In this study, in addition to exploring the impact of lactylation modification on the survival of patients with EOC, we also identified several factors affecting the prognosis of these patients. These factors included the pathological stage, pathological grade, recurrence, lymph node metastasis, distant metastasis, and CA125 level (Figures 5A–6G and 6A–6F), which were consistent with previous studies [4–7]. Figures 5A–5G show that patients with advanced ovarian cancer have a worse prognosis compared to those at an early stage, and the prognosis is even poorer for ovarian cancer patients who experience recurrence, lymph node metastasis, or distant metastasis.

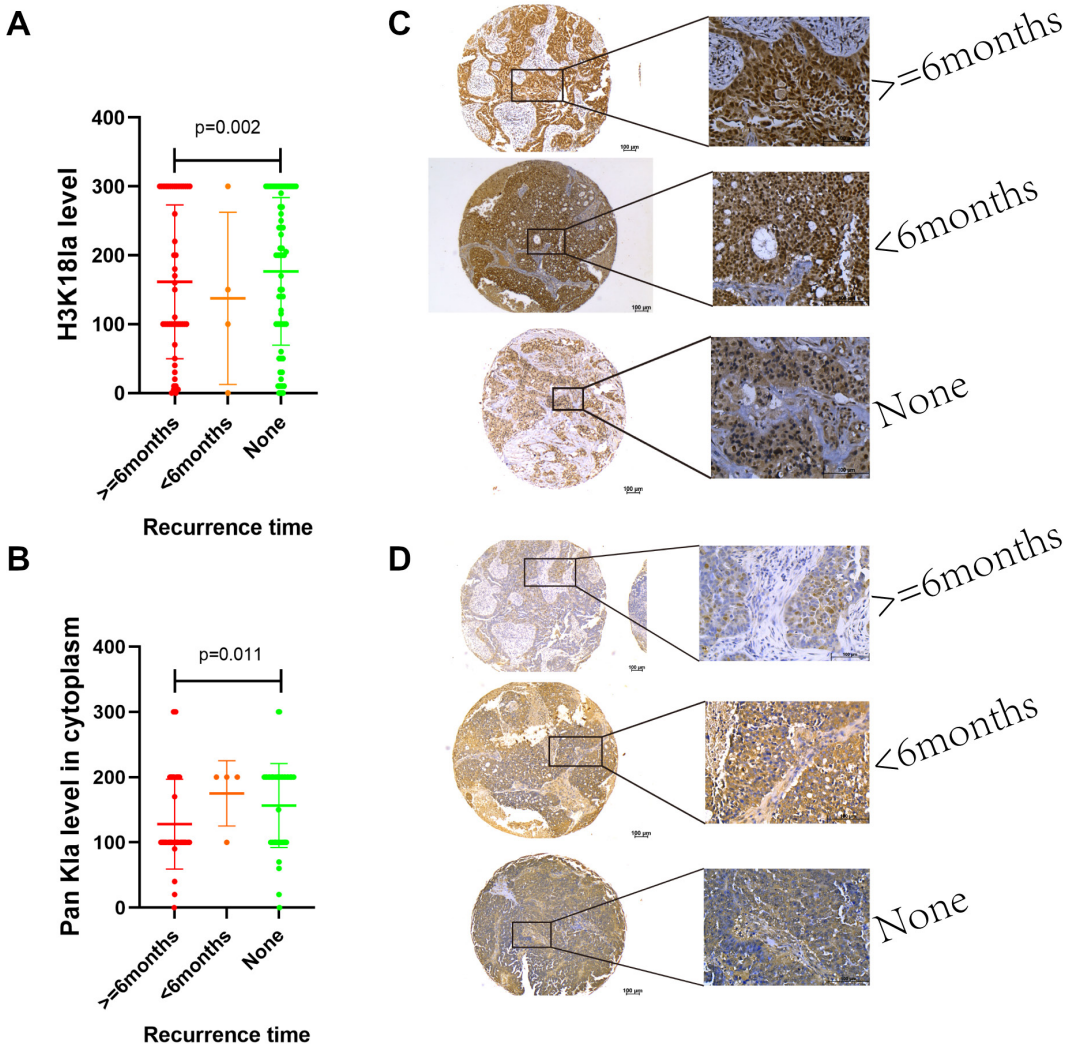

Figure 3. Correlation analysis between lactylation level and platinum recurrence (recurrence time ≥6 months refers to platinum-sensitive recurrence, <6 months refers to platinum-resistant recurrence. None means no recurrence). A) correlation analysis between H3K18la level and recurrence time; B) correlation analysis between Pan Kla level in the cytoplasm and recurrence time; C) H3K18la level in EOC tissue with different recurrence time; D) Pan Kla level in the cytoplasm in EOC tissue with different recurrence time (scale bar: 100 μm).

High level of histone H3K18la as an independent risk factor for PFS in EOC patients. We further evaluated the prognostic value of lactylation levels in patients with EOC. In the univariate analysis, we observed a correlation between higher levels of histone H3K18la and worse OS and PFS among patients with EOC (Figures 2A–2F). Although the level of histone H3K18la was not found to be an independent risk factor for OS in the multivariate Cox regression analysis (Supplementary Table S1), it was identified as an independent risk factor for PFS in patients with EOC (Table 5). Thus, we concluded that a high level of histone H3K18la was an independent prognostic factor for patients with EOC and may be associated with poor prognosis.

Protein lactylation may promote the migration of ovarian cancer cells. Based on the above results, we have observed a correlation between protein lactylation levels and the pathological stage of EOC. The level of lactylation was relatively high in patients with advanced ovarian cancer. Compared to patients at the early stage, most of the patients at the advanced stage have metastasized. Thus, we speculated that protein lactylation might be involved in the process of tumor migration. To verify this hypothesis, we performed a Transwell assay and wound healing assay on ovarian cancer cells. Based on the findings of previous studies, oxamate could inhibit the formation of lactic acid and reduce lactylation levels, while exogenous L-lactate could promote lactic acid production and increase lactylation levels. Consequently, we employed oxamate as an inhibitor and exogenous L-lactate as a promoter, creating experimental groups: negative control group, inhibitor group, promoter group, and

Table 5. Cox regression multivariate analysis of PFS prognostic factors in EOC.

Characteristics		Number of cases	Multivariate analysis		
			p-value	HR	95% CI
Pan Kla in the cytoplasm			**0.030**	0.459	0.228–0.926
	Low*	58			
	High	54			
Pan Kla in the nucleus			0.883	0.951	0.488–1.853
	Low*	38			
	High	74			
H3K18-lactylation			**0.001**	2.836	1.491–5.396
	Low*	58			
	High	54			
FIGO stage			**0.047**	2.397	1.011–5.687
	I+II*	35			
	III+IV	77			
Grade			0.088	2.910	0.853–9.927
	Low*	20			
	High	92			
Lymph node metastasis			0.985	1.008	0.425–2.394
	No*	76			
	Yes	36			
Distant metastasis			**0.007**	3.149	1.363–7.276
	No*	95			
	Yes	17			
CA125 (U/ml)			0.822	1.078	0.561–2.072
	<882*	57			
	≥882	55			
Age (years)			0.344	1.387	0.704–2.731
	≥50*	59			
	<50	53			

Notes: *control group; p-values in bold indicate significance (p<0.05)

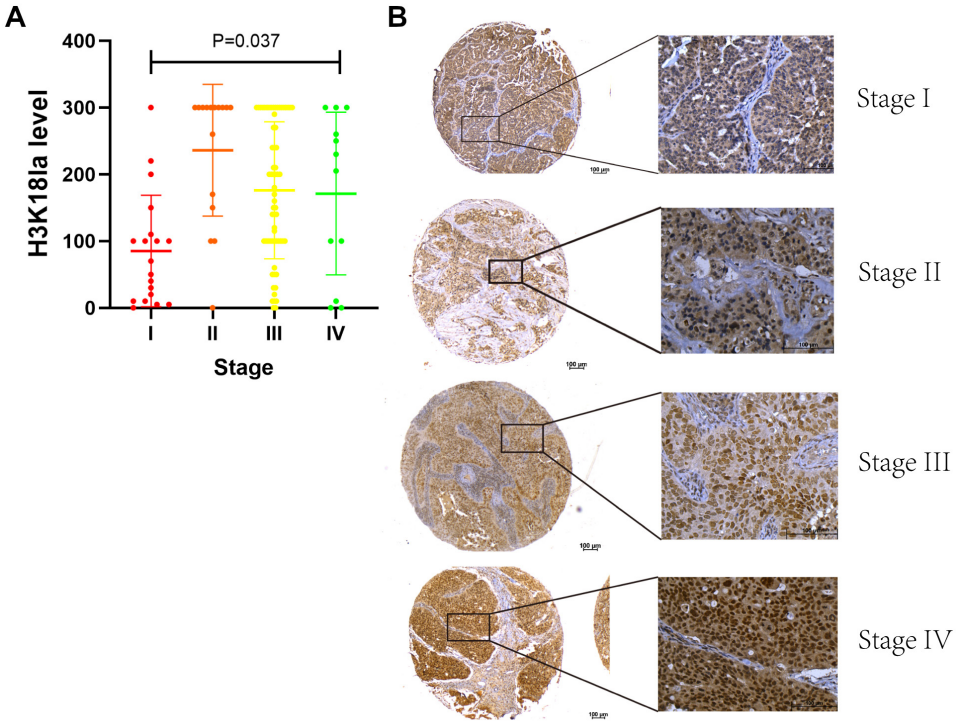

Figure 4. Correlation analysis between H3K18la level and tumor stage. A) correlation analysis between H3K18la level and tumor stage; B) representative IHC (scale bar: 100 μm).

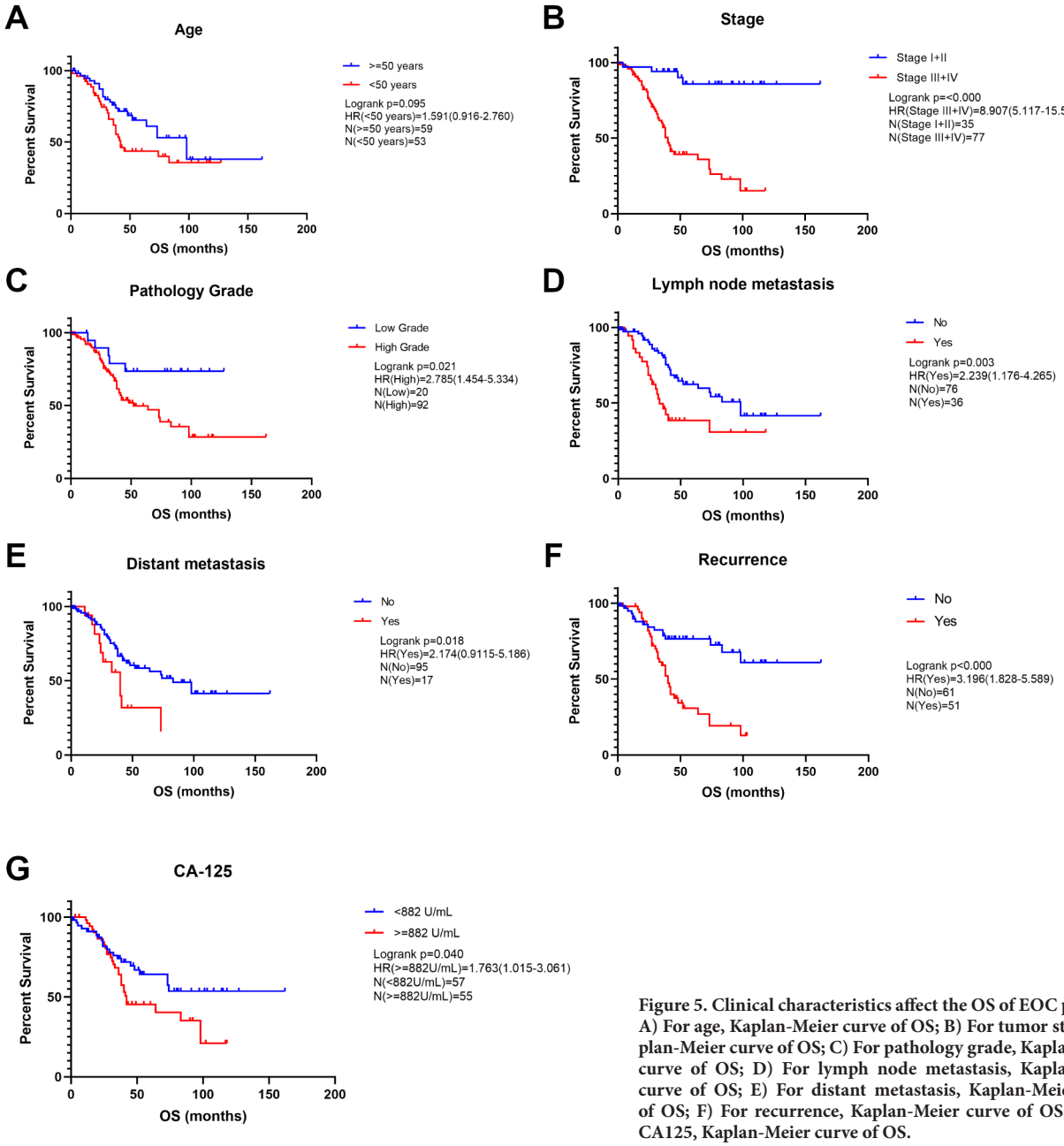

Figure 5. Clinical characteristics affect the OS of EOC patients. A) For age, Kaplan-Meier curve of OS; B) For tumor stage, Kaplan-Meier curve of OS; C) For pathology grade, Kaplan-Meier curve of OS; D) For lymph node metastasis, Kaplan-Meier curve of OS; E) For distant metastasis, Kaplan-Meier curve of OS; F) For recurrence, Kaplan-Meier curve of OS; G) For CA125, Kaplan-Meier curve of OS.

inhibitor+promoter group. The results indicate a significant increase in migration in the promoter group compared to the negative control group and inhibitor group. The inhibitor group also showed increased migration relative to the negative control group, although not as significantly as that of the promoter group (Figures 7A, 7B and 8A, 8B).

Discussion

The Warburg effect has been recognized as a unique characteristic of energy metabolism within tumors. It is characterized by a preference for glycolysis to meet energy demands and the production of large amounts of lactic acid, even under aerobic conditions. Numerous studies have revealed the significant role of lactate in tumor growth, immune tolerance, migration, and other processes [18–20]. Lactate can act as a metabolic carbon source, a regulator of the tumor microenvironment, a signaling molecule, and an inhibitor of PHD2/VHL ubiquitination degradation. These roles contribute to the regulation of tumor growth, invasion, metastasis, angiogenesis, immune escape, and other behaviors [21–25]. Recently, it has been discovered that lactic

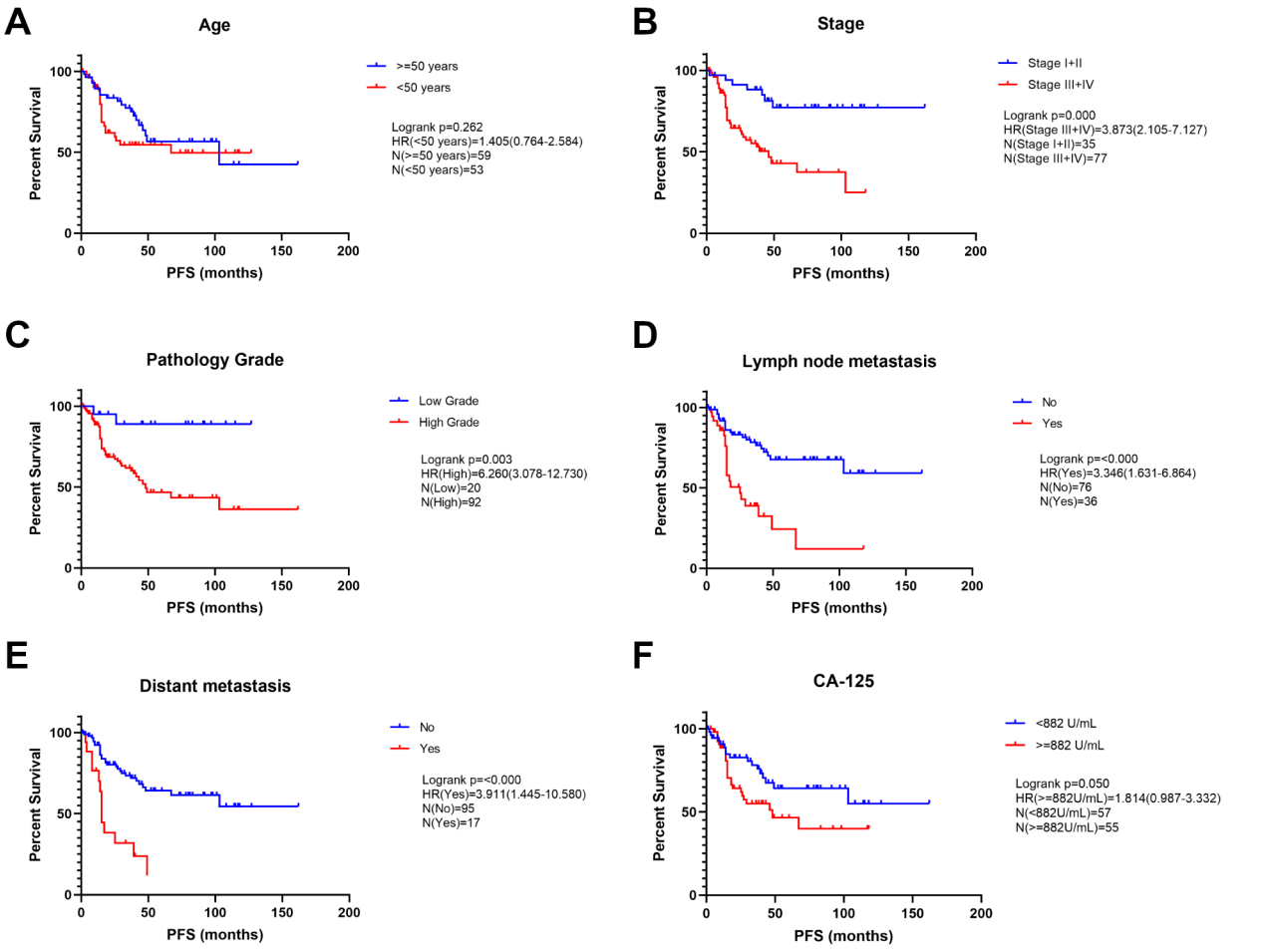

Figure 6. Clinical characteristics affect the PFS of EOC patients. A) For age, Kaplan-Meier curve of PFS; B) For tumor stage, Kaplan-Meier curve of PFS; C) For pathology grade, Kaplan-Meier curve of PFS; D) For lymph node metastasis, Kaplan-Meier curve of PFS; E) For distant metastasis, Kaplan-Meier curve of PFS; F) For CA125, Kaplan-Meier curve of PFS.

acid can exert its effects through protein post-translational modification known as lactylation. This novel type of modification has been shown to be associated with tumor prognosis. For instance, one study found elevated levels of lactylation modification in ocular melanomas compared to normal sentinel tissues, potentially contributing to the poor prognosis of patients. Similar findings have been reported in non-small cell lung cancer and colon cancer.

Therefore, this paper aims to investigate the impact of protein lactylation modification on EOC. Initially, we conducted immunohistochemical staining on 112 EOC tissues and 3 normal ovarian tissues using lactylation modification-related antibodies. Our findings revealed higher levels of both pan lactylation modification and H3K18la modification in tumor tissues compared to normal ovarian tissues, suggesting a potential promotional effect of lactylation modification on the development of EOC. Subsequently, we examined whether the level of lactylation modification influenced the prognosis of patients with EOC. KM analysis demonstrated that patients with high levels of histone H3K18la modification showed poorer OS and PFS when compared to those with low levels (Figures 2A, 2D). Conversely, the level of Pan Kla, both in the cytoplasm and the nucleus, did not show a statistically significant difference in OS for patients with EOC (Figures 2B, 2C, 2E, 2F). However, patients with high cytoplasmic lactylation modification levels experienced longer PFS. Hence, we hypothesized that lactylation of proteins in the cytoplasm and histone H3K18la in the nucleus play different roles in EOC. The latter may influence tumor occurrence and development by impacting the expression of oncogenes or tumor suppressor genes. In a study conducted by Professor Yingming Zhao's team, 28 lactylation modification sites were identified, all of which occurred on histones, with H3K18 being particularly important. As the research progressed, it was discovered that Pan Kla existed not only in the cytoplasm and nucleus but also in other proteins [26]. Therefore, it was hypothesized that lactylation modifications on histones could potentially

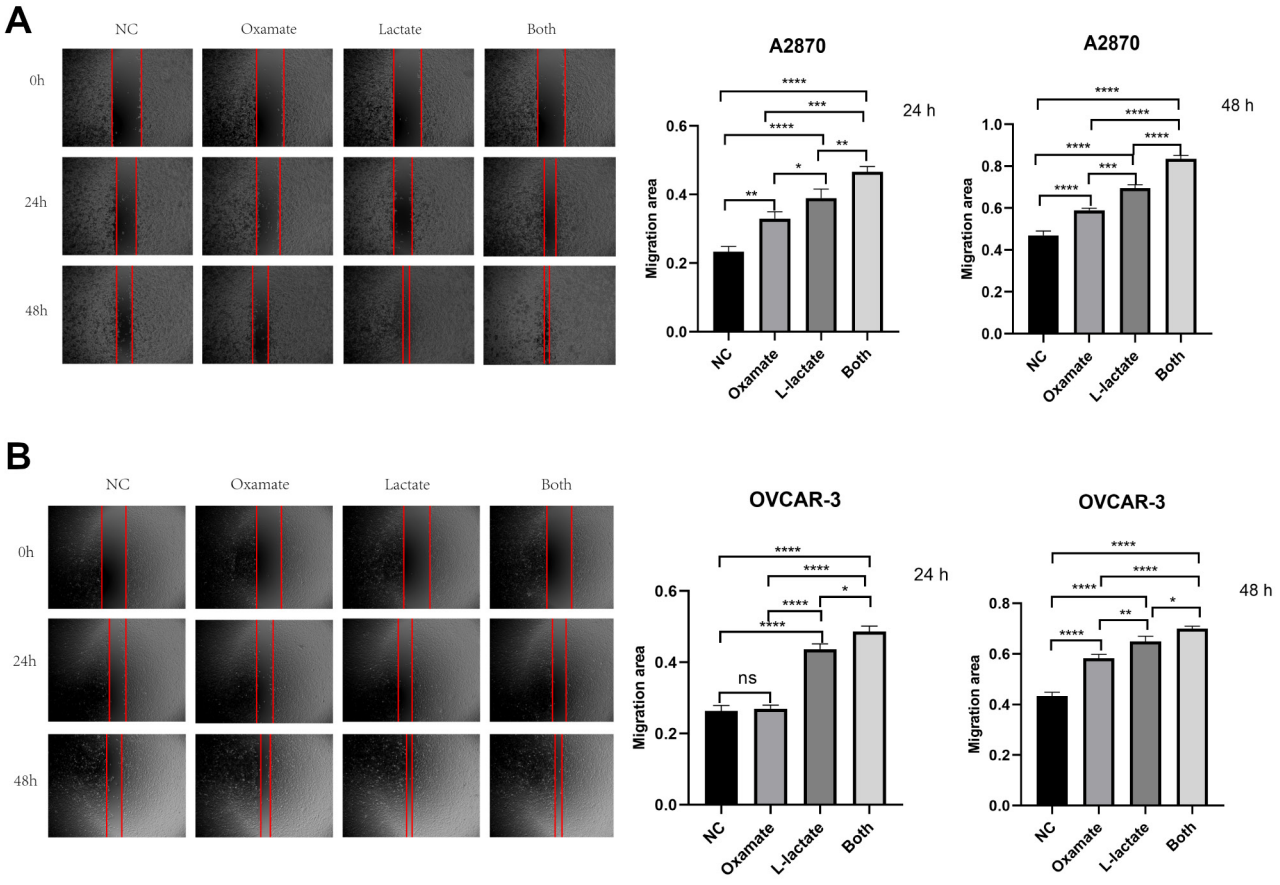

Figure 7. Wound healing assay for ovarian cancer cells with different conditions. A) Wound healing assay for A2780 cells with NC, oxamate, L-lactate, both oxamate and L-lactate; B) Wound healing assay for OVCAR3 cells with NC, oxamate, L-lactate, both oxamate and L-lactate. Abbreviations: NC-negative control; Both-both oxamate and lactate; Notes: ns-not significant, *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001

facilitate or inhibit gene expression, thus affecting tumor growth and potentially leading to a poor prognosis in cancer patients. Previous studies have also demonstrated the influence of histone lactylation modifications on gene expression [27–30], marking the first time the connection between metabolism and gene expression has been established.

In addition, we explored the relationship between protein lactylation modifications and clinical characteristics of patients with EOC. We found that both the level of protein Pan Kla occurring in the cytoplasm and histone H3K18la level correlated with recurrence time in patients with EOC. It is well known that approximately 75% of patients with ovarian cancer will experience a recurrence. Depending on the recurrence time, which is generally defined as 6 months, ≥6 months is considered platinum-sensitive recurrence, while <6 months is defined as platinum-resistant recurrence. From these results, we observed that although the level of lactylation at both sites correlated with recurrence time in patients with EOC, the cytoplasmic lactylation level was higher in patients with platinum-resistant recurrence than in patients with platinum-sensitive recurrence. On the other hand, the histone H3K18la was lower in patients with platinum-resistant recurrence of ovarian cancer. Therefore, this also suggests that different parts of the protein lactate modification play different roles, or they may have a combined effect. However, this study did not further investigate the specific role of lactylation on which proteins, which was a limitation of this paper. The specific mechanism needs to be elucidated by future research.

Furthermore, we also found that the level of histone H3K18la was related to the pathological stage of EOC patients (Table 4). The lactylation level in stages II, III, and IV was generally higher than that in stage I (Figures 4A, 4B). Compared with early-stage ovarian cancer patients, the five-year survival rate of advanced ovarian cancer patients is less than 40%, indicating a worse prognosis. It may be attributed to the large tumor load and metastasis in advanced stages. Therefore, we conducted experiments related to the migration of ovarian cancer cells (wound healing assay, Transwell assay). The results indicated that

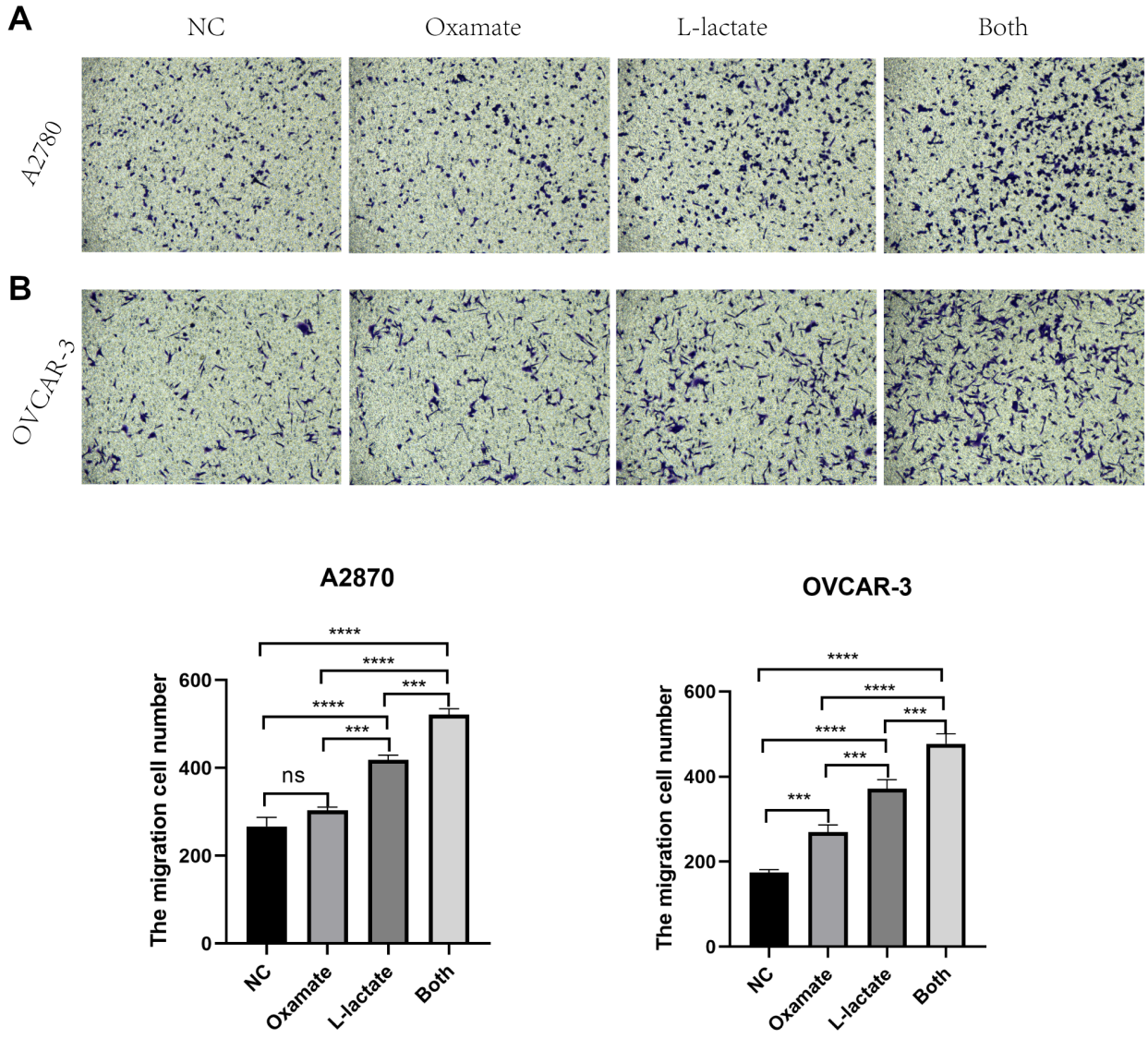

Figure 8. Transwell assay for ovarian cancer cells with different conditions. A) Transwell assay for A2780 cells with NC, oxamate, L-lactate, both oxamate and L-lactate; B) Transwell assay for OVCAR-3 cells with NC, oxamate, L-lactate, both oxamate and L-lactate. Abbreviations: NC-negative control; Both-both oxamate and L-lactate; Notes: ns-not significant, *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001

the migration of the promoter group significantly increased compared to the negative control group and inhibitor group. The inhibitor group also showed an increase compared to the negative control group, but not as significant as the promoter group. It is possible that as time progresses, ovarian cancer cells themselves produce more endogenous lactic acid, which serves as a precursor substance for lactylation. Although oxamate has an inhibitory effect, it is not a specific inhibitor. Currently, there are no specific inhibitors of protein lactylation. Hence, maybe its inhibitory effect is not prominent, leading to an overall increase in migration. We knew the previous research which explored how histone lactylation affected ocular melanoma. They found that when oxamate was added, protein lactylation level decreased and the migration and proliferation ability of cancer cells was weaker. However, when they added lactate, they did not observe significant changes in the migration and proliferation of tumor cells. Thus, multi-factor cooperation may play an important role in tumorigenesis. Glycolysis, which produces lactic acid, is a complex process regulated by many key enzymes. The regulation of lactic acid, as a precursor of lactylation, is also influenced by various factors. It has been shown that histone acetyltransferase P300 can mediate the lactylation of proteins, and HDAC1-3 (histone deacetylase) [31] can mediate de-lactylation. In addition, this result also showed an increase in migration in the group with the

addition of the inhibitor and promotor, and speculate that the reason for this is that oxamate is not a specific inhibitor of lactylation, but is an inhibitor of glycolysis as a whole, and therefore inhibits the glycolytic pathways, and the effect of lactylation is highlighted and amplified, suggesting that lactylation has a more important role to play. However, these studies lack sufficient evidence to prove that they are the specific writers and erasers of lactylation. Further research is required in the future.

In conclusion, our findings indicated that a high level of histone H3K18la was an independent prognostic factor in patients with EOC. Consequently, protein lactylation modification might have a significant impact on EOC and potentially serve as a target for future therapy. In addition, further *in vivo* and *in vitro* experimental studies are necessary to explore the effects of protein lactylation modification on EOC and its underlying molecular mechanisms.

Supplementary information is available in the online version of the paper.

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High histone H3K18 lactylation level is correlated with poor prognosis in epithelial ovarian cancer

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Supplementary Information

Supplementary Table S1. Cox Regression multivariate analysis of overall survival prognostic factors in EOC.

Characteristics		Number of cases	Multivariate analysis		
			p-value	HR	95% CI
Pan Kla in cytoplasm			0.980	1.009	0.516–1.974
	Low*	58			
	High	54			
Pan Kla in nucleus			0.677	0.870	0.453–1.673
	Low*	38			
	High	74			
H3K18-lactylation			0.108	1.650	0.895–3.042
	Low*	58			
	High	54			
FIGO stage			**0.001**	6.748	2.184–20.850
	I+II*	35			
	III+IV	77			
Grade			0.498	1.453	0.493–4.286
	Low*	20			
	High	92			
Lymph node metastasis			0.996	0.998	0.500–1.992
	No*	76			
	Yes	36			
Distant metastasis			0.566	1.270	0.561–2.875
	No*	95			
	Yes	17			
CA125 (U/ml)			0.287	0.692	0.351–1.363
	<882*	57			
	≥882	55			
Age (years)			0.065	1.950	0.960–3.961
	≥50*	59			
	<50	53			
Platinum recurrence			0.163	1.622	0.822–3.203
	No*	61			
	Yes	51			

Notes: *control group; p-values in bold indicates significance (p<0.05)