European Journal of Gynaecological Oncology

IMR Press

Original Research

Exploration on correlation of high DLX2 expression with poor prognosis and cellular proliferation in epithelial ovarian cancers

Meng-Hui Huang[1,†], Yun Han[1,†], Qing-Hua Xi[2], Yun-Feng Jin[2], Ying-Lei Liu[1], Yu-Wen Han[1], Hai-Li Kai[1], Qian Zhang[1], Yan-Li Zheng[1,*]

[1] *Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Nantong University, NO. 6 North Hai-er-xiang Road, 226001 Nantong, China*
[2] *Department of Obstetrics and Gynecology, Affiliated Hospital of Nantong University, NO. 20 Xi-si Road, 226001 Nantong, China*

*Correspondence: gaoshan1189@sina.com (Yan-Li Zheng)
† These authors contributed equally.

DOI:10.31083/j.ejgo.2021.03.2093

Submitted: 22 March 2020 Revised: 26 May 2020 Accepted: 17 June 2020 Published: 15 June 2021

Objective: It has been found that overexpression of distal-less homeobox 2 (DLX2) is closely correlated with multiple cancers. However, the role of DLX2 in the pathogenesis of ovarian cancers is not known. This study was designed to explore the mechanism of action of DLX2 on cellular proliferation and apoptosis in epithelial ovarian cancers (EOCs). *Methods*: A total of 119 EOC tissue specimens were analyzed immunohistochemically, and the correlation between DLX2 expressional level and clinicopathological characteristics was determined. Moreover, western blot method was used to measure DLX2 protein in EOC specimen of different grades and EOC cell lines and explore its molecular mechanism of action. Kaplan-Meier survival analysis revealed that overexpression of DLX2 was obviously correlated with an adverse clinical outcome in EOCs ($P < 0.01^*$). *Results*: DLX2 expressional level had an obvious association with histopathological grade, FIGO stage, ascites and ki-67 expressional level in EOC patients and DLX2 exerted a crucial effect in regulating cellular proliferation in EOCs. Knocking down DLX2 using shRNA-DLX2 reduced the proliferation and enhanced the apoptosis in EOC cells. *Conclusion*: DLX2 might act as a new prognostic indicator and have an important value for molecular targeted therapeutic drugs in EOCs.

Keywords

Epithelial ovarian cancer (EOC); DLX2; Proliferation

1. Background

Epithelial ovarian cancers (EOCs) are most commonly seen in females, which exhibit the highest mortality among all gynecology malignant tumors around the world [1]. Although surgical treatment, chemotherapy or radiotherapy has obviously promoted qualities of life in EOC patients, the early diagnostic and curative ratios remain low [2]. Therefore this is still a big challenge in the treatment of EOC patients. It is important to establish a new strategy for the diagnosis and treatment of EOCs to identify novel molecular mechanisms during the disease development.

Distal-less homebox (DLX) genes, a homolog of Drosophila distal-less, compose a family of homebox transcription factors participating in regulation of cellular differentiation and morphogenesis. Some evidences have showed that the DLX gene family plays a vital role in regulation of embryogenesis, tissue homeostasis, cellular cycle and apoptosis [3]. Members of DLX gene family exert critical effects on cancer progression. For example, the inhibition of DLX4 enhances the apoptosis of choriocarcinoma cells [4]. Besides, researchers also found that up-regulated expression of DLX4 can promote migration, invasion and metastasis of tumor cells [5]. DLX5 can enhance cellular proliferation in tumors by transcriptionally regulating MYC [6]. Moreover, it has been found that DLX5 is closely correlated with cellular proliferation in ovarian cancers [7]. Those findings indicated that DLX gene family might participate in cancer development. It has been reported that one of DLX gene family members-DLX2 can activate mitogenic epidermal growth factor receptor (EGFR) signaling through direct induction of EGFR ligand betacellulin expression [8]. In addition, it has also been reported that DLX2 is abnormally detected in many human carcinomas such as, prostate carcinoma, hepatocellular carcinoma and breast carcinoma [9–11]. However, the effect of DLX2 on the progression of EOCs remain unexplored In our study, IHC was firstly applied to explore the correlation of DLX2 with clinicopathologic indicators in EOC patients. The results revealed that a high-level expression of DLX2 could predict adverse clinical outcome in EOC patients. Thereafter, short hairpin RNA (shRNA) was used to knockdown the expression of DLX2 to decrease the specific effect of DLX2 on EOC cells. CCK-8 and apoptosis assay were used for further exploration. Short hairpin RNA (shRNA) was used to knock down the expression of DLX2 to decrease the specific effect of DLX2 on EOC cells. It was found that low DLX2 expressional level could decrease proliferation and promote apoptosis in EOC cells. The above results indicated that DLX2 participated in the pathogenic process of EOCs and might predict a adverse clinical outcome in EOC patients.

Table 1. DLX2 and Ki-67 Expression and Clinicopathological Parameters in 119 OC Specimens.

Parameters	DLX2		*P*-value	χ^2	Ki-67		*P*-value	χ^2
	Low	High			Low	High		
Age (years)			0.354	0.860			0.659	0.195
< 50	11	24			14	21		
> 50	34	50			30	54		
Histological grade			0.038*	6.561			< 0.001*	30.622
Well	5	2			7	0		
Moderate	17	19			24	14		
Poor	23	53			15	61		
FIGO stage			0.046*	8.004			0.265	3.969
I	27	25			24	28		
II	4	9			3	10		
III	13	36			16	33		
IV	1	4			1	4		
Menopause			0.457	0.554			0.168	1.902
Absence	14	28			29	23		
Presence	31	46			25	52		
Ascites			0.009*	6.860			0.037*	4.365
Absence	34	38			32	40		
Presence	11	36			12	35		
Metastasis to lymph node			0.077	3.133			0.228	1.450
Absence	40	56			38	58		
Presence	5	18			6	17		
Metastasis to other organ			0.076	3.143			0.006*	7.447
Absence	27	32			29	30		
Presence	18	42			15	45		
Cancer cells in ascites			0.110	2.557			0.017*	5.743
Absence	38	53			39	52		
Presence	7	21			5	23		
Ki-67			0.004*	8.310				
Low	24	20						
High	21	54						

Statistical analyses were performed by Pearson χ^2 test. *$P < 0.05$ was considered significant.

2. Materials and methodology

2.1 Subject and specimen selection

A total of 119 EOC tissue specimens were acquired from the patients treated in the Obstetrics and Gynecology Department of the Second Affiliated Hospital of Nantong University. This study achieved an approval from the medical ethics committee of the above hospital, and all subjects had signed a written informed consent form. Major clinicopathological characteristics of patients such as age, cancer classification, FIGO stage, menstrual status, lymph node metastasis and organic metastases, ascitic fluid, cancer invasiveness and overall survival (OS) were presented in Table 1.

All specimens were quickly frozen in liquid nitrogen and stored at -80 °C until being used for Western blotting detection.

2.2 Immunohistochemistry staining

All slices with 5 MM thickness were prepared and then placed on 10% polysine-coated glass slides. The slices were rinsed twice with xylene (15 min each time) and then rehydrated in graded ethanol. Thereafter the slice were added with antibodies such as DLX2 (diluting ratio 1 : 200, Invitrogen, Carlsbad, CA, USA), Ki-67 (diluting ratio, 1 : 500, Invitrogen, Carlsbad, CA, USA) and cultivated for 2 h at indoor temperature; subsequently the slices were rinsed thrice with PBS; the signal development was performed using diaminobenzidine; all slices were dyed with 20% hematoxylin-eosin; finally all slices were dried, cleaned and assessed.

At least 500 cells or more per visual field were observed to calculate the label index, which was the ratio of positively dyed cells to total cells. The numbers of DlX2 and Ki67 positive cells were calculated, and the proportion of positive cells was calculated. The dying status was evaluated according to the proportion of positive cancer cells: 0 point indicated negative result and positively dyed cells $< 5\%$; 1 point indicated 5–25% positively dyed cells; 2 points indicated 25–50% positively dyed cells; 3 points indicated 50–75% positively dyed cells and 4 points indicated $> 75\%$ positively dyed cells. The immunoreactivity intensity was evaluated for each

cancer slice: 0 point indicated negative staining; 1 point indicated weak intensity; 2 points indicated moderate intensity; 3 points indicated strong intensity. The scores for percentage and intensity were multiplied to obtain the final scores 0, 1, 2, 3, 4, 6, 9, or 12. Statistical analysis showed that a score $\leq$ 4 points indicated low expression, and a score $>$ 4 points indicated a strong overexpression [12].

2.3 Western blotting assay

EOC tissues and cells were analyzed with Western blotting assay. The tissues were lysed in lysis buffer comprising protease inhibitor mixture solution. The lysates were centrifuged at 12,000 rpm for 20 min at 4 °C. Thirty mcg total proteins were analyzed using SDS-PAGE and then transferred onto a PVDF membrane (Immobilon, Millipore, Birrica, MA, USA), which was immersed in blocking buffer containing skimmed milk powder and cultivated with appropriate primary and secondary antibodies. Enhanced chemiluminescence (ECL; Pierce Company, Rockford, IL, USA) was used to measure protein signals. The antibodies included DLX2 (Santa Cruz, Delaware Ave, CA, USA, diluted at 1 : 200), PCNA (Santa Cruz, Delaware Ave, CA, USA, diluted at 1 : 500), GAPDH (Santa Cruz, Delaware Ave, CA, USA, diluted at 1 : 3000), CDK2 (Santa Cruz, Delaware Ave, CA, USA, diluted at 1 : 1000), cyclin A (Santa Cruz, Delaware Ave, CA, USA, diluted at 1 : 500), caspase-3 (Cell Signaling Technology, Beverly, MA, USA, diluted at 1 : 100), goat-anti-rabbit (Santa Cruz, Delaware Ave, CA, USA, diluted at 1 : 10000), goat-anti-mouse (Santa Cruz, Delaware Ave, CA, USA, diluted at 1 : 10000).

2.4 Celluar cultivation and total protein content detection

The human EOC cell lines were prepared and cultivated with 1640 medium in a humidified atmosphere with 5% CO2 in our laboratory. The cells were added with protease inhibitor mixture solution and 1 mM PMSF and then lysed with ice-cold RIPA lysis buffer. Thereafter, the lysates were centrifuged for 10 min at 4 °C, and a protein assay kit (G-Biosciences, Louis, MO, USA) was used to detect the total protein content of the cell lysates. All specimens were kept in a -20 °C refrigerator.

2.5 Flow cytometric analysis

In order to analyze the cell cycle, the cells were collected properly and fixed in 70% ethanol for over 24 h at -20 °C and then cultivated using 1 mg/mL Rnase A for 20 min at 37 °C. Thereafter, the cell solution was centrifuged at 1200 rpm for 5 min to collect cells , and the cells were stained using PBS containing 50 μg/mL PI (Becton-Dickinson, Franklin Lakes, NJ, USA) and 0.5% Tween-20. Finally, the cells were analyzed using a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and Cell Quest software (BD Biosciences, Franklin Lakes, NJ, USA).

2.6 Reproduction assay

Cellular activity was detected using Cell Counting Kit-8 (CCK-8) assay according to the user's guide. The cells were inoculated on a 96-well plate (Corning Inc., New York, NY, USA) at a density of 1×10^4 cells/well, the plate was immersed in 100 μL medium and cultivated overnight. CCK-8 reagents (Dojindo, Kumamoto, Japan) were added into a subset of wells under different treatments and the cells were cultivated for 1 h at 37 °C. Finally, an appropriate amount of reaction solution was taken to detect the absorbance at 490 nm.

2.7 Transfection of short hairpin RNA

Short hairpin RNAs (shRNAs) were provided by Shanghai Genechem Co. Ltd (Shanghai, China). DLX2 gene expression was decreased by shRNA deplex targeting the sequence, designated shRNA-1: 5'-GAACGGGAAGCCAAAGAAA-3', designated shRNA-2: 5'-CTGAAATTCGGATAGTGAA-3' and, designated shRNA-3: 5'-ATATGCACTCGACCAGAT-3'. The shRNA 5'-UUCUCCGAACGUGUCACGU-3' was used as a negative control (Neg) of mRNAs. DLX2 shRNAs were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) referring to the procedure described in the manual.

2.8 Apoptosis analysis

The cultured cells were co-transfected with control-shRNA and DLX2-shRNA, respectively. Thereafter, the cells were dissociated into single cell suspension with EDTA-free trypsin and rinsed twice using PBS. EOC cells were dyed using Annexin-V-FITC/PI, and then the cellular apoptosis was quantified by a flow cytometer.

2.9 Statistical treatment

The data were statistically analyzed by SPSS 10.0 (SPSS Inc, Chicago, IL, USA). The correlations of DLX2 and Ki-67 with clinicopathological characteristics were determined by chi-square test. The survival was ananlyzed using Kaplan-Meier survival curve and the log-rank test. Cox model was used to conduct multivariate analysis, and $P < 0.05$ indicated an obvious difference.

3. Results

3.1 Association between DLX2 expressiona level and survival of EOC tissues and cells

To investigate the clinicopathological effect of DLX2 on EOCs, immunohistochemistry analysis was firstly conducted to examine DLX2 protein expressional level in 119 EOC specimens. DLX2 and Ki-67 expressional level in EOCs of various histopathological grades were presented in Fig. 1. DLX2 and Ki-67 were not or lowly expressed in low grade EOC specimens (Fig. 1A,D), meanwhile it was confirmed that DLX2 and ki-67 were strongly expressed in poor grade EOC specimens (Fig. 1C,F). The results indicated that DLX2 is closely correlated with prognosis in EOC patients.

To confirm this viewpoint, the expressions of DLX2 in additional specimens were detected using western blotting (three normal ovarian tissue specimens and three different specimens for each of three grades of EOC tissue specimens). The findings of this study revealed that the expression level of

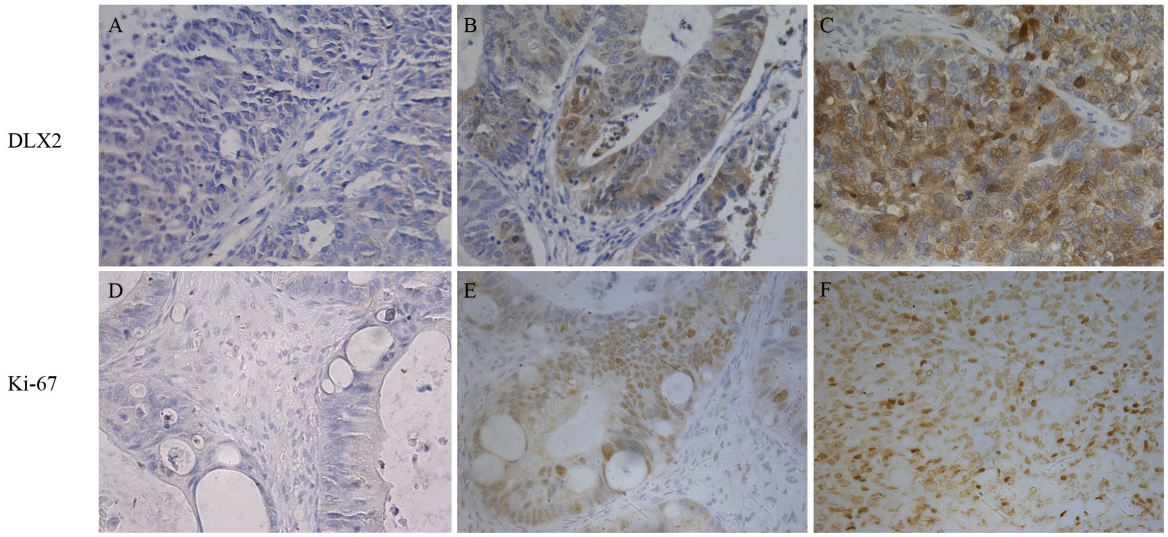

Fig. 1. Immunohistochemical detection of DLX2 and Ki-67 expressions in EOCs with various histopathological grades. A and D indicated DLX2 and Ki-67 expressions in stage I EOCs, respectively. B and E indicated DLX2 and Ki-67 expressions in stage II EOCs, respectively. C and F indicated DLX2 and Ki-67 expressions in stage III EOCs, respectively.

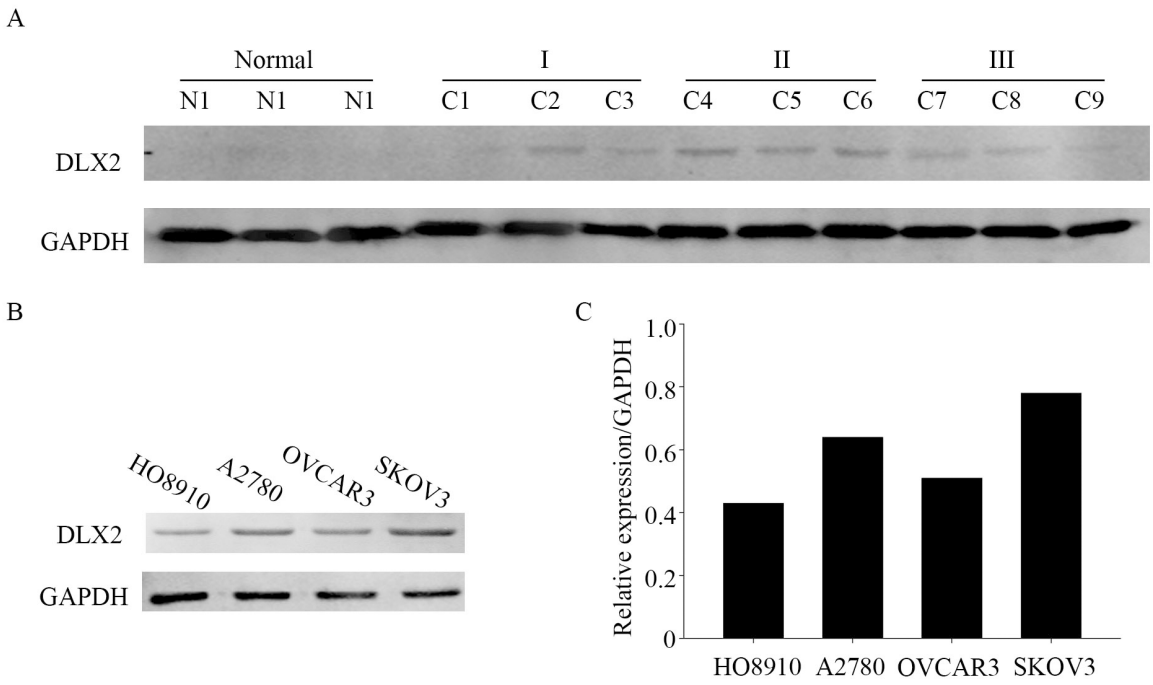

Fig. 2. Expressional statuses of DLX2 in EOCs. (A) Western blotting detection of DLX2 expressions in EOCs with different histopathologicalgrades. (B) Western blotting indicated DLX2 protein expressions in 4 kinds of EOC cells. (C) The bar chart indicated a ratio of DLX2 to GAPDH measured by a densitometer. The results were presented as mean ± SEM. GAPDH was applied as a loading control. Each experiment was performed thrice.

DLX2 was significantly higher in tumors in comparison with normal ovarian tissues (Fig. 2A). Thereafter, a difference in DLX2 expressional level was detected among 4 EOC cell lines, indicating that DLX2 was highly expressed in SKOV3 cells (Fig. 2B). Therefore, SKOV3 cell line was used for subsequent studies.

3.2 Association between DLX2 expression and clinicopathlogical characteristics of EOCs

In order to explore the pathogenic effect of DLX2 in EOCs, clinicopathological indicators were detected to assess the relationship between DLX2 and Ki-67 expressions. The histopathological characteristics of patients were showed in

Table 1. The study results revealed that DLX2 expressional level had an obvious association with histopathological grade, FIGO stage, ascites and Ki-67 expressional level in EOC patients, but had no association with other prognosis indicators. In addition, Ki-67 expression had a close association with histopathological grade, ascites, lymph-node metastases and ascetic cancer cells. Thereafter, scatter plot further indicated an association of DLX2 with Ki-67. As shown in Fig. 3, DLX2 positive cancer cells accounted for 8-93%, and Ki-67 positive cancer cells accounted for 9-98%. Therefore, it could be concluded that the expressional level of DLX2 was close to that of Ki-67 in EOCs, which indicated that EOC tissues with a poorer histopathological grade had higher expressional levels of DLX2 and Ki-67 compared with those with better and moderate histopathological grades. The above results revealed a close association of DLX2 expressional level with Ki-67-defined proliferative activity ($P < 0.01$; Fig. 3).

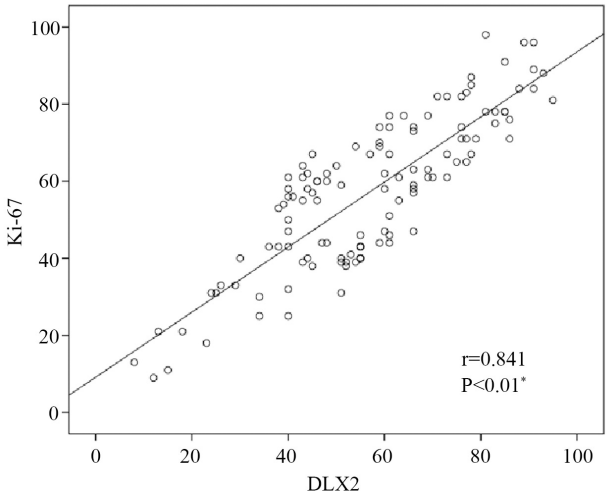

Fig. 3. Association of DLX2 with Ki-67 expressions in EOCs. Scatter plot with linear regression line revealed an association between DLX2 and Ki-67 expressions, which was determined by Spearman rank correlation coefficients.

3.3 Prognosis effect of DLX2 expression

The OS of 119 EOC patients was analyzed using immunohistochemistry, the results were presented in Table 2. Only 19 of 74 (25.7%) EOC patients with a high DLX2 expression still survived, while 31 of 45 (68.9%) EOC patients with a low DLX2 expression still survived at 5 years. All influencing factors of survival of EOC patient were analyzed separately, and the results reveal that the histopathological grade, FIGO stage, organic metastases, DLX2 and Ki-67 expressional levels obviously affected the OS (Table 2). Furthermore, Kaplan-Meier analysis was conducted to determine the association of DLX2 expression with OS in EOC patients, the results indicated that a higher DLX2 expressional level had a close association with a poorer OS ($P < 0.01$; Fig. 4). Thereafter, a multivariate analysis was conducted by Cox model,

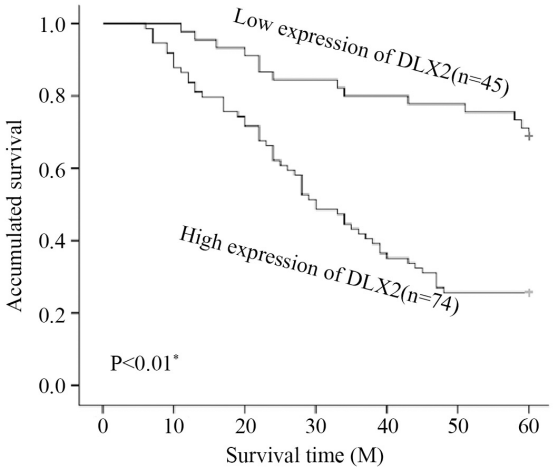

Fig. 4. Kaplan-Meier survival analysis indicated a very obvious difference in OS between EOC patients respectively with low and high DLX2 expressions ($P < 0.01$).

the results revealed that DLX2was an independent prognosis index for OS in EOC patients (Table 3).

3.4 Expression of DLX2 in proliferating EOC cells

This study revealed that a higher DLX2 expressional level might be correlated with oncogenesis in EOCs. To further confirm the role of DLX2 in cell-cycle progression, the cell cycle was analyzed after serum deprivation and re-feeding with serum, respectively. Fig. 5F indicated G1 phase cell cycle arrest in EOCs after 48 hours of serum deprivation, and cell cycle progression from G1 to S phase in EOCs after re-feeding with serum, which indicated DLX2 expressional level was increased after re-feeding with serum. In addition, CDK2 and Cyclin A were lowly expressed at G0/G1 phase and highly expressed at S phase after serum stimulation (Fig. 5G). These studies suggested that DLX2 exerted a crucial effect in regulating cellular proliferation in EOCs.

3.5 Effect of DLX2 on EOC cells

The effect of DLX2 on the progression of EOC cells was investigated through shRNA knockdown of DLX2 in SKOV3 cells, which were transiently transfected with DLX2-shRNA and control-shRNA, respectively. Fig. 6A showed that DLX2 expressional level in the DLX2 shRNA-transfected cells was obviously decreased in comparison with that in the control-shRNA-transfected cells It was also confirmed that DLX2-shRNA-3 showed the greatest knockdown efficiency among three shRNAs (Fig. 6B). Therefore, DLX2-shRNA-3 was applied in the following experiments.

In this study, CCK-8 assay was conducted to assess the effect of DLX2 on cellular proliferation of EOCs, and the results indicated that the cellular proliferation in DLX2-shRNA-transfected EOC cells was obviously decreased in comparison with that in control-shRNA transfected EOC cells (Fig. 6C). Therefore, the invasive ability of DLX2-shRNA-transfected EOC cells were obviously decreased, while the proliferat-

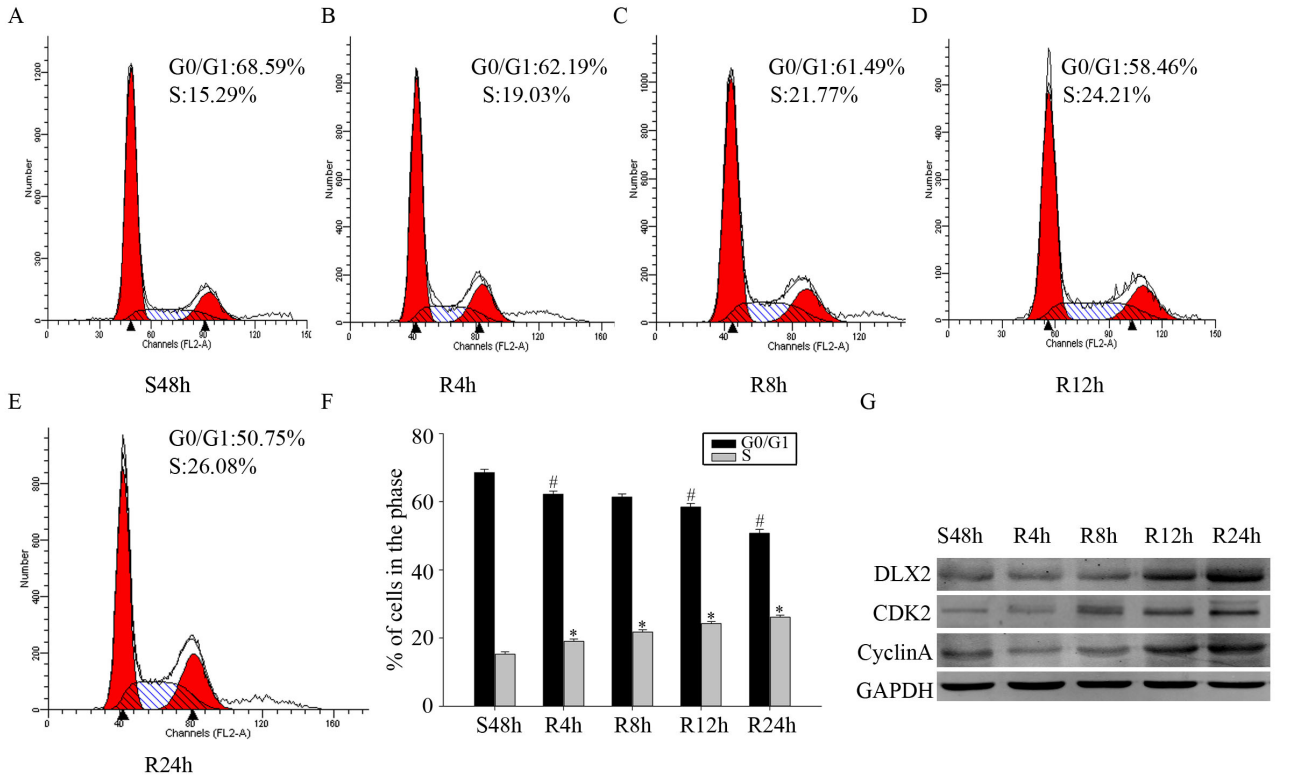

Fig. 5. Expressions of SGTA and cell cycle-related indicators in EOC cells. (A, B, C, D, E) The cell cycle in SKOV3 cells was analyzed by Flow cytometry, the results indicated that the cells were staying at G1 phase after 48 hours of serum starvation, and then passed from G1 phase to S phase after being cultured in MEM supplemented with 10% FBS . (F) The bar chart revealed a ratio of S to G0/G1 phase cells. (G) SKOV3 cells were synchronized after 48 hours of serum starvation or after serum stimulation, the cellular lysates were evaluated by Western blotting using antibodies against CDK2 and Cyclin A. GAPDH served as a loading control. The results were presented as means ± SEM (n = 3, *, #: $P < 0.05$, in comparison with control group after 48 hours of serum starvation). S: serum starvation; R: serum release.

ing cell nuclear antigen (PCNA) was also down-regulated (Fig. 6D). In addition, knocking down DLX2 led to decreased expressions of CDK2 and Cyclin. Moreover, the cell cycle analysis indicated that increased EOC cells were arrested at the G0/G1 phase, and the EOC cells at S phase were decreased in comparison with the control group (Fig. 6E), which indicated that DLX2 might enhance the transition from G0/G1 to S phase. All above findings suggested that DLX2 exerted an irreplaceable effect in cellular proliferation in the progression of EOCs.

3.6 Knockdown of DLX2 induces apoptosis in EOC cells

Because some evidences showed that DLX2 can regulate cellular apoptosis [13], whether DLX2 could affect the EOC cellular apoptosis was explored in this study. At first, the effect of DLX2 on the apoptosis of EOC cells was determined by a flow cytometer. The results indicated that the apoptosis was greater in DLX2-shRNA-transfected EOC cells in comparison with the control group (Fig. 6G). Furthermore, the expression of apoptosis index caspase 3 in EOC cells transfected with DLX2-shRNA or control shRNA were detected. The results suggested that caspase 3 was highly expressed in DLX2-shRNA-transfected EOC cells (Fig. 6H). All of these studies revealed that DLX2 might be an essential factor in regulating the apoptosis of EOC cells.

4. Discussion

EOCs are caused by various molecular factors, and the death rate of EOC patients is still very high. Hence, it is crucial to investigate the molecular pathogenesis of EOCs, and this study aimed to explore the effect of DLX2 in the progression of EOCs.

A previous study has indicated that DLX2 protects cells from transforming growth factor β (TGF β) induced cell-cycle arrest and apoptosis [13]. Furthermore, previous studies have confirmed the effect of DLX2 in multiple human carcinomas such as breast carcinoma, prostate carcinoma and hepatocellular carcinoma [9–11]. It was found that DLX2 has a close association with clinicopathological characteristics of cancers. In this study, the correlation of DLX2 with the clinicopathological characteristics of EOCs was investigated, the results indicated that a high expressional level of DLX2 had a obvious correlation with histopathological grade, FIGO stage, ascites and Ki-67 in EOCs. In addition, DLX2 expressional level was positively associated with Ki-67 expressional level it was found that histopathological grade was closely

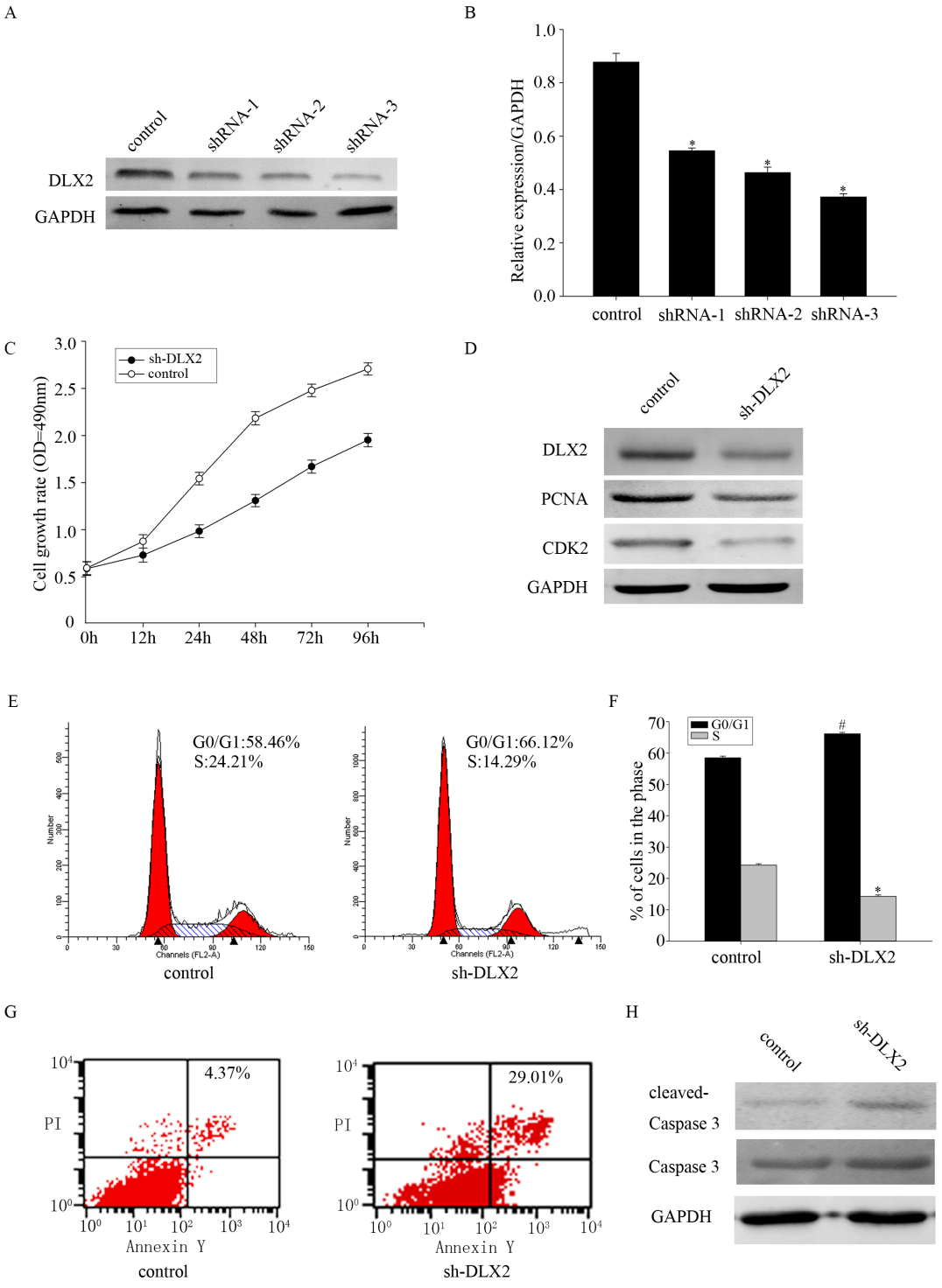

Fig. 6. Knocking down DLX2 suppressed he cellular proliferation and induced cell cycle arrest at G2/M phase and enhanced cellular apoptosis in EOCs. (A) CDK1 expression was measured by Western blotting at 48 h after shRNA transfection of SKOV3 cells, and the results indicated that CDK1-shRNA-3 showed the greatest knockdown efficiency among three shRNAs. (B) The bar chart indicated a ratio of DLX2 to GAPDH measured by a densitometer. The results were presented as mean ± SEM ($P < 0.01$. in comparison with the control group). (C) Cellular proliferation was detected by CCK-8 assay. Absorbance of shRNA-transfected SKOV3 cells was measured. The results were presented as mean ± SEM; each experiment was repeated thrice (**$P < 0.01$, in comparison with the control group). (D) PCNA and CDK2 expressions in SKOV3 cells wherein DLX2 was knocked down were analyzed using Western blotting. (E) At 48 h after transfection, the cells were dyed by PI, and DNA content was analyzed by a flow cytometer. (F) The bar chart indicated a ratio of S to G0/G1 phase cells. *, #: $P < 0.05$ indicated a obvious difference. (G) apoptosis status in SKOV3 cells transfected with or without DLX2-shRNA was detected by a flow cytometer. (H) Caspase 3 expressions in control-transfected cells, and DLX2-shRNA were detected by a flow cytometer.

Table 2. Survival Status and Clinicopathological Parameters in 119 EOC Specimens.

Parameters	Total	Survival status		*P*-value	χ^2
		Dead, n (%)	Alive, n (%)		
Age (years)				0.080	3.064
< 50	35	16	19		
> 50	84	53	31		
Histological grade				0.007*	9.793
Well	7	1	6		
Moderate	36	17	19		
Poor	76	51	25		
FIGO stage				< 0.001*	21.258
I	52	18	34		
II	13	11	2		
III	49	36	13		
IV	5	4	1		
Menopause				0.091	2.862
Absence	43	20	23		
Presence	77	49	28		
Ascites				0.296	1.090
Absence	72	39	33		
Presence	47	30	17		
Metastasis to lymph node				0.085	2.970
Absence	96	52	44		
Presence	23	17	6		
Metastasis to other organ				< 0.001*	14.384
Absence	59	24	35		
Presence	60	45	15		
Malignant tumor Cells in ascites				0.099	2.717
Absence	91	49	42		
Presence	28	20	8		
DLX2				< 0.001*	21.449
Low	45	14	31		
High	74	55	19		
Ki-67				0.004*	8.354
Low	43	18	26		
High	76	51	24		

Statistical analyses were performed by Pearson χ^2 test.

* $P < 0.05$ was considered significant.

Table 3. Contribution of Various Potential Prognostic Factors to Survival by Cox Regression Analysis in 119 Specimens.

	Relative ratio	95% Confidence interval	*P*-value
Histological grade	1.635	0.968–2.762	0.066
FIGO stage	1.315	0.923–1.875	0.130
Metastasis to other organ	1.501	0.740–3.042	0.260
Ki-67	0.911	0.479–1.736	0.778
DLX2	0.325	0.173–0.611	< 0.001*

Statistical analyses were performed by Cox test.

* $P < 0.05$ was considered significant.

correlated with OS of EOC patients. Kaplan-Meier survival analysis indicated that a higher DLX2 expressional level predicted a poorer OS in EOC patients. Furthermore, multivariate analysis indicated that DLX2 was associated with high-risk clinical parameter and served as an independent prognosis index for OS in EOC patients. This study showed that DLX2 was a significant gene affecting the progression of EOCs.

Furthermore, it was determined in this study that DLX2 exerted an effect on cellular proliferation in EOCs. Cell-cycle analysis indicated that DLX2 was highly expressed during transition from G1 phase to S phase. Thereafter, it was confirmed that the cyclin A and CDK2 expressional levels were obviouly increased in the progression of cell-cycle. In addition, how exogenous DLX2 affected the biologic behaviors of EOC cells was investigated. The protein expression levels of CDK2 and PCNA were decreased through shDLX2 down-regulation of DLX2 expression. Effect of DLX2 on apoptosis of EOC cells transfected with DLX2-shRNA or control-shRNA was detected to determine the effect of DLX2 on the progression of EOCs. The flow cytometry analysis indicated that the apoptosis rate of DLX2-shRNA-transfected EOC cells was higher than that in the control group. This study also indicated that knocking down DLX2 expression increased the apoptosis rate of EOC cells. However, more studies are needed to investigate the specific effect of DLX2 on the progression of EOCs.

To sum up, our study confirms the effect of DLX2 on cellular proliferation of EOCs, and it is indicated that knocking down DLX2 expression induces cell cycle deregulation and has a close association with the occurrence of EOCs. DLX2 may serve as a new therapeutic or preventive target for EOCs. It is very meaningful for resolving the debate on the role of DLX2 in progression of EOCs as a prognosis indicaor. Therefore, it is necessary to clarify the role of DLX2 in EOC progression in the future studies.

Author contributions

MHH, YLZ and YH designed the research study. QHX performed the research. YFJ and YWH provided help and advice on the cell and tissue experiments. YLL analyzed the data. MHH, HLK and QZ wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Nantong University (approval number: 0196 (2016)).

Acknowledgment

We are grateful to the Department of Pathology of The Second Affiliated Hospital of Nantong University for providing immunohistochemical pathological sections for our experiment.

Funding

Thanks to the The Science and Technology Projects Fund of Nantong City (No. JC2020006, MS12018007, MS12019004), The Maternal and Child Health Research Projects Fund of Jiangsu Province (NO. F201836) for supporting this research.

Conflict of interest

The authors declare no conflict of interest.

References

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA: A Cancer Journal for Clinicians. 2012; 62: 10–29.

[2] Konstantinopoulos PA, Matulonis UA. Current status and evolution of preclinical drug development models of epithelial ovarian cancer. Frontiers in Oncology. 2013; 3: 296.

[3] Panganiban G, Rubenstein JL. Developmental functions of the Distal-less/Dlx homeobox genes. Development. 2002; 129: 4371–4386.

[4] Sun Y, Lu X, Yin L, Zhao F, Feng Y. Inhibition of DLX4 promotes apoptosis in choriocarcinoma cell lines. Placenta. 2006; 27: 375–383.

[5] Zhang L, Yang M, Gan L, He T, Xiao X, Stewart MD, *et al.* DLX4 Upregulates TWIST and enhances tumor migration, invasion and metastasis. International Journal of Biological Sciences. 2012; 8: 1178–1187.

[6] Xu J, Testa JR. DLX5 (Distal-less Homeobox 5) promotes tumor cell proliferation by transcriptionally regulating MYC. Journal of Biological Chemistry. 2009; 284: 20593–20601.

[7] Tan Y, Cheung M, Pei J, Menges CW, Godwin AK, Testa JR. Upregulation of DLX5 promotes ovarian cancer cell proliferation by enhancing IRS-2-AKT signaling. Cancer Research. 2010; 70: 9197–9206.

[8] Suh Y, Obernier K, Hölzl-Wenig G, Mandl C, Herrmann A, Wörner K, *et al.* Interaction between DLX2 and EGFR regulates proliferation and neurogenesis of SVZ precursors. Molecular and Cellular Neuroscience. 2009; 42: 308–314.

[9] Green WJ, Ball G, Hulman G, Johnson C, Van Schalwyk G, Ratan HL, *et al.* KI67 and DLX2 predict increased risk of metastasis formation in prostate cancer-a targeted molecular approach. British Journal of Cancer. 2016; 115: 236–242.

[10] Dai Q, Deng J, Zhou J, Wang Z, Yuan X, Pan S, *et al.* Long noncoding RNA TUG1 promotes cell progression in hepatocellular carcinoma via regulating miR-216b-5p/DLX2 axis. Cancer Cell International. 2020; 20: 8.

[11] Morini M, Astigiano S, Gitton Y, Emionite L, Mirisola V, Levi G, *et al.* Mutually exclusive expression of DLX2 and DLX5/6 is associated with the metastatic potential of the human breast cancer cell line MDA-MB-231. BMC Cancer. 2010; 10: 649.

[12] Xi Q, Huang M, Wang Y, Zhong J, Liu R, Xu G, *et al.* The expression of CDK1 is associated with proliferation and can be a prognostic factor in epithelial ovarian cancer. Tumor Biology. 2015; 36: 4939–4948.

[13] Yilmaz M, Maass D, Tiwari N, Waldmeier L, Schmidt P, Lehembre F, *et al.* Transcription factor Dlx2 protects from TGFβ-induced cell-cycle arrest and apoptosis. The EMBO Journal. 2011; 30: 4489–4499.