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ORIGINAL ARTICLE

Prognostic Significance of Peroxisome Proliferator-activated Receptor-gamma (PPAR-γ) and Cyclin D1 in Bladder Urothelial Carcinoma.

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ABSTRACT

BBackground: stimulation of the PPAR-γ axis was efficient in inhibiting the propagation of human bladder cancer cells through various pathways, in part joining to Cyclin D1, so this study aimed to evaluate the role of PPAR-γ and Cyclin D1 expressions in both non-invasive and invasive muscle urothelial carcinoma patients. **Methods:** This study was carried out using 80 paraffin-embedded specimens of urothelial carcinoma of them non-muscle invasive carcinoma (N=44) and invasive muscle carcinoma (N=36). Non-invasive carcinoma shown (65.9%) with low grade and (34.1%) with high grade while invasive carcinoma showed (33.3%) with low grade and (66.7%). According to staging, non-invasive reported (75%) Ta, (25%) T1 while invasive carcinoma reported (61.1%) T2, (27.8%) T3, (11.1%) T4.Immunohistochemical staining was achieved to study the association between PPAR-γ and Cyclin D1 expressions with the clinicopathological parameters and its prognostic outcomes.

Results: positive expression of PPAR-γ and Cyclin D1 were significantly related to the higher tumor grade, further lymph node metastasis, and progressive tumor stage. Both positive PPAR-γ and Cyclin D1 expression were significantly connected with overall survival and disease-free survival . A significant positive association between PPAR-γ and Cyclin D1 expressions were found ($p<0.001$).

Conclusion: the expressions of PPAR-γ and Cyclin D1 are related to the progression of bladder urothelial carcinoma, as well as advanced-stage and presence of lymph node metastasis. Evaluation of PPAR-γ and Cyclin D1 expression could help is assessment of infiltrative detection ability of urothelial carcinoma and benefitted as prognostic factors for patients with bladder tumor.

Keywords: Bladder cancers, PPAR-γ, Cyclin D1, urothelial carcinoma.

INTRODUCTION

Cancer of urinary bladder is the highest ninth diagnosed tumors worldwide **[1].** The most incidence rates of bladder cancer in world records in Egypt; it accounts for 30% of all cancers, 40% of male cancers, and 16% of female cancers **[2].** The high mortality rates recorded in Egyptian men (12.6%), while females have fewer mortality rates (3.3%) **[3].**

Urinary bladder cancer is the maximum diagnosed malignancies in the urinary system; about 90% of bladder cancer cases affected by urothelial carcinoma **[4].**

The recurrence rates after surgical treatment are high, and its mortality rate class first between cancers of the urinary system. The main reason for mortality in urothelial carcinoma patients is invasion and metastasis of tumors, so the detection of new therapeutic markers may lead to improve survival of bladder cancer patients **[5].**

Transcription factors such as nuclear receptors and their triggered are one such approach targets. Interruption of these transcription factors is the main factor in the beginning and progression of malignancy. Mainly for this analysis, the (PPAR-γ), peroxisome proliferator-activated receptor gamma, is an element of the nuclear family receptors that activated transcription factors ligand **[6].**

Once PPAR-γ activated, it will particularly combine with a retinoid receptor X and indicate antiangiogenic, antiproliferative, and

differentiation pathways in numerous types of tissue. As a result, assembly it has a hugely useful goal for the downregulation of tumorigenesis **[7].** PPAR-γ is articulated in healthy tissues and takes part in different roles in differentiation, metabolism, and anti-inflammatory responses **[8].** Various cancers were detected with PPAR-γ expression as well as advanced prostate cancer **(9)**, ovarian carcinoma **([10]**, and testicular carcinoma [**11**]. Even if it has been confirmed to production an essential function in tumorigenesis, it is blurred whether higher expression of PPAR-γ associated with preferred consequence in tumor cases. Although, further scientific explanation shows that high expression PPAR-γ protein promotes colonic cancer, esophageal cancer, and cervical carcinoma **[12]**. The observations recommend that PPAR-γ may be an impending target for the treatment of malignancies **[6].** Thus, we are paying attention in evaluating the reliability of PPAR-γ in urothelial carcinoma of the bladder

Dysregulation of cell cycle has a leading role in the occasion of cancer **[13].** Cyclin D1 represents one of the regulatory factors of cell cycle, and it has a crucial goal in signals of proliferative (G1 phase) By combining with Cyclin-dependent kinase 4, One time activated, the compound Cyclin-dependent kinase 4 Cyclin D1 is designed, and the cycle of a cell is wholly controlled **[14].**

Expressions of Cyclin D1 are changed in different cancers, signifying that its dysregulation donates to carcinogenesis **[15].** So, Cyclin D1 is an efficient therapeutic target in the prevention of the growth of the tumor by reducing progression risk **[16].**

Overexpression of Cyclin D1 independently predicted an unfortunate effect in prostate cancer metastases patients **[17]** Also, higher expressions of Cyclin D1 have been significantly related to shorter survival in ovarian cancer. Signifying the Cyclin D1 expression is clinically essential for the progression of cancer **[18].**

In this work, we used an immunohistochemical method to assay the expression of PPAR-γ and Cyclin D1 in urothelial carcinoma of bladder to discover its prognostic roles and relationship with their clinicopathological parameters**.**

METHODS

This is a retrospective study involved 80 cases of primary bladder urothelial carcinoma. Cases were regained from Pathology archives records, clinical oncology and urology Department of Faculty of Medicine, Zagazig University in the period from (June 2012 to-June 2017). Inclusion criteria were patients with primary urothelial bladder carcinoma who underwent TURB-T either as complete or maximal resection or radical cystectomy as source of bladder samples. Samples from Patients with previous intravesical chemotherapy or BCG for non-muscle invasive bladder treatment or samples from patients undergoing palliative cystectomy after radiotherapy were excluded from the study. The studied patients' groups [80 cases] of primary bladder urothelial carcinoma were subdivided into two subgroups [44 cases with non-muscle invasive carcinoma, 36 cases with muscle-invasive carcinoma]. Of these 80 cases 50 cases were sampled by TURB-T and 30 cases by radical cystectomy, functional MRI was done for all 36 cases with muscle-invasive bladder carcinoma before cystectomy or chemoradiotherapy. Patients were followed up by cystourethroscopy and pelvi-abdominal contrast CT for all cases, according patients risk stratification criteria, oncological and urosurgical consultation.

Sections of Hematoxylin and eosin were studied to approve the diagnosis. Cases were graded depending on the cystectomy sample, and TURB samples were contained muscle fibers according to the WHO (2016) published criteria **[19]** and were assessed the invasion depth of urothelial carcinoma according to the classification of the TNM system was used for pathologic staging **[20].**

Two skilled pathologists instinctively and independently established the histopathological diagnosis of each section and decided on the grading and staging. Other lesions were riding on positively-charged slides, an immunohistochemically stained with rabbit polyclonal antibodies against PPAR gamma (ChIPGade ab45036, Abcam, Cambridge, U.K.) and Cyclin D1 using (EB2 clone, Dako, ready to use).

Immunohistochemistry

Immunohistochemical staining was performed by using indirect streptoavidin-biotin immunoperoxidase techniques. Sections of lesions (4–5 µm) were deparaffinized in xylene and rehydrated in graded alcohol. Sections were incubated for 10 minutes in hydrogen peroxide (0.3 %) of absolute methanol to block endogenous peroxidase action. Antigen retrieval was made using pH 6.0 Dako Target recovery solution (Dako, CA, USA). The sections were then hatched for 60 minutes at temperature room using the antibody against PPAR gamma and Cyclin D1. Negative panels were created by the replacement of the primary antibody with a corresponding an unrelated specificity primary antibody.

Immunohistochemical evaluation:

Interpretation of immunohistochemical staining of PPAR-γ

A semiquantitative scoring scheme (Remmele score or IR-score) was working to measure immunostaining. The IR-score calculated

immunostaining by staining intensity multiplication categorized as (0; none, 1;weak, 2;moderate and 3; intense staining) and percentage of cells with positive stains (0;no staining, 1; ≤ 10% of positive cells, 2; 11–50% of positive cells, 3;51–80% of positive cells and 4; ≥ 81% of positive cells). Tissue sections had been given an IR-Score (≥)higher or equal to 4 were recorded as positive **[21].**

Interpretation of immunohistochemical staining of Cyclin D1

Nuclear staining for Cyclin D1were recorded. Scoring of Cyclin D1 expression calculated according to **[15]:**

A semiquantitative scoring based on the sum of the percentage of positive cells was performed according to the following staining criteria: 0 (no positive tumor cells); 1 (<10% positive tumor cells); 2 (10-50% positive tumor cells); 3 (>50% positive tumor cells) and The staining intensity was scored as 0 (no staining); 1 (weak); 2 (moderate); 3 (strong). The cyclin D1 expression is defined as positive when the score is more than three and as negative when the score is less than or equal to 3.

Statistical analysis

Continuous parameters were articulated by way of the mean ± SD & (range) median, and the definite parameters were articulated such as a number (ratio). Continuous parameters were patterned for regularity by means of Shapiro-Wilk tests. The test of Mann Whitney U was performed for comparing two sets of non-normally disseminated parameters. Percentage of definite parameters was associated with Fisher's exact test or Pearson's Chi-square test when it was suitable. The tendency of variation in the dispersal of comparative incidences between ordinal information was related to the used Chi-square test for tendency. The (OS) Overall Survival was considered such as the period from diagnosis to death or the greatest new follow-up contact. The (DFS) Disease-Free Survival was considered as the time from the begin of treatment to date of deterioration or the greatest new follow-up connection that the patient was identified such as relapse-free. The OS and DFS stratification was completed conferring to markers. These time-to-event supplies were assessed by the Kaplan-Meier plot method and related to use a two-pronged particular log-rank test. Wholly tests were two-sided. A p-value <0.05 was measured significant. Wholly statistics were achieved by SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA) and Med Calc windows (Med Calc Softwares bvba 13, Ostend, Belgium).

RESULTS

The mean age was 61.03 ± 6.25 years (range 46-74), 66 males and 14 females were involved in the study. Non-muscle invasive urothelial carcinomas were 44 cases, while muscle-invasive UC were 36 cases. Histological grading showed 41 cases (51.2%) with low grade, 39 cases (48.8%) with high-grade urothelial carcinoma. Conferring to the staging system TNM were categorized into 33 cases (41.2%) of pathological stage PTa, 11 cases (13.8%) of stage PT1, 22 cases (27.5%) of stage PT2, 10 cases (12.5%) of stage PT3 and 4 cases (5%) of stage PT4. As regards lymph node metastasis, 10 (100%) positive lymph node in the muscle-invasive tumor as shown in table 1

Expression PPAR-γ and Cyclin D1 in non-muscle invasive urothelial carcinoma

The positive percentage of PPAR-γ expressions in non-invasive urothelial carcinoma (pTa/pT1) were (17 out of 44) cases (38.6%), while expressions of PPAR-γ were not observed in the adjacent tissue tumor (Fig. 1A). The expressions of PPAR-γ were significantly in the highest expression of high-grade urothelial carcinoma (93.3%) than low-grade carcinoma (10.3%) (Fig. 1B) With p<0.001. As regards multicentricity, The expressions of PPAR-γ were highly articulated in multiple cancer (100%) than the solitary one (25%) with p-value < 0.001. PPAR-γ expressions were excessive significant in tumors more than 3 cm (57.1%) than tumors less than 3 cm with p -value <0.016 (table 3).

The positive expressions of cyclin D1 in non-invasive urothelial carcinoma (pTa/pT1) cases were(34.1 %) while it gave negative expression in tissue adjacent tumor. Cyclin D1 was a highly significant expression in high-grade carcinoma (86.7%) (Fig. 1E) than low grade carcinoma (6.9%) (Fig. 1D) with p < 0.001. As well as Cyclin D1 expression was highly significant in multiple tumors (100%) than a solitary one (14.3%) p-value < 0.001. As regards tumor size cyclin D1 was a significant expression in cancer more than 3 cm (52.4%) than the tumors less than 3 cm (17.4%) with p-value = 0.014 (table 3)

Relationship of PPAR-γ and Cyclin D1 expression with muscle invasiveness among bladder carcinoma cases (table 2).

The expression of PPAR-γ was significant in muscle-invasive urothelial carcinoma (69.4%) concerning non-muscle invasive urothelial carcinoma (38.6%) with p = 0.006. There is a significant expression of Cyclin D1 in muscle-invasive urothelial carcinoma (72.2%) concerning non-muscle invasive urothelial carcinoma (34.1%) with p-value = 0.001. In bladder urothelial cancer, the expressions of PPAR-γ were positively associated with the expressions of Cyclin D1 protein.

Association of PPAR-γ and Cyclin D1 and treatment outcome among non-muscle invasive bladder carcinoma: (Table 1 and 2)

As regards to the relationships of PPAR-γ, Cyclin D1, and recurrence, it was shown that among 44 cases with non-muscle invasive urothelial carcinoma recurrence were detected in 20 patients (45.5%), and 24 patients (54.5%) had no relapse throughout follow up dated. It was clear that disease recurrence was significantly present in the positive expressions of PPAR-γ, Cyclin D1, and significantly absent in cases in which PPAR-γ and Cyclin D1 are negatively expressed (p =0.001).

Association between survival with PPAR-γ and Cyclin D1 expressions:

We analyzed overall survival and disease-free survival of non-muscle invasive urothelial carcinoma using Kaplan Meier method (fig2). Regarding DFS, a significant difference existed between patients with negative versus those with positive expressed PPAR-γ and CyclinD1, the DFS for negative expressed was significantly longer than with positive expressed (58.79 months versus 38.96 months p=0.001), and (59.63 months versus 40.02 months p= 0.001, respectively.

Expression PPAR-γ and Cyclin D1 in muscle-invasive urothelial carcinoma (Table 4).

The positive expression of PPAR-γ in muscle-invasive carcinoma (pT2-pT4) was cases (25 out of 36) cases (69.4%). PPAR-γ expression was significant in high-grade urothelial carcinoma (83.3%) (Fig 1C) than low grade (41.7) carcinoma with p<0.02.

We detected that PPAR-γ was expressively higher in cases with positive lymph nodes than those without (p< 0.016) (a higher number of cases were significant (100 %) of the invasive bladder. Higher PPAR-γ expressions were significantly correlated with the cancer TNM stage, AJCC stage (P= 0.06 and P= 0.008, respectively).

The positive expression of Cyclin D1 invasive carcinoma (pT2- pT4) cases were (72.2%) (26 out of 36) cases. Cyclin D1 expression was a significant expression in high grade UC (91.7%) than low grade UC (33.3%) with p = 0.02.To evaluate whether expressions of Cyclin D1 was related to cancer invasiveness, We detected that expressions of Cyclin D1 were significantly greater (100%) in cases with metastasis lymph node related to those without metastasis (p< 0.03).

Association of PPAR-γ and Cyclin D1 with recurrence among muscle-invasive bladder carcinoma (table1and 2):

Regarding recurrence, it was clear that it occurred in cases with high expression of Cyclin D1 13 cases (100%) and 2cases (20%) in negative expression with a extremely significant difference (p = 0.001). Also, a vast significant difference in recurrence was present among cases with positive expression of PPAR-γ 12 cases (100%) and 3cases (27.3%) negative PPAR-γ cases (p =0.001).

Association between Cyclin D1 and PPAR-γ expressions with survival in muscle-invasive bladder carcinoma (fig;2 A ,B,Cand D):

According to analyzed survival, we found that patients with negative expressions of Cyclin D1 survived more in DFS means as 57.20 months and 46.53 months in positive expressed cases (p = 0.001) and OS mean was60 months and 52.03 months in negative and positive expressed cases respectively (p = 0.001). Also, there were a significantly difference in survival among cases with a negative expression of PPAR-γ which was longer than positive expressed cases survived in which DFS mean as 57.27 months and 45.58 months in positive expressed cases (p 0.001) and OS mean 60 months in negative expressed cases and 51.72 months in positive expressed cases, respectively (p =0.013).

Table (1): Basic characteristics of the studied bladder urothelial carcinoma patients.

	All Patients		**Non muscle invasive**		**Muscle Invasive**	
	(n = 80)		**(n = 44)**		**(n = 36)**	
	No.	**%**	**No.**	**%**	**No.**	**%**
Age						
Mean±SD	61.03	±6.25	60.27	±5.55	61.97	±6.97
Median (range)	60	(46-74)	60	(46-74)	61	(47-73)
<=60 years	40	50%	25	56.8%	15	41.7%
>60 years	40	50%	19	43.2%	21	58.3%
Sex						
Male	66	82.5%	37	84.1%	29	80.6%
Female	14	17.5%	7	15.9%	7	19.4%
Grading						

	All Patients		Non muscle invasive		Muscle Invasive	
	(n = 80)		(n = 44)		(n = 36)	
	No.	%	No.	%	No.	%
Low grade	41	51.2%	29	65.9%	12	33.3%
High grade	39	48.8%	15	34.1%	24	66.7%
Lymph Node						
Node negative	70	87.5%	44	100%	26	72.2%
Node positive	10	12.5%			10	27.8%
PT stage						
PTa	33	41.2%	33	75%		
PT1	11	13.8%	11	25%		
PT2	22	27.5%			22	61.1%
PT3	10	12.5%			10	27.8%
PT4	4	5%			4	11.1%
N stage						
N0	70	87.5%	44	100%	26	72.2%
N1	4	5%			4	11.1%
N2	4	5%			4	11.1%
N3	2	2.5%			2	5.6%
Recurrence	(n=67)		(n=44)		(n=23)	
No recurrence	32	47.8%	24	54.5%	8	34.8%
Recurrence	35	52.2%	20	45.5%	15	65.2%
Type of recurrence			(n=20)			
Non-invasive recurrence			6	30%		
Invasive recurrence			14	70%		
Mortality						
Alive	65	81.2%	40	90.9%	25	69.4%
Died	15	18.8%	4	9.1%	11	30.6%
Follow-up (months)						
Mean SD	56.30	8.04	57.98	6.21	54.25	9.52
Median (range)	60	(31-60)	60	(31-60)	60	(31-60)

Categorical variables were expressed as number (percentage). Continuous variables were expressed as mean ± SD & median (range).

Table (2): Relation between muscle invasiveness and IHC staining for PPAR-γ and Cyclin D1 among bladder urothelial carcinoma patients (n=80).

	All patients		Non muscle invasive		Muscle Invasive		p-value‡
	(n = 80)		(n = 44)		(n = 36)		
	No.	%	No.	%	No.	%	
PPAR-γ							
Negative	38	47.5%	27	61.4%	11	30.6%	0.006
Positive	42	52.5%	17	38.6%	25	69.4%	
Cyclin D1							
Negative	39	48.8%	29	65.9%	10	27.8%	0.001
Positive	41	51.2%	15	34.1%	26	72.2%	

Categorical variables were expressed as number (percentage); ‡ Chi-square test; $p<0.05$ is significant.

Table (3): Relationship between basic characteristics and IHC staining for PPAR-γ and Cyclin D1 among non-muscle invasive bladder urothelial carcinoma patients (n=44).

Basic characteristics	**Nonmuscle invasive (n = 44)**		**Cyclin D1**					**PPAR-γ**				
			Negative (n=29)		Positive (n=15)		p-value	Negative (n=27)		Positive (n=17)		p-value
	No.	%	No.	%	No.	%		No.	%	No.	%	
Age												
Mean±SD	60.27	±5.55	59.13	±5.78	62.46	±4.47	0.022•	59.25	±5.10	61.88	±5.99	0.052•
Median (range)	60	(46-74)	59	(46-72)	62	(57-74)		59	(46-72)	61	(47-74)	
<=60 years	25	56.8 %	20	80%	5	20%	0.024‡	18	72%	7	28%	0.096‡
>60 years	19	43.2 %	9	47.4 %	10	52.6 %		9	47.4 %	10	52.6 %	
Sex												
Male	37	84.1 %	25	67.6 %	12	32.4 %	0.594‡	23	62.2 %	14	37.8 %	1.000‡
Female	7	15.9 %	4	57.1 %	3	42.9 %		4	57.1 %	3	42.9 %	
Grading												
Low grade	29	65.9 %	27	93.1 %	2	6.9%	<0.001 ‡	26	89.7 %	3	10.3 %	<0.001 ‡
High grade	15	34.1 %	2	13.3 %	13	86.7 %		1	6.7%	14	93.3 %	
Multicentricity												
Solitary	36	81.8 %	29	80.6 %	7	19.4 %	<0.001 ‡	27	75%	9	25%	<0.001 ‡
Multiple	8	18.2 %	0	0%	8	100 %		0	0%	8	100 %	
Tumor size												
<=3 cm (small)	23	52.3 %	19	82.6 %	4	17.4 %	0.014‡	18	78.3 %	5	21.7 %	0.016‡
>3 cm (large)	21	47.7 %	10	47.6 %	11	52.4 %		9	42.9 %	12	57.1 %	
Risk stratification (AUA)												
Low risk	18	40.9 %	18	100 %	0	0%	<0.001 §	17	94.4 %	1	5.6%	<0.001 §
Intermediate risk	12	27.3 %	11	91.7 %	1	8.3%		10	83.3 %	2	16.7 %	
High risk	14	31.8 %	0	0%	14	100 %		0	0%	14	100 %	
CyclinD1												
Negative	29	65.9 %						26	89.7 %	3	10.3 %	<0.001 ‡
Positive	15	34.1 %						1	6.7%	14	93.3 %	
PPAR-γ												
Negative	27	61.4 %	26	96.3 %	1	3.7%	<0.001 ‡					
Positive	17	38.6 %	3	17.6 %	14	82.4 %						

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); • Mann Whitney U test; ‡ Chi-square test; § Chi-square test for trend; p<0.05 is significant.

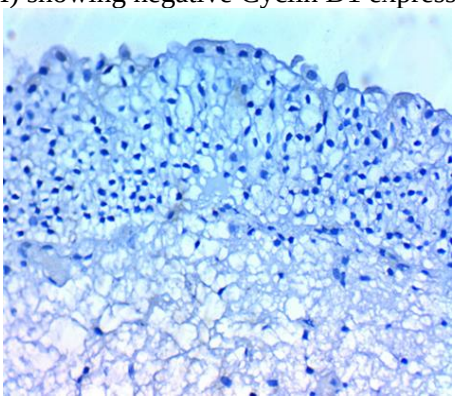
Table (4): Relationship between basic characteristics and IHC staining for Cyclin D1 and PPAR-γ among muscle invasive bladder urothelial carcinoma patients (n=36).

Basic characteristics	**Muscle Invasive (n = 36)**		**Cyclin D1**					**PPAR**				
			Negative (n=10)		Positive (n=26)		p-value	Negative (n=11)		Positive (n=25)		p-value
	No.	%	No.	%	No.	%		No.	%	No.	%	
Age												
Mean±SD	61.97	±6.97	58.30	±6.66	63.38	±6.68	0.028•	57.09	±4.41	64.12	±6.86	0.003•
Median (range)	61	(47-73)	57	(47-69)	63	(48-73)		57	(47-62)	66	(48-73)	
<=60 years	15	41.7 %	7	46.7 %	8	53.3 %	0.058‡	8	53.3 %	7	46.7 %	0.025‡
>60 years	21	58.3 %	3	14.3 %	18	85.7 %		3	14.3 %	18	85.7 %	
Sex												
Male	29	80.6 %	7	24.1 %	22	75.9 %	0.370‡	8	27.6 %	21	72.4 %	0.650‡
Female	7	19.4 %	3	42.9 %	4	57.1 %		3	42.9 %	4	57.1 %	
Grading												
Low grade	12	33.3 %	8	66.7 %	4	33.3 %	0.001‡	7	58.3 %	5	41.7 %	0.020‡
High grade	24	66.7 %	2	8.3%	22	91.7 %		4	16.7 %	20	83.3 %	
Tumor size												
<=5 cm	19	52.8 %	9	47.4 %	10	52.6 %	0.008‡	11	57.9 %	8	42.1 %	<0.001‡
>5 cm	17	47.2 %	1	5.9%	16	94.1 %		0	0%	17	100 %	
Lymph Node												
Node negative	26	72.2 %	10	38.5 %	16	61.5 %	0.035‡	11	42.3 %	15	57.7 %	0.016‡
Node positive	10	27.8 %	0	0.0%	10	100 %		0	0%	10	100 %	
PT stage												
PT2	22	61.1 %	7	31.8 %	15	68.2 %	0.286§	9	40.9 %	13	59.1 %	0.069§
PT3	10	27.8 %	3	30%	7	70%		2	20%	8	80%	
PT4	4	11.1 %	0	0%	4	100 %		0	0%	4	100 %	
N stage												
N0	26	72.2 %	10	38.5 %	16	61.5 %	0.041§	11	42.3 %	15	57.7 %	0.029§
N1	4	11.1 %	0	0%	4	100 %		0	0%	4	100 %	
N2	4	11.1 %	0	0%	4	100 %		0	0%	4	100 %	
N3	2	5.6%	0	0%	2	100 %		0	0%	2	100 %	
AJCC Stage group												

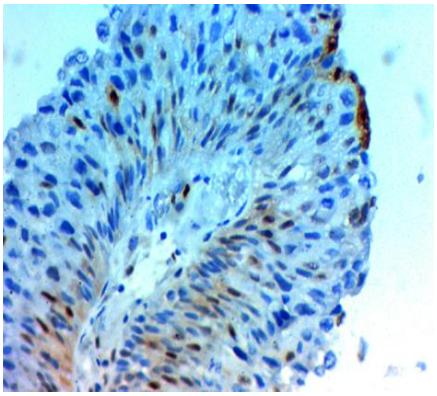
Basic characteristics	**Muscle Invasive (n = 36)**		**Cyclin D1**					**PPAR**				
			Negative (n=10)		Positive (n=26)		p-value	Negative (n=11)		Positive (n=25)		p-value
	No.	%	No.	%	No.	%		No.	%	No.	%	
Stage II	14	38.9 %	6	42.9 %	8	57.1 %	0.056§	8	57.1 %	6	42.9 %	0.008§
Stage IIIA	16	44.4 %	4	25%	12	75%		3	18.8 %	13	81.2 %	
Stage IIIB	4	11.1 %	0	0%	4	100 %		0	0%	4	100 %	
Stage IVA	2	5.6%	0	0%	2	100 %		0	0%	2	100 %	
Cyclin D1												
Negative	10	27.8 %						8	80%	2	20%	<0.001‡
Positive	26	72.2 %						3	11.5 %	23	88.5 %	
PPAR-γ												
Negative	11	30.6 %	8	72.7 %	3	27.3 %	<0.001‡					
Positive	25	69.4 %	2	8%	23	92%						

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); • Mann Whitney U test; ‡ Chi-square test; § Chi-square test for trend; $p<0.05$ is significant

Figure (1): Immunohistochemical expression of PPAR-γ and Cyclin D1 sections counterstained with hematoxylin: A) normal urothelium of bladder showing negative expression of PPAR-γ. B) Non-invasive urothelial carcinoma (Ta, low grade, stage I) showing negative PPAR-γ staining. C) Invasive urothelial carcinoma (T2, high grade, stage II) showing positive nuclear staining of PPAR-γ.. D)Non-invasive urothelial carcinoma (Ta, low grade, stage I) showing negative Cyclin D1 expression. E) Non-invasive urothelial carcinoma (high grade) showing positive Cyclin D1 expression F) Invasive urothelial carcinoma (T2, high grade, stage II) showing negative Cyclin D1 expression.



(A)



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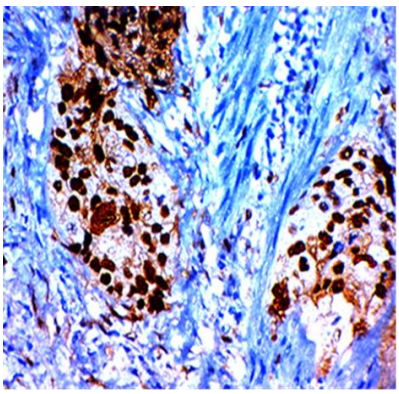

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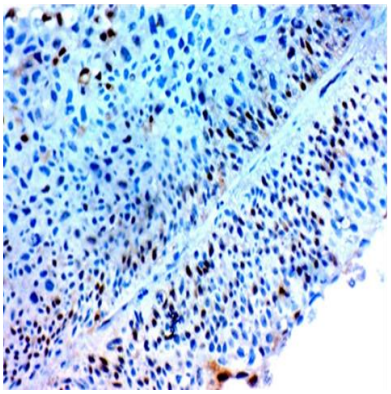
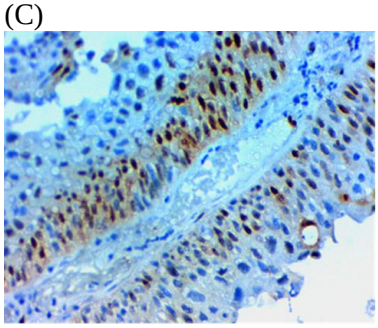
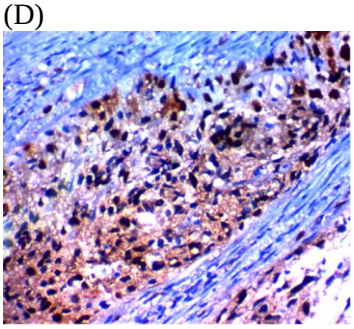


(D)



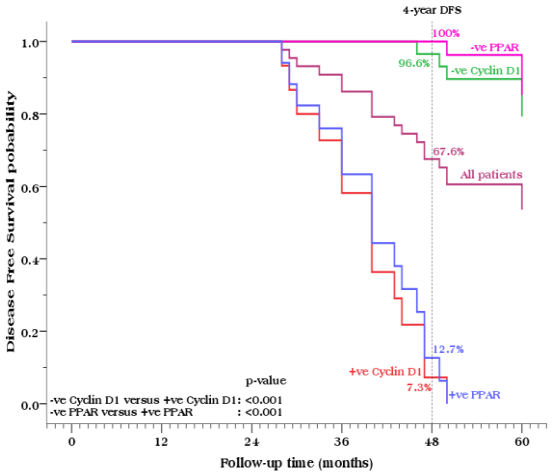
(E)



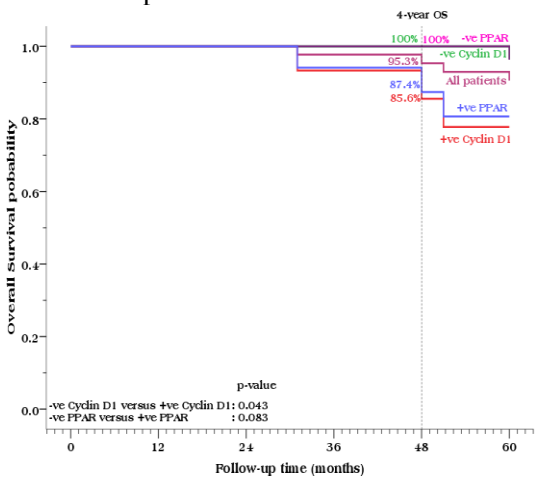
(F)

Figure (2): Kaplan Meier plot;

(A) Disease Free Survival for non-muscle invasive bladder carcinoma patients.
(B) Overall Survival for non-muscle invasive bladder carcinoma patients.
(C) Disease Free Survival for muscle invasive bladder carcinoma patients.
(D) Overall Survival for muscle invasive bladder carcinoma patients.



(A)



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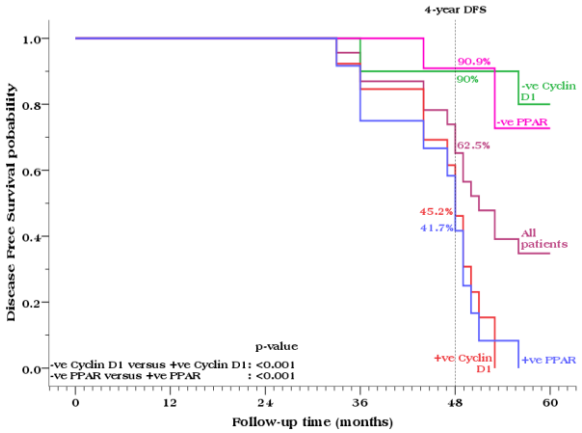
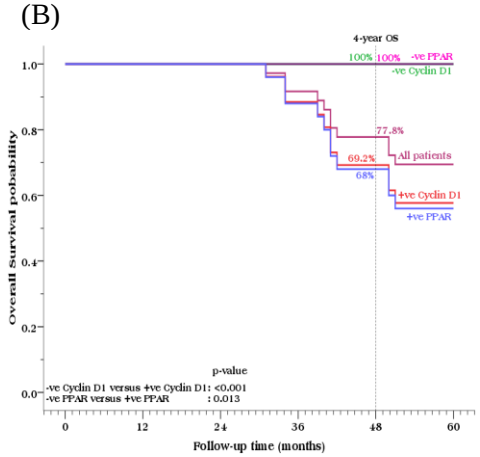


(C)



(D)

DISCUSSION

- Urinary bladder cancer was ordered into two main sets for predictive purposes and management, non-muscle invasive urothelial carcinoma (Tis ,Ta, T1) and muscle-invasive urothelial carcinoma (T2, T4). An excessive case of urothelial carcinoma existing with non-muscle invasive that granting heterogeneous clinically were characteristically related to a satisfactory prognosis and a comparatively low risk for relapse after cystectomy. Although, non-muscle invasive urothelial carcinoma was related to higher risk (60 -75%) of relapse in cases with remained bladders **[22].**
- Stimulation of the PPAR-γ axis were both active in expressively hindering propagation of human carcinoma cells through diverse pathways, in part joining to Cyclin D1, and Cyclin-dependent kinase inhibitors **[23].** So we explored the role of PPAR-γ and Cyclin D1in urothelial carcinoma of the urinary bladder.
- In our work, PPAR-γ expressions were higher in bladder urothelial carcinoma than the adjacent tumor tissue, suggesting that PPAR-γ may participate in urothelial carcinoma tumorigenesis. This accord with **Inamoto et al., [7],** found that PPAR-γ was noticed only in bladder cancer. However, in contrast with **Yoshimura et al. [24].** Moreover, **Varley et al**., **[25]** found PPAR-γ expression in normal urothelium, which responsible for the differentiation of urothelium.
- As regards to the association between PPAR-γ expression and pathological stage (T) of urothelial carcinoma, PPAR-γ expressions were detected in 38.6% of non-muscle invasive urothelial carcinomas and 69.4 % of muscle-invasive UC. There were a statistical significantly relationship between PPAR-γ expressions and muscle invasiveness of urothelial carcinoma (pathological stage) (P= 0.006). In agreement with other studies, **Yang et al., [26], Inamoto et al., [7],** and **Nakashiro et al., [27]** found that the expressions of PPAR-γ were high in cases with invasive carcinoma than those with non-muscle-invasive carcinoma. It is suggested that PPAR-γ had an important role in the progression of urothelial carcinoma.
- To provide the effect of PPAR-γ on progression of bladder cells , **Yang et al., [26]** investigated samples to bladder cancer and benign bladder mucosal samples by (FISH) fluorescence in situ hybridization analyze for expressions of PPAR-γ, and they found 31% (8/21 samples) samples of bladder cancer exhibited amplification while 4.3% (1/23 samples) of benign bladder samples exhibited amplification. Also, minor level of PPAR-γ amplification was noticed in samples of non-muscle invasive bladder cancer compared to samples of muscle-invasive carcinoma (167% against 46.7%, respectively) In dissimilarity to **Mylona et al., [28]** who found that the expressions of PPAR-γ more commonly in the superficial carcinoma (Ta,T1) than invasive carcinoma (T2-T4) tumors. This difference may be due to the small numbers of sample size, heterogeneity of tumors, collecting samples, and method used and subjective errors in assessing the stage.
- In this present study, analyzed PPAR-γ expressions conferring to grade of the non-invasive urothelial carcinoma, a positive rate of PPAR-γ immunoreactivity was detected in 3 % of low-grade carcinoma and 93.3% of high-grade carcinoma. PPAR-γ expression exhibited a highly statically significant relationship with grade of the non-muscle invasive urothelial carcinoma ($p<0.001$). Also, PPAR -γ expression according to the grade of muscle-invasive urothelial carcinoma, a positive rate of PPAR-γ immunoreactivity was observed in 41.7 % of low-grade carcinoma and 83.3% of high-grade carcinoma. PPAR-γ expression showed a highly statically significant correlation with muscle-invasive urothelial carcinoma. The PPAR-γ expression may be associated with differentiation, the progression of tumors, so the PPAR -γ can be used as prognostic factors.
- Similar results were obtained by **Yoshimura et al., [24].** Furthermore, **Inamoto et al., [7]** who detected more prevalent PPAR-γ immunoreactivity in cases of high histological grade more than low grade and disagreed with other studies were completed by **Varley et al., [29]** who found PPAR-γ was expressed in normal urothelium and its occurrence was related to lower risk of tumor relapse and progression of tumors . Also, our results disagree with **Mylona et al., [28].** PPAR -γ immunoreactivity was in reverse with tumor grade (p = 0.007), such as it was observed more expressed in lower grade carcinoma than those of higher grades carcinoma and **Nakashiro et al., [27]** who detected all cases of urothelial carcinomas of low grade were more positive staining than high-grade carcinomas. This different due to a small number of cases and improper staging of the specimens and variation in the scoring method for detecting PPAR-γ immunohistochemical marker expression

- Regarding to disease-free survival (DFS) and overall survival (OS) ,analysis revealed PPAR-γ immunopositivity to be associated significantly with short duration of OS, p= 0.013, worse DF, P= 0.001 and significantly with carcinoma recurrence, p= 0.001and mortality p=

0.015. However, **Possati et al., [30]** studied PPARγ expression in human bladder cancer (75) specimens, and the outcomes were related to the pathological and clinical features of tumor . They observed that the PPARγ expression was related to a significantly lower incidence of tumor progression and recurrence

- Reduction of PPAR-γ activity, whether by genetic ablation or pharmacologic inhibition, repressed proliferation of PPAR-γ -activated tumor cells of bladder. So it could be utilized as a therapeutic goal in bladder cancer **[31].** PPAR-γ as a therapeutic goal in bladder tumor could be realized as equivalent to the androgen receptor targeting in prostate cancer or targeting estrogen receptors in breast cancer **[32].**
- **Rochel et al., [33]** reported that increased PPAR-γ transcriptional activity had an outstanding result in bladder cancer. It reduces growth of tumor cells PPAR-γ-dependent and controls the microenvironment of tumors to favor ejection from immuno-surveillance with activation of the pathway associated with amplifications or gains of the PPAR-γ gene resulting in PPARγ overexpression. Also, **Korpal et al., [34]** reported muscle-invasive bladder carcinoma was a potentially deadly cancer with various genetic changes that produce constitutive activation of PPAR-γ a and effect in escape from immunosurveillance by CD8+ T-cell recruitment inhibition.
- Cyclin D1 is a crucial regulator of cell cycle that panels the progression of G0/G1 and donates to proliferation of cells . It is the greatest significant conclusive cell cycle regulatory factor , woks a vital role in the development of cancers **[35].**
- In the present work, the Cyclin D1 expressions were detected in urothelial carcinoma in 56.5% (47 out of 80 cases) and negative expression in tumor-adjacent tissue. These results were union with **Songtao et al., [36],** who detected Cyclin D1 in bladder cancer cells. This might be attributed to the Cyclin D1 roles in urothelial carcinoma tumorigenesis. Also, **Kopparapu et al., [37]** were reported that bladder carcinoma had been significantly expressions of Cyclin D1 elevated levels with a 3- fold enhance contrasted to normal urothelium (p=0.003). **Tut et al., [38]** were detected positive staining for Cyclin D1 in 83% of tumors, while normal urothelium sections established nuclear staining in < 5 % of basal cells.
- In the present work, expressions of Cyclin D1 , according to grade of non-muscle-invasive urothelial carcinoma, a positive rates of Cyclin D1 immunoreactivity were observed in 6.9% of low grade carcinoma and 86.7% of high grade carcinoma. Cyclin D1 expression exhibited a highly statically significant relationship to urothelial carcinoma grade (p<0.001). The Cyclin D1 protein may be associated with differentiation, a progression of tumors, and could expect prognosis of cases with non-invasive urothelial carcinoma.
- Similar outcomes were found by **Songtao et al., [36]** who detected more prevalent Cyclin D1 immunoreactivity in cases with high histological grades. However, disagree with **Tut et al., [38]** who observed Cyclin D1 staining had a highly significantly in low grade carcinoma compared with high grade carcinoma. This difference may be due to the change in several diagnosed cases and different methods of procedure for taking samples.
- As regards the connection between Cyclin D1 expressions and pathological stage of urothelial carcinoma, Cyclin D1 expressions was detected in 34.2% non muscle invasive urothelial carcinoma and 72.2% of invasive urothelial carcinoma. There were a highly significantly association between Cyclin D1 expression and pathological stage of urothelial carcinoma (depth of invasion of tumors) (P= 0.001) which was with similarity to studies of **Songtao et al., [36]** and **Kopparapu et al., [37]** whose found Cyclin D1 expression in invasive urothelial carcinoma is much greater than non-invasive urothelial carcinoma. These suggested that the expression of Cyclin D1 is related to the advanced bladder cancer. In contrast to **Tut et al., [38]** and **Shan and Tang [14],** who found no significant association between immunohistochemical expressions of Cyclin D1 and the pathological T-staging of urothelial carcinoma. Our study also showed that cases with a positive Cyclin D1 expressions had significantly worse survival and poor prognosis contrasted with those cases with a negative Cyclin D1 expressions. Our study also showed that patients with positive Cyclin D1 expression showed significantly worse survival, both (OS), and (DSF) P=0.001 and 0.001 and also recurrence p=0.001 and poor prognosis as regards mortality p=0.016 compared with those patients with negative Cyclin D1 expression. In agreement with **Songtao et al., [36]** but disagree with another study done by **Lopez-Beltran et al., [39].** Cyclin D1were independent predictors of disease-free survival this difference due to the different cut off point Cyclin D1 (%), <15 vs. ≥15. Similar results also obtained by **Kopparapu et al., [37],** there was a strong tendency that cases with a positive expression of Cyclin D1 and metastasis of lymph node had poor disease- free survival contrasted to those had lesser levels of Cyclin D1expression with no lymph node metastasis .

Cases with higher levels expressions of Cyclin D1 had a tendency to have a shorter time to cancer relapse, even if statistical significance was not realized.

Our work presented that expressions of PPAR -γ protein in bladder urothelial carcinoma increased with higher expressions of Cyclin D1, which destined that they are positively correlated PPAR–γ protein may through up-regulating the Cyclin D1 expression, indicating the cell cycle disorder and then promoting tumor cell proliferation.

Gou et al., [6] reported that PPAR-γ was an essential target for cancer chemotherapy because of its higher expression in cancer, and it affected cell proliferation and apoptosis.

CONCELUSION

Overexpression PPAR -γ and Cyclin D1 protein in bladder urothelial carcinoma and their correlation with poor prognosis in cancer patients. Identifying PPAR -γ and cyclin D1 may help in detecting the advancement of urothelial carcinoma of bladder and predicting the prognosis of patients. Further research about PPAR -γ to be the new target for gene therapy of bladder urothelial carcinoma

Conflict of Interest: None.

Financial Disclosures: None

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