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NR2F6, LOXL2 and DMBT1 expression in cervical cancer tissues, prognostic and clinicopathological implications

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ABSTRACT

Background. Detection of novel predictive markers for early detection of lymph nodes metastases in cervical cancer is very important. Disturbed NR2F6 expression was found in many cancers playing different roles according to cancer type. LOXL2 was incriminated in cancer progression and unfavorable survival in many cancer types. Decreased DMBT1 expression was found in many cancers during their progression.
Aim of the present work was to assess expression of NR2F6, LOXL2 and DMBT1 in cervical cancer tissues using immunohistochemistry then correlating their expression with grade and stage of the tumor, occurrence of lymph nodes and distant metastases in addition to evaluating their prognostic roles.
Materials and methods. We assessed the expression of NR2F6, LOXL2 and DMBT1 in samples retrieved from sixty two cervical cancer patients in cancer tissues and corresponding adjacent normal tissues and we correlated markers expression with clinical data, pathological findings and patients' prognosis.
Results. High expression of NR2F6, LOXL2 and low expression of DMBT1 was up-regulated in cervical cancer tissues more than in adjacent non-neoplastic cervical tissues and was associated with high grade ($p = 0.005$), lymphovascular space involvement, advanced FIGO stage, resistance to chemotherapy, tumor recurrence, and shorter survival rates ($p < 0.001$).
Conclusions. Up regulation of NR2F6, LOXL2 and down regulation of DMBT1 were associated with unfavorable pathological parameters, bad clinical findings and dismal outcome of cervical cancer patients.

SOMMARIO

Contesto. La rilevazione di nuovi marcatori predittivi per la diagnosi precoce delle metastasi linfonodali nel cancro cervicale è molto importante. L'espressione distorta di NR2F6 è stata trovata in molti tumori che svolgono ruoli diversi a seconda del tipo di cancro. LOXL2 è stato incriminato nella progressione del cancro e nella sopravvivenza sfavorevole in molti tipi di cancro. La diminuzione dell'espressione di DMBT1 è stata trovata in molti tumori durante la loro progressione. Lo scopo del presente lavoro era valutare l'espressione di NR2F6, LOXL2 e DMBT1 nei tessuti del cancro cervicale mediante immunoistochimica, quindi correlando la loro espressione con il grado e lo stadio del tumore, la presenza di linfonodi e metastasi a distanza, oltre a valutare i loro ruoli prognostici.
Materiali e metodi. Abbiamo valutato l'espressione di NR2F6, LOXL2 e DMBT1 in campioni prelevati da sessantadue pazienti con cancro cervicale nei tessuti tumorali e nei corrispondenti tessuti normali adiacenti e abbiamo correlato l'espressione dei marcatori con i dati clinici, i risultati patologici e la prognosi dei pazienti.
Risultati. L'alta espressione di NR2F6, LOXL2 e la bassa espressione di DMBT1 erano sovraregolate nei tessuti del cancro cervicale più che nei tessuti cervicali adiacenti non neoplastici ed erano associate ad alto grado ($p = 0{,}005$), coinvolgimento dello spazio linfovascolare, stadio FIGO avanzato, resistenza a chemioterapia, recidiva del tumore e tassi di sopravvivenza più brevi ($p < 0{,}001$).
Conclusioni. La sovraregolazione di NR2F6, LOXL2 e la sottoregolazione di DMBT1 sono state associate a parametri patologici sfavorevoli, risultati clinici negativi e risultati negativi dei pazienti con cancro cervicale.

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Key words
Cancer cervix; NR2F6; LOXL2; DMBT1; prognosis, immunohistochemistry.

INTRODUCTION

Cancer cervix is the 4th commonest cancer and 4th cause of malignancy associated fatalities in women worldwide (1). Squamous cell carcinoma of the cervix is the commonest histopathological subtype of cancer cervix forming about 75-80% of all diagnosed cervical carcinoma cases (2).

Staging of cervical cancer is performed preoperatively (3). Presence of lymph node metastasis is the most important predictive factor of cancer recurrence and dismal outcome in cervical cancer (4). Therefore, detection of novel predictive markers for early detection of lymph nodes metastases in cervical cancer is very important.

Nuclear receptor subfamily 2 group F member 6 (NR2F6) is mapped on chromosome 19p13.1.1; and it encodes a conserved 43-kDa protein (5). NR2F6 was found to be responsible for regulating many biological processes as organogenesis and cellular differentiation during embryogenesis (6). Disturbed NR2F6 expression was found in many cancers playing different roles according to cancer type (7, 8). Lysyl oxidase-like 2 (LOXL2) is a lysyl oxidase (LOX) family member that plays an essential role in making cross-links of collagen and elastin in the extracellular matrix (9). LOXL2 was incriminated in cancer progression and unfavorable survival in many cancer types (10). Additionally, LOXL2 was found to be one of the recent therapeutic targets for cancer treatment (11).

Deleted in malignant brain tumor 1 (DMBT1), which is mapped on chromosome 10q25.3-26.1, was primarily discovered in glioblastoma and medulloblastoma as deleted, hence responsible for their progression and dismal outcome (12).

Recently, decreased DMBT1 expression was found in many cancers during their progression (13, 14) NR2F6, LOXL2 and DMBT1 expression in cervical cancer was not sufficiently clarified.

Aim of the present work was to assess expression of NR2F6, LOXL2 and DMBT1 in cervical cancer tissues using immunohistochemistry then correlating their expression with grade and stage of the tumor, occurrence of lymph nodes and distant metastases in addition to evaluating their prognostic roles.

MATERIALS AND METHODS

The present prospective study was performed on 62 cervical cancer patients where we acquired 124 specimens from both malignant cervical cancer tissues and corresponding adjacent normal tissues. Patients who were admitted and operated in Gynecology and Obstetrics Department and General Surgery Department, Faculty of Medicine, Zagazig University. Samples were processed and evaluated in Pathology Department, Faculty of Medicine, Zagazig University and patients were followed up for 5 years in Clinical Oncology and Nuclear Medicine Department and in Medical Oncology Department in the period from January 2013 to May 2018.

All included samples were stained with NR2F6, LOXL2 and DMBT1 using immunohistochemistry. We assessed the relationship between NR2F6, LOXL2 and DMBT1 tissue protein expression with disease progression and survival outcome of included cervical cancer patients.

The inclusion criteria were:

1. patients with a sure diagnosis of cervical squamous cell carcinoma and adenocarcinoma with accurate staging,
2. patients with complete clinical and pathological data and
3. patients with complete records of about 30 months follow-up.

Exclusion criteria were:

1. patients with other histopathological subtypes of cervical cancer,
2. patients with preoperative administration of radiotherapy, chemotherapy or hormonal therapy and
3. inoperable patients.

We acquired consents from included patients and an ethical approval for performing the study from the local ethical committee of Faculty of Medicine, Zagazig University.

Immunohistochemical (IHC) analysis

Primary antibodies used were: primary rabbit polyclonal anti- NR2F6 antibody (ab137496), anti-LOXL2 antibody (ab96233) and DMBT1 (MyBioSource, MBS9416387). Human gastric carcinoma tissue, human esophageal cancer tissue and non-neoplastic mucosa of the colon were used as positive controls for NR2F6, LOXL2 and DMBT1 respectively. IHC analysis was done as previously described (15).

Evaluation of IHC of NR2F6, LOXL2 and DMBT1

We assessed nuclear NR2F6 expression, cytoplasmic LOXL2 expression and cytoplasmic and membranous DMBT1 expression in cervical cancer tissues and adjacent non-neoplastic cervical mucosa. Evaluation of the stain was done according to staining extent and intensity.
The extent was scored as follows: 0 (negative tumor cells); 1 (< 10% positive tumor cells); 2 (10%–50% positive tumor cells); 3 (> 50% positive tumor cells). The intensity was scored as follows: 0, negative; 1, weak stain (light yellow); 2, moderate stain (yellow brown); 3, strong stain (brown). The final stain index was calculated by multiplying scores of extent and intensity, yielding results from 0–9. The suitable cut-off value was 4. Values above that value are considered high expression and below this value were considered low expression.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 (SPSS, Chicago, IL, USA).
The relationship between NR2F6, LOXL2 and DMBT1expression and the clinicopathologic features of cervical cancer was analyzed using the chi-square test and Fisher's exact test. We used Spearman's rank correlation coefficients to calculate the bivariate correlations between the studied variables. We plotted survival curves using the Kaplan–Meier method and compared them using log-rank testing. Relative risk ratios were calculated using the Cox proportional hazard model. Univariate and multivariate survival distributions were compared using log-rank testing. We considered $p < 0.05$ statistically significant.

NR2F6 expression in cervical cancer and adjacent non-neoplastic tissues

Tissue protein expression of NR2F6 was up-regulated in cervical cancer tissues more than in adjacent non-neoplastic cervical tissues and was negative in all samples of non-neoplastic cervical mucosa ($p < 0.001$) (**table I**).
High expression of NR2F6 was present in 34 (54.8%) of cervical cancer tissues and was associated with old age of the patient ($p = 0.002$), large tumor size ($p = 0.004$), high grade ($p = 0.005$), lymphovascular space involvement, para-uterine organ infiltration, advanced FIGO stage, resistance to chemotherapy, tumor recurrence ($p < 0.001$).
Patients with high NR2F6 expression had shorter recurrence free survival (RFS) and overall survival (OS) rates ($p < 0.001$) (**figures 1, 4, tables II-V**).

Table I. *Association between expression of NR2F6, LOXL2 and DMBT1 in cervical carcinoma and adjacent normal mucosa tissues.*

	Cervical cancer (N = 62)		Adjacent normal (N = 62)		p-value‡
	No.(%)		No. (%)		
NR2F6					
Low	28	(45.2%)	62	(100%)	< 0.001
High	34	(54.8%)	0	(0%)	
LOXL2					
Low	28	(45.2%)	50	(80.6%)	0.001
High	34	(54.8%)	12	(19.3%)	
DMBT1					
Low	40	(61.3%)	0	(0%)	< 0.001
High	22	(38.7%)	62	(100%)	

C‡ Chi-square test; $p < 0.05$ is significant.

LOXL2 expression in cervical cancer and adjacent non-neoplastic tissues

Tissue protein expression of LOXL2 was up-regulated in cervical cancer tissues more than in adjacent non-neoplastic cervical tissues ($p = 0.002$) (**table I**).
High expression of LOXL2 was present in 34 (54.8%) of cervical cancer tissues and was associated with old age of the patient ($p=0.002$), large tumor size ($p = 0.004$), high grade ($p = 0.005$), lymphovascular space involvement, para-uterine organ infiltration, advanced FIGO stage, resistance to chemotherapy, tumor recurrence ($p<0.001$).

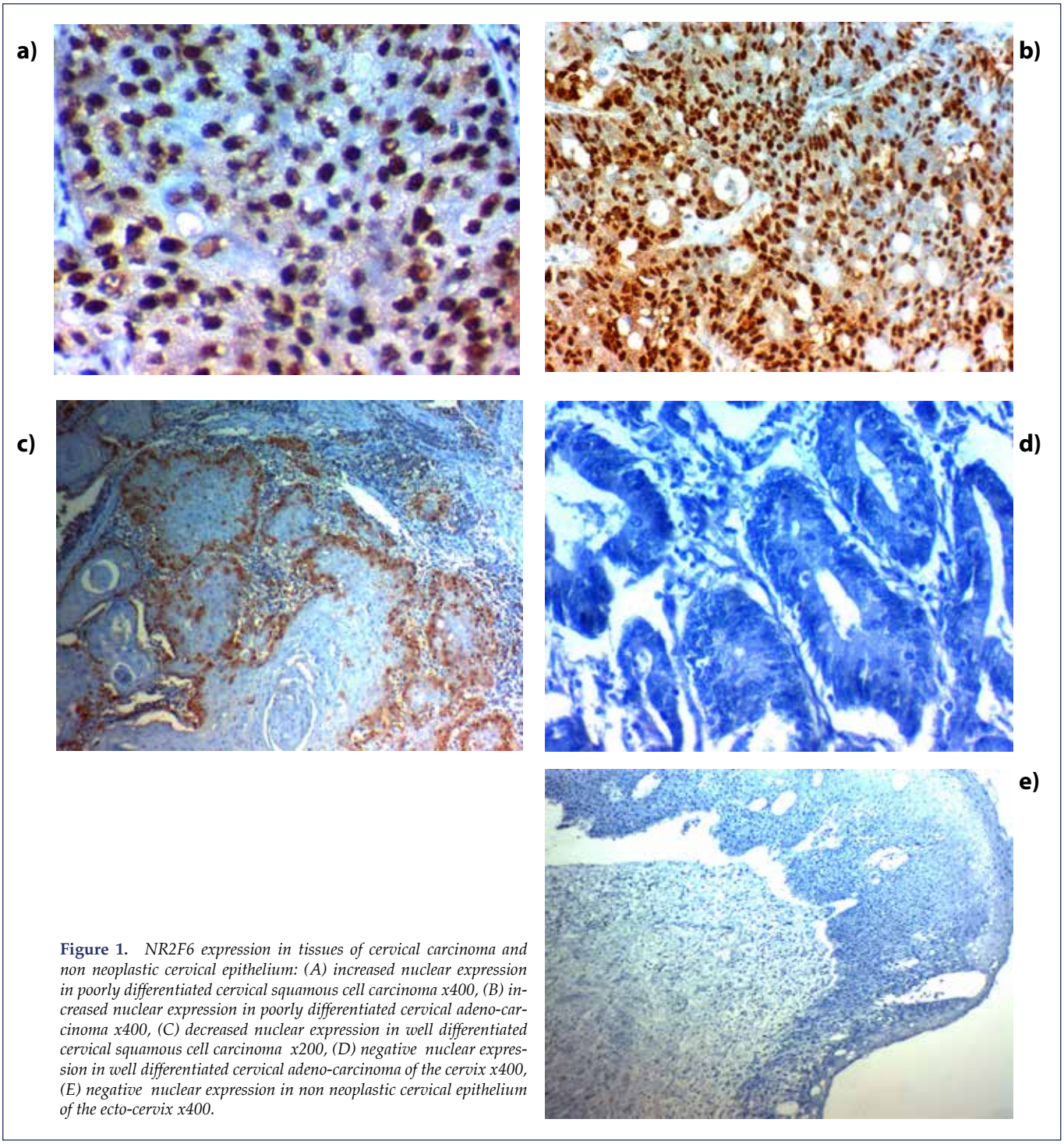

Figure 1. *NR2F6 expression in tissues of cervical carcinoma and non neoplastic cervical epithelium: (A) increased nuclear expression in poorly differentiated cervical squamous cell carcinoma x400, (B) increased nuclear expression in poorly differentiated cervical adeno-carcinoma x400, (C) decreased nuclear expression in well differentiated cervical squamous cell carcinoma x200, (D) negative nuclear expression in well differentiated cervical adeno-carcinoma of the cervix x400, (E) negative nuclear expression in non neoplastic cervical epithelium of the ecto-cervix x400.*

Patients with high LOXL2 expression had shorter recurrence free survival (RFS) and overall survival (OS) rates ($p < 0.001$) (**figures 2, 4, tables II-V**).

DMBT1expression in cervical cancer and adjacent non-neoplastic tissues

Tissue protein expression of DMBT1 was markedly down regulated in cervical cancer tissues than in adjacent non-neoplastic cervical tissues and high expression was found in all samples of non-neoplastic cervical mucosa ($p < 0.001$) (**table I**).

High expression of DMBT1 was associated with low grade ($p = 0.049$), absence of myometrium invasion ($p = 0.039$), absence of lymphovascular space involvement, absence of para-uterine infiltration, early FIGO stage ($p = 0.022$), response to chemotherapy ($p = 0.33$) and lower incidence of tumor recurrence ($p = 0.028$).

Table II. *Association between patients demographics, prognostic parameters and expression of NR2F6, LOXL2 and DMBT1 in the cervical cancer patients.*

	Total Patients		NR2F6			LOXL2			DMBT1		
			Low	High	P¥	Low	High	P¥	Low	High	P¥
	N = 62	%	N = 28 (45.2%)	N=34 (54.8%)		N = 28 (45.2%)	N = 34 (54.8%)		N = 40 (61.3%)	N = 22(38.7%)	
Age group:											
≤ 55 years old	25	40.3	26 (92.7)	11 (32.4)	0.002	16 (57.1)	9 (26.5)	0.002	9 (22.5)	16 (72.7)	< 0.001*
> 55 years old	37	59.7	2 (7.1)	23 (67.6)		12 (42.9)	25 (73.5)		31 (77.5)	6 (27.3)	
Histopathology:											
Squamous cell	44	71	19 (67.9)	25 (73.5)		19 (67.9)	25 (73.5)	0.78	28 (70)	16 (72.7)	
carcinoma	18	29	9 (32.1)	9 (26.5)	0.78	9 (32.1)	9 (26.5)		12 (30)	6 (27.3)	0.821
Adenocarcinoma											
Size:											
< 4 cm	6	9.7	6 (21.4)	0 (0)	0.004	6 (21.4)	0 (0)	0.004	3 (7.5)	3 (13.6)	0.657
≥ 4 cm	56	90.3	22(78.6)	34 (100)		22 (78.6)	34 (100)		37 (92.5)	24 (86.4)	
Grade:											
I	10	16.1	10 (35.7)	0 (0)	0.005	10 (35.7)	0 (0)	0.005	4 (10)	6 (27.3)	0.049
II	38	61.3	17 (60.7)	21 (61.8)		16 (57.1)	22 (64.7)		27 (67.5)	11 (50)	
III	14	22.6	1 (3.6)	13 (38.2)		2 (7.1)	12 (35.3)		9 (22.5)	5 (22.7)	
LVSI:											
Absent	44	71	27 (96.4)	17 (50)	< 0.001*	26 (92.9)	18 (52.9)	0.001*	28 (70)	16 (72.7)	0.022
Present	18	29	1 (3.6)	17 (50)		2 (7.1)	16 (47.1)		12 (30)	6 (27.3)	
Lymph node:											
Absent	30	48.4	22 (78.6)	8 (23.5)	< 0.001*	22 (78.6)	8 (23.5)	0.002*	17 (42.5)	13 (59.1)	0.211
Present	32	51.6	6 (21.4)	26 (76.5)		6 (21.4)	26 (76.5)		23 (57.5)	9 (40.9)	
Distant metastasis:											
Absent	46	74.2	27 (96.4)	19 (55.9)	< 0.001*	20 (71.4)	10 (29.4)	0.003*	29 (72.5)	17 (77.3)	0.681
Present	16	25.8	1 (3.6)	15 (44.1)		8 (28.6)	24 (70.6)		11 (27.5)	5 (27.7)	
Stage:											
I	6	9.7	6 (21.4)	0 (0)		6 (21.4)	0 (0)		3 (7.5)	3 (13.6)	
II	24	38.7	16 (57.1)	8 (23.5)	< 0.001*	14 (50)	10 (29.4)	0.001*	14 (35)	10 (45.5)	0.286
III	16	25.8	5 (17.9)	11 (32.4)		6 (21.4)	10 (29.4)		12 (30)	4 (18.2)	
IV	16	25.8	1 (3.6)	15 (44.1)		2 (7.1)	14 (41.2)		11 (27.5)	5 (22.7)	
NR2F6:											
Low	28	45.2				26 (92.9)	2 (5.9)	<	14 (35)	14 (63.6)	0.03*
High	34	54.8				2 (7.1)	32 (94.1)	0.001*	26 (65)	8 (36.4)	
LOXL2:											
Low	28	45.2	26 (92.9)	2 (5.9)	< 0.001*				13 (32.5)	15 (68.2)	0.007*
High	34	54.8	2 (7.1)	32 (94.1)					27 (67.5)	7 (31.8)	
DMTB1:											
Low	40	61.3	28 (100)	10 (29.4)	< 0.001*	28 (100)	10 (29.4)	<			
High	22	38.7	0 (0)	24 (70.6)		0 (0)	24 (70.6)	0.001*			

*p < 0.05 is statistically significant; ¥Chi square test.

Patients with high DMBT1 expression had longer recurrence free survival (RFS) and overall survival (OS) rates (p < 0.001) (**figures 3, 4, tables II-V**).

DISCUSSION

We showed that high NR2F6 expression was found in cancer tissues more than in adjacent non-neoplastic tissues and its high expression was associated with higher grade and advanced stage of cervical cancer.
Similarly Niu *et al*., (1) reported for the first time that high NR2F6 expression is correlated with poor prognosis, unfavorable clinical characteristics and dismal outcome.

NR2F6 might be considered a novel prognostic biomarker and therapeutic target for cervical cancer. NR2F6 was found to be overexpressed and associated with poor prognosis in patients with many cancers (7). These results denoted that it promoted tumor development and progression.
Niu *et al*., (1) showed that there was upregulation of NR2F6 mRNA and its tissue protein expression in cervical cancer and they have found a significant correlation between its tissue expression and advanced FIGO stage, tumor recurrence, resistance to chemotherapy, and presence of lymph nodes metastases.
Lymph nodes metastases is the most important predictive prognostic parameter and determining

Table III. *Association between treatment-related results and cervical cancer patients outcome.*

	N = 62	%
Treatment:		
Surgery	13	21
Surgery and radiotherapy	10	16.1
Surgery and chemotherapy	17	27.4
Surgery, radiotherapy and chemotherapy	14	22.6
Radiotherapy	4	6.5
Chemotherapy	4	6.5
Response:		
PD	37	59.7
SD	4	6.5
PR	7	11.3
CR	14	22.6
Response:		
OAR	41	66.1
NR	21	33.9
Outcome:		
Alive	37	59.7
Dead	25	40.3
Disease free survival (months) (N = 38):		
Mean ± SD	29.03 ± 6.49	
Range	16 - 36	
Overall survival (months):		
Mean ± SD	27.68 ± 9.18	
Range	10 – 36	

factor for treatment of early-stage cervical cancer (16).

Predicting occurrence of lymph node metastases before treatment of cervical cancer allows selecting the best management options (1). In the current study we found that NR2F6 was highly expressed in cervical cancer with positive lymph nodes metastases so it could be considered a predictive biomarker for lymph nodes metastases in early stage cervical cancer.

We also found that increased tissue protein expression was related to poor survival of patient hence considering it as a novel prognostic biomarker.

Despite discovering the values of using cancer cervix screening programs using Pap smear in early diagnosis of cancer cervix which in turn led to improvements in management, yet, the rate of recurrence is still high reaching up to 15%–30% (17). Although using adjuvant treatment reduced recurrence rate but it till now has many complications (1). Thus, it is essential to discover novel biomarker for early prediction of recurrence which in succes-

Table IV. *Association between patients treatment-response, outcome and expression of NR2F6, LOXL2 and DMBT1 in the cervical cancer patients.*

	NR2F6			LOXL2			DMBT1		
	Low	High	p	Low	High	p	Low	High	p
	N = 28 (45.2%)	N = 34 (54.8%)		N = 28 (45.2%)	N = 34 (54.8%)		N = 40 (64.5%)	N = 22 (35.5%)	
Treatment response:									
CR	27 (96.4)	10 (29.4)		26 (92.9)	11 (32.4)		32 (57.5)	14 (63.6)	
PR	1 (3.6)	3 (8.8)	< 0.001*	2 (7.1)	2 (5.9)	< 0.001*	3 (7.5)	1 (4.5)	0.033
SD	0 (0)	7 (20.6)		0 (0)	7 (20.6)		4 (10)	3 (13.6)	
PD	0 (0)	14 (41.2)		0 (0)	14 (41.2)		10 (25)	4 (18.2)	
Response:									
OAR	28 (100)	13 (38.2)	< 0.001*	28 (100)	13 (38.2)	< 0.001*	26 (65)	15 (68.2)	0.033
NR	0 (0)	25 (61.8)		0 (0)	21 (61.8)		14 (35)	7 (31.8)	
Recurrence (n=38):									
Absent	16 (59.3)	1 (9.1)	< 0.001*	17 (63)	0 (0)	< 0.001*	7 (30.4)	10 (66.7)	0.028*
Present	11 (40.7)	10 (90.9)		10 (37)	11 (100)		16 (69.6)	5 (33.3)	

*p < 0.05 is statistically significant; ¥Chi square test.

sion will lead to discovering new targeted therapies against recurrence. We showed that NR2F6 expression was significantly associated with recurrence and it might be considered a predictive factor of recurrence and a cancer therapeutic target (18).

In the present study we assessed the expression of LOXL2 a novel cancer prognostic biomarker and we found that its expression was positively associated with NR2F6 expression and was related to unfavorable pathological and clinical findings, in addition, patients with high expression of LOXL-2 had poor survival rates.

We have chosen LOXL-2 in cervical cancer tissues due to previous studies about its association with EMT induction in cancer through interaction with Snail-1 and E-cadherin (19).

Cao *et al*., (10) found that LOXL2 expression was positively related to EMT phenotype in cancer cervix and they found that cancer cells proliferation and migration were reduced after inhibition of LOXL2.

Cao *et al*., (10) found similar results regarding association between high LOXL2 expression and poor OS and DFS in cancer cervix patients.

Aberrant activation of EMT in many types of cancer was found during tumor progression and metastasis (20). LOXL2 overexpression induced EMT by increasing expression of many EMT inducing factors as SNAIL, ZEB, FAK/SRC and IRE1-XBP1

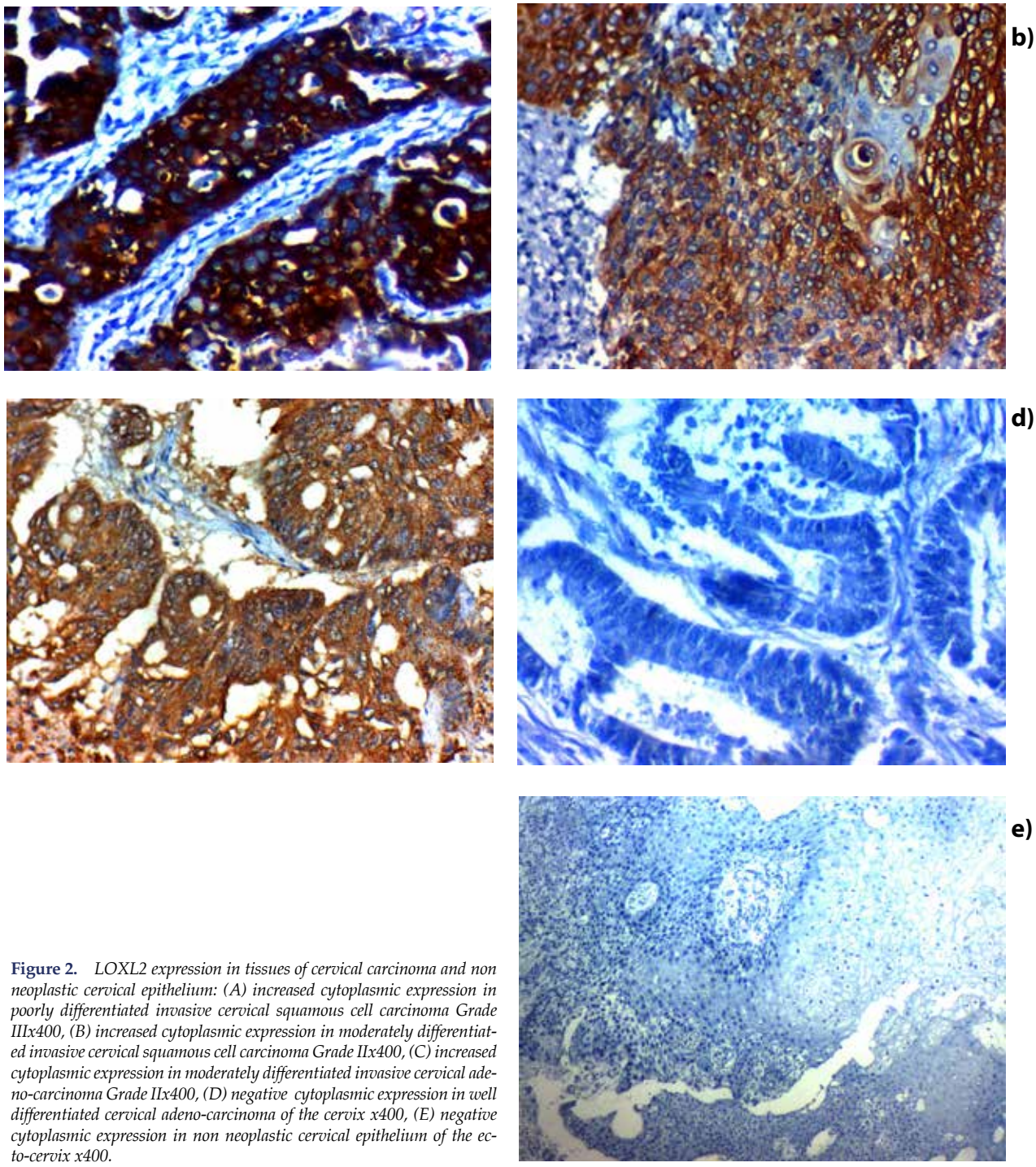

Figure 2. *LOXL2 expression in tissues of cervical carcinoma and non neoplastic cervical epithelium: (A) increased cytoplasmic expression in poorly differentiated invasive cervical squamous cell carcinoma Grade IIIx400, (B) increased cytoplasmic expression in moderately differentiated invasive cervical squamous cell carcinoma Grade IIx400, (C) increased cytoplasmic expression in moderately differentiated invasive cervical adeno-carcinoma Grade IIx400, (D) negative cytoplasmic expression in well differentiated cervical adeno-carcinoma of the cervix x400, (E) negative cytoplasmic expression in non neoplastic cervical epithelium of the ecto-cervix x400.*

signaling pathways in cancer cells (21, 22). Additionally, LOXL2 has a role in remolding process of the extracellular matrix which facilitated cancer cells invasion (23). Due to the scarcity of studies that assessed the expression of LOXL2 in cancer in addition to plenty of recent studies which were not significant particularly in regards to the grade and stage relation with its expression Cao *et al*., (10), we assessed another marker DMBT1 expression and its association with NRF2 and LOXL2 in cancer cervix.

In the present report we showed that DMBT1 expression levels were down regulated in cervical cancer tissues and its low expression was associated with poor prognosis and dismal outcome. Similar results were found by previous studies (14). Zhang (14) showed that mRNA and tissue protein DMBT1 levels were downregulated in cervical cancer tissues in comparison to normal cervical mucosa.
These findings collectively showed DMBT1 tumor suppressor role in cervical cancer.

Table V. *Association between patients survival and expression of NR2F6, LOXL2 and DMBT1 in the cervical cancer patients.*

Patients' outcome	NR2F6		LOXL2		DMBT1	
	High	Low	High	Low	High	Low
	N = 34 (%)	N = 28 (%)	N = 34 (%)	N = 28 (%)	N = 22 (%)	N = 40 (%)
Outcome:						
Dead	23 (67.6)	2 (7.1)	24 (70.6)	1 (3.6)	18 (81.8)	19 (47.5)
Alive	11 (32.4)	26 (92.9)	10 (29.4)	27 (96.4)	4 (18.2)	21 (52.5)
P	< 0.001**		< 0.001**		< 0.008*	
Odds ratio	27.18		64.8		4.97	
95% confidence interval	5.45 – 136.68		7.72 – 544.14		1.43 – 17.34	

OR odds ratio; CI confidence interval; **p ≤ 0.001 is statistically highly significant.

DMBT1 inhibition and association with cancer progression was found in many tumors (24).
Additionally high expression of DMBT1 was associated with early FIGO stage, small tumor size, absence of lymph node metastasis, and low grade of the tumor. Sousa JF, (25) and Dodurga *et al.* (26) and Du *et al.* (27) agreed with our result and showed that low expression of DMBT1 was associated with advanced disease and poor prognosis of many cancers.

Loss of DMBT1 expression in colorectal cancer was associated with unfavorable prognosis and was a predictive factor for disease recurrence (28). Moreover, Zhang (14) found that overexpression of DMBT1 promotes apoptosis and inhibits proliferation of cervical cancer cells which might be a possible explanation of our results.
Zhang (14) found both a marked elevation in the proapoptosis proteins as Bax and caspase-3 and

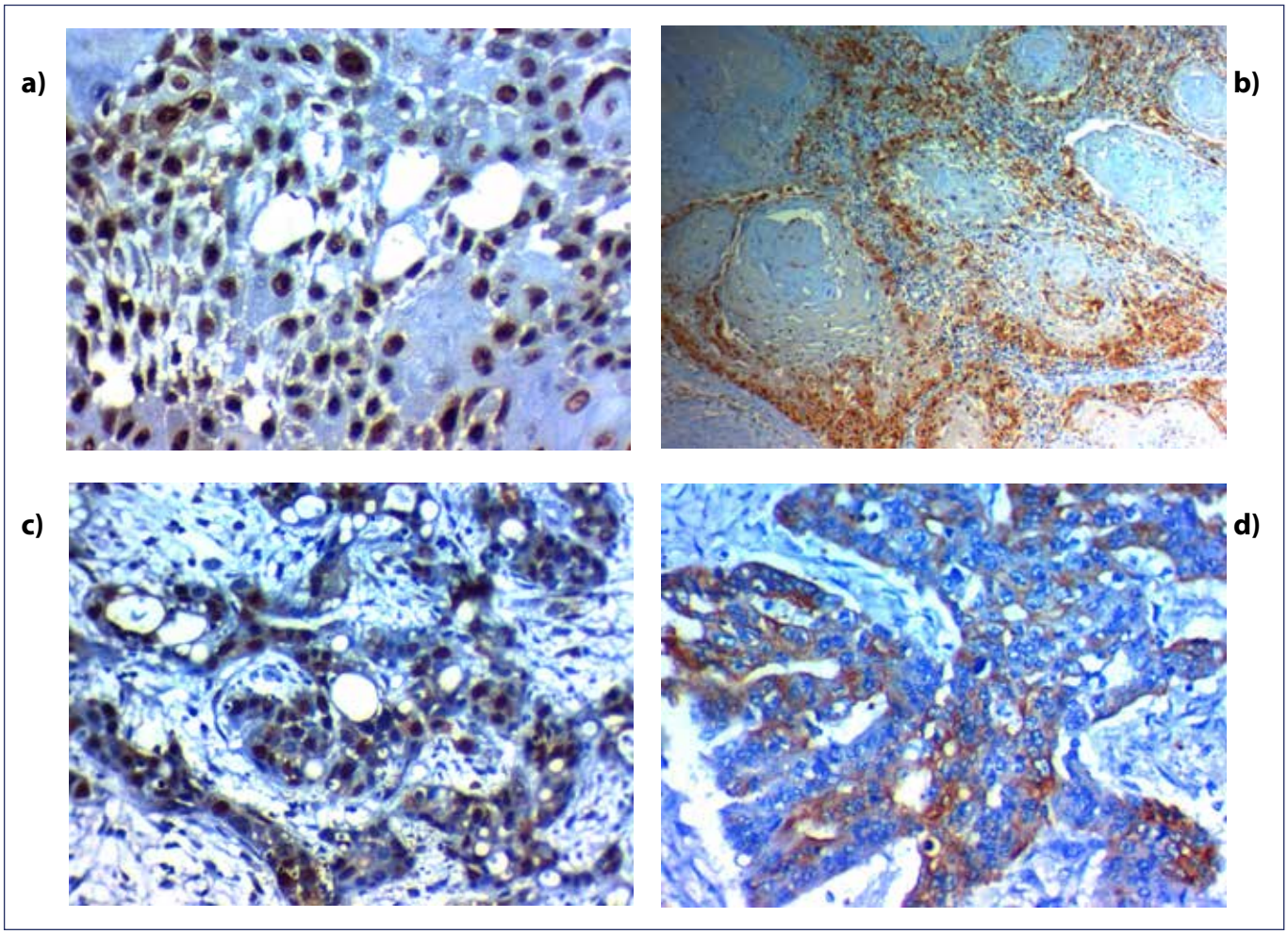

Figure 3. *DMBT1in tissues of cervical carcinoma and non neoplastic cervical epithelium: (A) High nuclear expression in non neoplastic cervical epithelium of the ecto-cervix x400, (B) High nuclear expression in well differentiated invasive squamous cell carcinoma of the cervix x400, (C) Low nuclear expression in poorly differentiated cervical squamous cell carcinoma x400, (D) Low nuclear expression in poorly differentiated cervical adeno-carcinoma x400.*

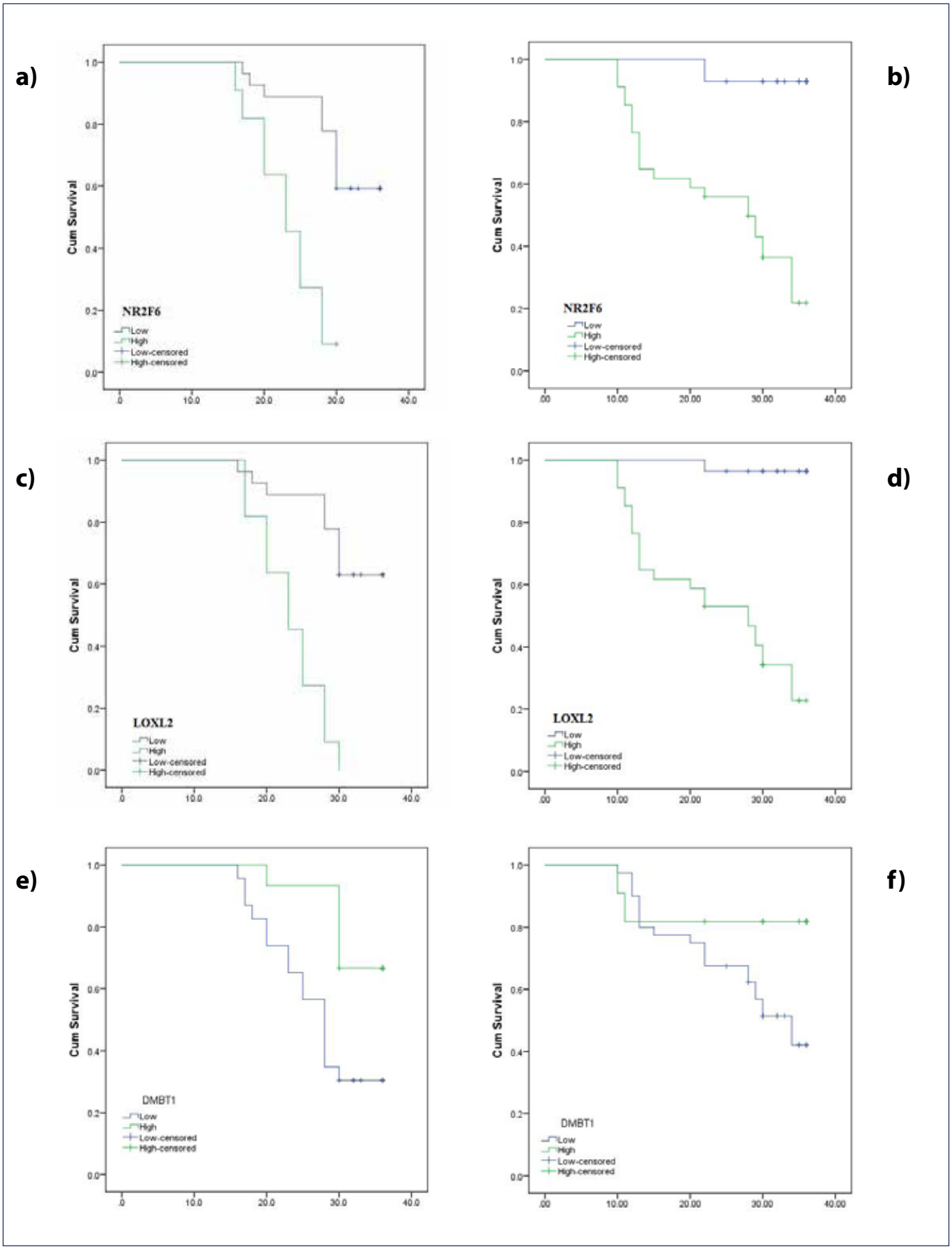

Figure 4. *Kaplan Meir survival curves of Disease free survival (DFS) and overall survival rate (OS) of cervical carcinoma patients: (A and B) DFS and OS rates stratified according to NR2F6 expression respectively, (C and D) DFS and OS rates stratified according to LOXL2 expression respectively, (E and F) DFS and OS rates stratified according to DMBT1 expression respectively.*

a marked reduction in the expression of the anti-apoptosis protein (Bcl-2) in cancer cells with higher DMBT1 expression.
Moreover, they showed that overexpression of DMBT1 could inhibit cervical cancer cells invasion and metastasis.
It was found that disturbances in the PI3K-AKT signaling pathway was incriminated in EMT induction and in controlling cancer progression (29)
Shen *et al.* (13) showed that higher expression of DMBT1 could be able to control PI3KAKT pathway by which it inhibits gall bladder cancer migration and invasion. Zhang *et al*., (14) showed that increased DMBT1 expression led to reduction in the expression of Vimentin and N-cadherin and up-regulation of E-cadherin so DMBT1 was able to inhibit EMT reducing cancer cells invasion and spread.

CONCLUSIONS

In the current study we assessed the expression of NR2F6, LOXL2 and DMBT1 which were novel markers incriminated in controlling EMT in cancer cells generally and particularly in cancer cervix cells. We concluded that up regulation of NR2F6, LOXL2 and down regulation of DMBT1 were associated with unfavorable pathological parameters, bad clinical findings and dismal outcome of patients.

Strengths of the study

First, the study was a prospective study that was the first in evaluation of 3 novel biomarkers that have not been previously evaluated together. Second, the three markers were previously found to have similar roles regarding induction of EMT in variable cancers, but they were not sufficiently evaluated in cancer cervix.
Moreover, previous studies assessed each marker in squamous cell carcinoma (SCC) which is the commonest histopathological subtype of cancer cervix but we assessed the expression of the markers in both SCC and adenocarcinoma.

Limitations of the study

First, the study included small cohort. Second, evaluation of the markers was done by only immunohistochemistry for evaluation of tissue protein expression of the assessed markers without genetic assessment.

So further future studies are needed to prove our findings in a larger cohort of cervical cancer patients and evaluation of genetic levels of the markers was needed to detect the mechanism of action of NR2F6, LOXL2 and DMBT1 in cervical cancer progression aiming at discovering novel therapies to decrease cervical cancer recurrence and progression.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

REFERENCES

1. Niu Ch, Sun X, Zhang W, *et al*. NR2F6 Expression Correlates with Pelvic Lymph Node Metastasis and Poor Prognosis in Early-Stage Cervical CancerInt. J. Mol Sci 2016;17:1694.
2. Furuta Y, Todo Y, Yamazaki H, *et al*. Radiation therapy versus surgery for patients with cervical squamous cell carcinoma who have undergone neoadjuvant chemotherapy revisited. Int J Clin Oncol 2018;23(1):126–133.
3. Iida M, Banno K, Yanokura M, *et al*. Candidate biomarkers for cervical cancer treatment: Potential for clinical practice (Review). Mol Clin Oncol 2014;2(5):647-55.
4. Asano H, Todo Y, Watari H. Adjuvant chemotherapy for early-stage cervical cancer. Chin J Cancer Res 2016;28:228–34.
5. Safe S, Jin UH, Hedrick E, Reeder A, Lee SO. Minireview: role of orphan nuclear receptors in cancer and potential as drug targets. Mol Endocrinol 2014;28(2):157-72.
6. Tsai SY, Tsai MJ. Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): Coming of age. Endocr Rev 1997;18:229–40.
7. Li XB, Jiao S, Sun H, *et al.* The orphan nuclear receptor EAR2 is overexpressed in colorectal cancer and it regulates survivability of colon cancer cells. Cancer Lett 2011;309(2):137-44.
8. Ichim CV, Atkins HL, Iscove NN, Wells RA. Identification of a role for the nuclear receptor EAR-2 in the maintenance of clonogenic status within the leukemia cell hierarchy. Leukemia 2011;25(11):1687-96.
9. Smith-Mungo LI, Kagan HM. Lysyl oxidase: properties, regulation and multiple functions in biology. Matrix Biol 1998;16:387–98.

10. Cao C, Lin S, Zhi W, *et al*. LOXL2 Expression Status Is Correlated With Molecular Characterizations of Cervical Carcinoma and Associated With Poor Cancer Survival via Epithelial-Mesenchymal Transition (EMT) Phenotype. Front Oncol2020;10:284.
11. Barker HE, Cox TR, Erler JT. The rationale for targeting the LOX family in cancer. Nat Rev Cancer 2012;12:540–52.
12. Mollenhauer J, Wiemann S, Scheurlen W, *et al*. DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. Nat Genet 1997;17(1):32–9.
13. Shen S, Liu H, Wang Y, *et al*. Long non-coding RNA CRNDE promotes gallbladder carcinoma carcinogenesis and as a scaffold of DMBT1 and C-IAP1 complexes to activating PI3K-AKT pathway. Oncotarget 2016;7(45):72833–44.
14. Zhang CH. The protective role of DMBT1 in cervical squamous cell carcinomaKaohsiung J Med Sci 2019;35:739–49.
15. Hsu SM, Soban E. Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. J Histochem Cytochem 1981;30:1079–82.
16. Liu L, Xia M, Wang J, Zhang W, Zhang Y, He M. CISD2 expression is a novel marker correlating with pelvic lymph node metastasis and prognosis in patients with early-stage cervical cancer. Med Oncol 2014;31(9):183.
17. Ahn HK, Shin JW, Ahn HY, et al. Metabolic components and recurrence in early-stage cervical cancer. Tumour Biol 2015;36(3):2201-7.
18. Wang SD, Li HY, Li BH, *et al*. The role of CTLA-4 and PD-1 in anti-tumor immune response and their potential efficacy against osteosarcoma. Int Immunopharmacol 2016;38:81-9.
19. Peinado H, Del Carmen Iglesias-de la Cruz M, Olmeda D, *et al*. A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. EMBO J 2005;24:3446–58.
20. Jolly MK, Ware KE, Gilja S, Somarelli JA, Levine H. EMT and MET: necessary or permissive for metastasis? Mol Oncol 2017;11:755–69.
21. Cuevas EP, Eraso P, Mazon MJ, *et al*. LOXL2 drives epithelial-mesenchymal transition via activation of IRE1-XBP1 signalling pathway. Sci Rep 2017;7:44988.
22. Peng DH, Ungewiss C, Tong P, *et al*. ZEB1 induces LOXL2-mediated collagen stabilization and deposition in the extracellular matrix to drive lung cancer invasion and metastasis. Oncogene 2017;36:1925–38.
23. Park PG, Jo SJ, Kim MJ, *et al*. Role of LOXL2 in the epithelial-mesenchymal transition and colorectal cancer metastasis. Oncotarget 2017;8(46):80325-35.
24. Imai MA, Moriya T, Imai FL, *et al*. Down-regulation of DMBT1 gene expression in human oral squamous cell carcinoma. Int J Mol Med 2005;15(4):585–9.
25. Sousa JF, Ham AJ, Whitwell C, *et al*. Proteomic profiling of paraffin-embedded samples identifies metaplasiaspecific and early-stage gastric cancer biomarkers. Am J Pathol 2012;181(5):1560–72.
26. Dodurga Y, Avci CB, Satiroglu-Tufan NL, *et al*. Detection of deleted in malignant brain tumors 1 and runt-related transcription factor 3 gene expressions in bladder carcinoma. Mol Biol Rep 2012;39(4):4691–5.
27. Du J, Guan M, Fan J, Jiang H. Loss of DMBT1 expression in human prostate cancer and its correlation with clinical progressive features. Urology 2011;77(2):509.e9–5e13.
28. Park HS, Kim BC, Yeo HY, *et al*. Deleted in malignant brain tumor 1 is a novel prognostic marker in colorectal cancer. Oncol Rep 2018;39(5):2279–87.
29. Yan LX, Liu YH, Xiang JW, *et al*. PIK3R1 targeting by miR-21 suppresses tumor cell migration and invasion by reducing PI3K/AKT signaling and reversing EMT, and predicts clinical outcome of breast cancer. Int J Oncol 2016;48(2):471–84.