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Original Contribution

Fatty acid synthase, Her2/neu, and E2F1 as prognostic markers of progression in non-muscle invasive bladder cancer

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ABSTRACT

Non-muscle-invasive bladder cancer (NMIBC) is a heterogeneous disease which has an unpredictable risk of progression to muscle-invasive bladder cancer (MIBC). The selection of patients who may benefit from early radical intervention is a challenge. To define the useful prognostic markers for progression, we analyzed the immunohistochemical expression of fatty acid synthase (FASN), Her2/neu, and E2F1 in 60 cases of NMIBC who underwent TURBT and adjuvant intravesical bacillus-Calmette-Guérin (BCG). Their predicting role for tumor recurrence, progression, recurrence-free survival (RFS) and progression-free survival (PFS) was analyzed. High FASN expression was observed in 56.7% (34/60) of NMIBC cases, and FASN expression was significantly associated with the tumor size, grade, and tumor stage ($p = 0.003$, $p < 0.001$, $p < 0.0001$ respectively). Positive Her2/neu was noted in 18.3% (11/60) of the cases, and its expression was significantly associated with the tumor size, histologic grade, and tumor stage ($p = 0.001$, $p = 0.002$, $p = 0.011$ respectively). High E2F1 expression was detected in 40% of the cases, and it was associated with tumor size, histologic grade, and tumor stage ($p < 0.001$ for each). Analysis of follow-up period revealed that NMIBC with high FASN, positive Her2/neu, and high E2F1 expression exhibited a potent relation with tumor progression, shorter RFS, and poor PFS. Conclusions: High FASN, Her2/neu, and E2F1 are considered as adverse prognostic factors of tumor recurrence and progression in NMIBC and these patients should be followed carefully. Therefore, we suggest that FASN, Her2/neu, and E2F1 should be considered and evaluated during the selection of the appropriate management strategy for NMIBC patients.

1. Introduction

Bladder cancer (BC) is the commonest malignancy of the urinary tract and the sixth most common neoplasm of men worldwide [1]. Nearly 75% of these patients present with non-muscle-invasive BC (NMIBC) that may be limited to the mucosa as a papillary tumor (stage Ta) or carcinoma in situ (CIS, stage Tis) without stromal invasion or invasion limited to the submucosa (stage T1) [2]. NMIBC is a heterogeneous group of neoplasms with different rates of progression to Muscle invasive bladder cancer (MIBC), ranging from 0.8% to 45% in 5 years. Previous studies proposed that the main risk factors for the progression are the presence of concomitant CIS, higher grade, and the T1 stage. Moreover, multiplicity, large tumor size (≥3 cm), and a history of recurrence are also considered as risk factors for progression. Discrimination of NMIBC cases with progressive potential to MIBC is fundamental, considering the advantage of early radical intervention. However, there are a few tools to predict progression in NMIBC [3]. The present prognostic models suffer from limited predictive accuracy [4,5].

Several in vitro studies have reported that elevated lipogenesis is related to poor prognosis in certain tumors [6]. Fatty acid synthase (FASN), a multifunctional enzyme implicated in de-novo lipogenesis, is overexpressed in a variety of tumors with a significant prognostic value [7]. Furthermore, a decline of FASN activity markedly promotes apoptosis and inhibits tumor growth and metastasis [8].

FASN is the only enzyme having the ability to catalyze a reductive de-novo synthesis of long-chain fatty acids from acetyl-coenzyme A (CoA) and malonyl-CoA via NADP. In most normal tissue, except for the adipose and hepatic tissues, FASN expression is low [9]. However, the expression of

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FASN is often upregulated in rapidly proliferating cells. Inhibition of FASN expression could suppress cellular proliferation in various tumors. Accordingly, FASN has become an attractive target for cancer therapy in the preceding 15 years [10]. FASN can be phosphorylated by kinases such as mammalian target of rapamycin (mTOR) and Her2/neu and this regulation is important for both its activity and the subcellular localization [11].

Her2/neu is a tyrosine kinase receptor that belongs to the erbB family. Similar to FASN, Her2/neu is often low in normal tissues, but it is abnormally activated and overexpressed in various tumors [12]. Her2/neu activates multiple downstream pathways, as the PI3K/Akt and Ras/Raf/MAPK pathways, which are the upstream signals of FASN. Moreover, sufficient assembly of phospholipids for membrane microdomains will participate in the adjustment of receptor tyrosine kinases expressed on the membrane, as Her2/neu. Therefore, a significant correlation between FASN and Her2/neu which may accentuate carcinogenesis might be present [13,14]. The predictive value of Her2/neu status in BC is still controversial. Moreover, there are only a few studies of Her2/neu status in NMIBC [15].

Furthermore, to identify the correlation of FASN with tumor cell proliferation, we assessed a key factor in cellular proliferation. The transcription factor E2F1 plays a crucial role in lipogenesis alongside the cell-cycle progression and apoptosis induction in response to DNA damage under normal circumstances. Nevertheless, increased levels of E2F1 promote cellular proliferation [16]. Deregulated E2F1 mediates tumor progression throughout the upregulation of EGFR and activation of the cytoplasmic Ras/mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K)/AKT signaling cascades [17]. In a prior study, E2F1 overexpression and its associated target genes predicted progression from Non-MIBC to MIBC [18].

Accordingly, there is a real need for dependable molecular tumor markers to predict BCG response and to identify patients with high-risk superficial tumors that will likely progress to invasive tumors, and so individualize treatment choices for each patient. There are few studies that had investigated FASN expression or its association with Her2/neu or E2F1 in NMIBC. Therefore, the aim of the present study was to assess the immunohistochemical expression of FASN, Her2/neu, and E2F1 in 60 patients with NMIBC who were treated with TURBT and adjuvant intravesical BCG. Their predicting role of BCG response, tumor recurrence, progression and progression-free survival was analyzed.

2. Patients and methods

2.1. Patients' selection

This prospective study was performed in Urology, Pathology and Clinical Oncology departments, faculty of medicine, Zagazig University hospitals and Al-Ahrar teaching hospital, and included sixty patients with primary papillary superficial TCC (Stage Ta-T1) of the bladder (with or without associated CIS) who were registered in the period from January 2014 to December 2016. Formalin-fixed-paraffin-embedded tissue specimens were obtained from Pathology department, Zagazig university hospitals and Al-Ahrar teaching hospital. No cases with isolated Tis or recurrent tumor were included in this study. All hematoxylin and eosin-stained slides were assessed to verify the diagnosis, histological grade, and tumor stage. The tumors were graded according to the 2016 WHO tumor classification and assigned stages according to the AJCC Staging System, 8th edition [19,20]. Clinical staging was based on a combination of cystoscopy, computed tomography, ultrasound, and histopathological data. Patients' data, including age, sex, tumor size, multifocality, treatment strategy were obtained from the patients' records. Follow-up of the studied cases was recorded in Urology and Clinical Oncology departments. Written consent was obtained from each participant and the study was approved by the Research Ethics Committee of the faculty of medicine, Zagazig University.

2.2. Protocol of therapy

After the complete TURBT for all patients, intravesical BCG instillation therapy was started for intermediate or high-risk patients after 2–4 weeks post-endoscopy according to the current therapeutic guidelines [21]. BCG was continued with weekly instillation with a dose of 80 mg (Tokyo 172 strain) for six weeks. Intravesical maintenance of BCG instillation was then continued every 3 months for at least one year. Patients were followed-up with cystoscopy and urinary cytology for all patients every 3 months for 2 years and every 6 months thereafter. Computed tomography was performed for upper tract monitoring yearly till the end of the study. Tumor recurrence was defined as any pathological evidence of NMIBC during the period of follow-up following TURBT, whereas progression was defined as a pathological progression to MIBC or appearance of metastasis. Recurrence and progression-free survival time is the time interval between TURBT and the time that tumor recurrence or progression had occurred.

2.3. Immunohistochemistry protocol

Formalin-fixed paraffin-embedded tissue (FFPE) blocks were serially sectioned into 3–5 μm sections followed by deparaffinization in xylene and rehydration in descending series of alcohols. For antigen retrieval, 10 mM citrate buffer (pH 6.0) at the microwave for 20 min was used. Endogenous peroxidise activity was blocked by 3% hydrogen peroxide for 10 min. After repeat washing in PBS, the slides were incubated with Polyclonal FASN antibody (Clone H-300, dilution 1:50, Dako, California, USA), mouse monoclonal Her2/neu antibody (Clone e2-4001, catalog no. MS-730-R7, Lab vision, California, USA, ready to use), and monoclonal E2F1 antibody (Santa Cruz Biotechnology, USA, dilution 1:100). Binding site of primary antibodies was visualized by using the polymer detection system; the Dako EnVision™ kit (Dako, Copenhagen, Denmark). Finally, the tissue sections were counterstained with Meyer's hematoxylin, dehydrated and mounted. Negative controls were done by replacement of the primary antibodies with a non-immune serum. Positive and negative controls were stained in the same setting with the studied cases.

2.4. Interpretation of the immunohistochemistry

2.4.1. FASN scoring

Cytoplasmic FASN expression in tumor cells was evaluated and scored using a semiquantitative method. The intensity of its immunostaining was scored as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Whereas, the extent of staining was scored as follows: 0 (no staining), 1 (< 10%), 2 (10–50%) and 3 (> 50%). The sum of both the extent and intensity scores was the final score (0–6). Scores ≤ 3 were considered as low expression, while scores ≥ 4 were considered as a high expression [22].

2.4.2. Her2/neu scoring

Only the membranous Her2/neu immunostaining was assessed. A 4-point scale was used: 0 (no staining), 1 (weak membrane staining in < 10% of cells), 2 (moderate membrane staining in ≥10% of cells), 3 (strong membrane staining in ≥10% of cells). The tissue sections with score 2 or 3 staining were considered positive for Her-2/neu [23].

2.4.3. E2F1 scoring

Nuclear E2F1 expression in tumor cells was evaluated. Intensity of nuclear staining was scored as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Whereas, the pattern score was assessed as follows: (1 = 0–4% of positive tumor cells; 2 = 5–19; 3 = 20–39; 4 = 40–59; 5 = 60–79; 6 = 80–100%). Both the pattern and intensity scores were multiplied (0–18). A histological score value of 4 was used as a cut-off for the stratification of E2F1 expression into low (≤4) and high (> 4) [24].

2.5. Statistics

Continuous variables were expressed as the mean ± SD & median

(range), and the categorical variables were expressed as a number (percentage). Continuous variables were checked for normality by using the Shapiro-Wilk test. Percent of categorical variables were compared using Pearson's Chi-square test or Fisher's exact test when was appropriate. Recurrence Free Survival (RFS) was calculated as the time from diagnosis to reappearance of non-muscle invasive disease with no evidence of invasive disease or the most recent follow-up contact that the patient was known as recurrence free. Progression Free Survival (PFS) was calculated as the time from diagnosis to re-appearance of muscle invasive disease with or without evidence of non-muscle invasive disease recurrence or the most recent follow-up contact that the patient was known as progression free. Stratification of RFS and PFS was done according markers. These time-to-event distributions were estimated using the method of Kaplan-Meier plot, and compared using two-sided exact log-rank test. All tests were two sided. A p-value < 0.05 was considered significant. All statistics were performed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium).

3. Results

3.1. Clinicopathological features of the studied NMIBC cases

The clinicopathological features of the 60 patients with NMIBC were summarized in Table 1. The patients included 43 men (71.7%) and 17 women (28.3%). The mean age of our patients was 51.45 years (range 37–66 years). Tumor size of ≥3 cm was the predominant one (56.7%); and multifocality was present in 25% of the cases. Concomitant CIS was noted in 23.3% of NMIBC cases. Tumors were graded into low grade (40%) and high grade (60%) (Fig. 1). Pathologic stage before BCG consisted of pTa in 33.3% and pT1 in 66.7% of the studied cases. Mean follow-up duration was 40 months (range 12–48 months). Tumor recurrence and progression were observed in 24 (40%) and 15 (25%) patients respectively.

Table 1
Clinicopathological features, immunohistochemical markers and outcome of 60 patients with NMIBC.

Characteristics	All studied patients (N = 60)	
	No.	%
Age		
Mean ± SD	51.45 ± 7.75	
Median (range)	52 (37–66)	
< 50 years	24	40%
≥50 years	36	60%
Sex		
Male	43	71.7%
Female	17	28.3%
Tumor size		
≤3 cm	26	43.3%
> 3 cm	34	56.7%
T stage		
Ta	20	33.3%
T1	40	66.7%
Grade		
Low grade	24	40%
High grade	36	60%
CIS		
Absent	46	76.7%
Present	14	23.3%
Multiplicity		
Absent	45	75%
Present	15	25%
FASN		
Low	26	43.3%
High	34	56.7%
HER2/neu		
Negative	49	81.7%
Positive	11	18.3%
E2F1		
Low	36	60%
High	24	40%
Follow-up (months)		
Recurrence	40.01 ± 10.06	
Progression	44 (12–48)	
Outcome		
Recurrence	24	40%
Progression	15	25%

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

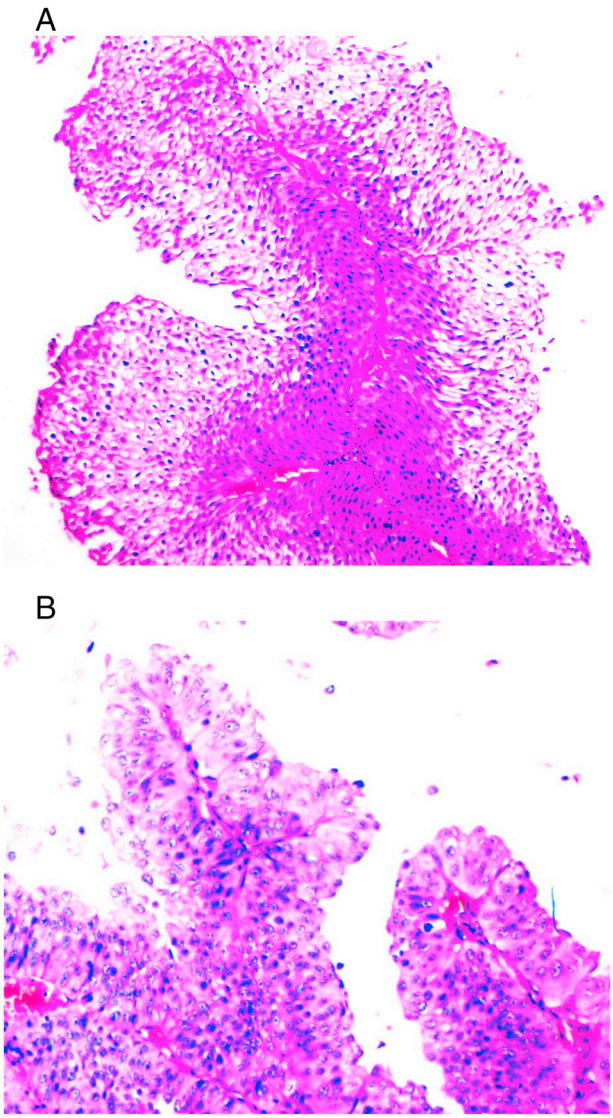

Fig. 1. (a) A case of low-grade non-muscle invasive bladder cancer, (b) a case of high-grade non-muscle invasive bladder cancer.

3.2. FASN, Her2/neu, and E2F1 immunoexpression in the studied NMIBC cases (Table 2)

The high cytoplasmic FASN immunoexpression was detected in 56.7% of the studied NMIBC cases (Fig. 2). There was a significant up-regulation of FASN expression with tumor size ($p = 0.003$), histological

Table 2
Relation between clinicopathological features and immunohistochemical staining for FASN, HER2/neu and E2F1 in NMIBC (N = 60).

Characteristics	All (N = 60)		FASN				*p*-Value[a]	HER2/neu				*p*-Value[a]	E2F1				*p*-Value[a]
			Low (N = 26)		High (N = 34)			Negative (N = 49)		Positive (N = 11)			Low (N = 36)		High (N = 24)		
	No.	(%)	No.	(%)	No.	(%)		No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Age																	
< 50 years	24	(40%)	11	(45.8%)	13	(54.2%)	0.750	19	(79.2%)	5	(20.8%)	0.741	17	(70.8%)	7	(29.2%)	0.162
≥ 50 years	36	(60%)	15	(41.7%)	21	(58.3%)		30	(83.3%)	6	(16.7%)		19	(52.8%)	17	(47.2%)	
Sex																	
Male	43	(71.7%)	21	(48.8%)	22	(51.2%)	0.171	38	(88.4%)	5	(11.6%)	0.059	28	(65.1%)	15	(34.9%)	0.198
Female	17	(28.3%)	5	(29.4%)	12	(70.6%)		11	(64.7%)	6	(35.3%)		8	(47.1%)	9	(52.9%)	
Tumor size																	
≤ 3 cm	26	(43.3%)	17	(65.4%)	9	(34.6%)	0.003	26	(100%)	0	(0%)	0.001	23	(88.5%)	3	(11.5%)	< 0.001
> 3 cm	34	(56.7%)	9	(26.5%)	25	(73.5%)		23	(67.6%)	11	(32.4%)		13	(38.2%)	21	(61.8%)	
T stage																	
Ta	20	(33.3%)	19	(95%)	1	(5%)	< 0.001	20	(100%)	0	(0%)	0.011	20	(100%)	0	(0%)	< 0.001
T1	40	(66.7%)	7	(17.5%)	33	(82.5%)		29	(72.5%)	11	(27.5%)		16	(40%)	24	(60%)	
Grade																	
Low grade	24	(40%)	20	(83.3%)	4	(16.7%)	< 0.001	24	(100%)	0	(0%)	0.002	21	(87.5%)	3	(12.5%)	< 0.001
High grade	36	(60%)	6	(16.7%)	30	(83.3%)		25	(69.4%)	11	(30.6%)		15	(41.7%)	21	(58.3%)	
CIS																	
Absent	46	(76.7%)	21	(45.7%)	25	(54.3%)	0.511	37	(80.4%)	9	(19.6%)	1.000	26	(56.5%)	20	(43.5%)	0.319
Present	14	(23.3%)	5	(35.7%)	9	(64.3%)		12	(85.7%)	2	(14.3%)		10	(71.4%)	4	(28.6%)	
Multiplicity																	
Absent	45	(75%)	19	(42.2%)	26	(57.8%)	0.764	34	(75.6%)	11	(24.4%)	0.051	26	(57.8%)	19	(42.2%)	0.543
Present	15	(25%)	7	(46.7%)	8	(53.3%)		15	(100%)	0	(0%)		10	(66.7%)	5	(33.3%)	
FASN																	
Low	26	(43.3%)						26	(100%)	0	(0%)	0.001	25	(96.2%)	1	(3.8%)	< 0.001
High	34	(56.7%)						23	(67.6%)	11	(32.4%)		11	(32.4%)	23	(67.6%)	
HER2/neu																	
Negative	49	(81.7%)	26	(53.1%)	23	(46.9%)	0.001						35	(71.4%)	14	(28.6%)	< 0.001
Positive	11	(18.3%)	0	(0%)	22	(100%)							1	(9.1%)	10	(90.9%)	
E2F1																	
Low	36	(60%)	25	(69.4%)	11	(30.6%)	< 0.001	35	(97.2%)	1	(2.8%)	< 0.001					
High	24	(40%)	1	(4.2%)	23	(95.8%)		14	(58.3%)	10	(41.7%)						

Categorical variables were expressed as number (percentage); $p < 0.05$ is significant.

[a] Chi-square test.

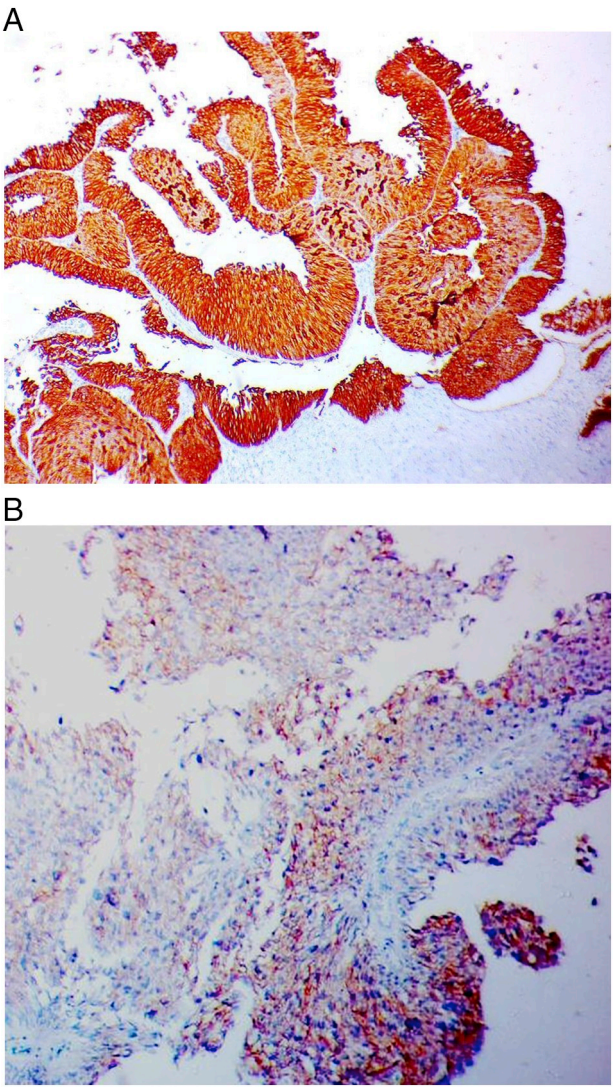

Fig. 2. (a) A case of high-grade non- muscle invasive bladder cancer (NMIBC) showing high cytoplasmic FASN expression (IHC ×100 magnification), (b) a case of low-grade NMIBC showing low cytoplasmic FASN (IHC ×200 magnification).

grade ($p < 0.001$), and tumor stage ($p < 0.001$). However, there was a non-significant association of FASN expression with the presence of CIS or the multiplicity of NMIBC.

The membranous Her2/neu positive expression was detected in 11 cases of the studied NMIBC cases (18.3%) (Fig. 3). There was a significant up-regulation of Her2/neu expression with tumor size ($p = 0.001$), histological grade ($p = 0.003$), and tumor stage ($p = 0.009$). On the other hand, there was a non-significant association of Her2/neu expression with the presence of CIS or the multiplicity of NMIBC.

High nuclear E2F1 expression was noted in 24 cases of NMIBC (40%) (Fig. 4). There was a significant up-regulation of E2F1 expression with tumor size, histological grade, and tumor stage ($p < 0.001$ for each). In contrast, there was a non-significant association of E2F1 expression with the presence of CIS or the multiplicity of NMIBC. A significant association of FASN immunoexpression with both Her2/neu and E2F1 expression was detected ($p = 0.001$ and $p < 0.001$ respectively).

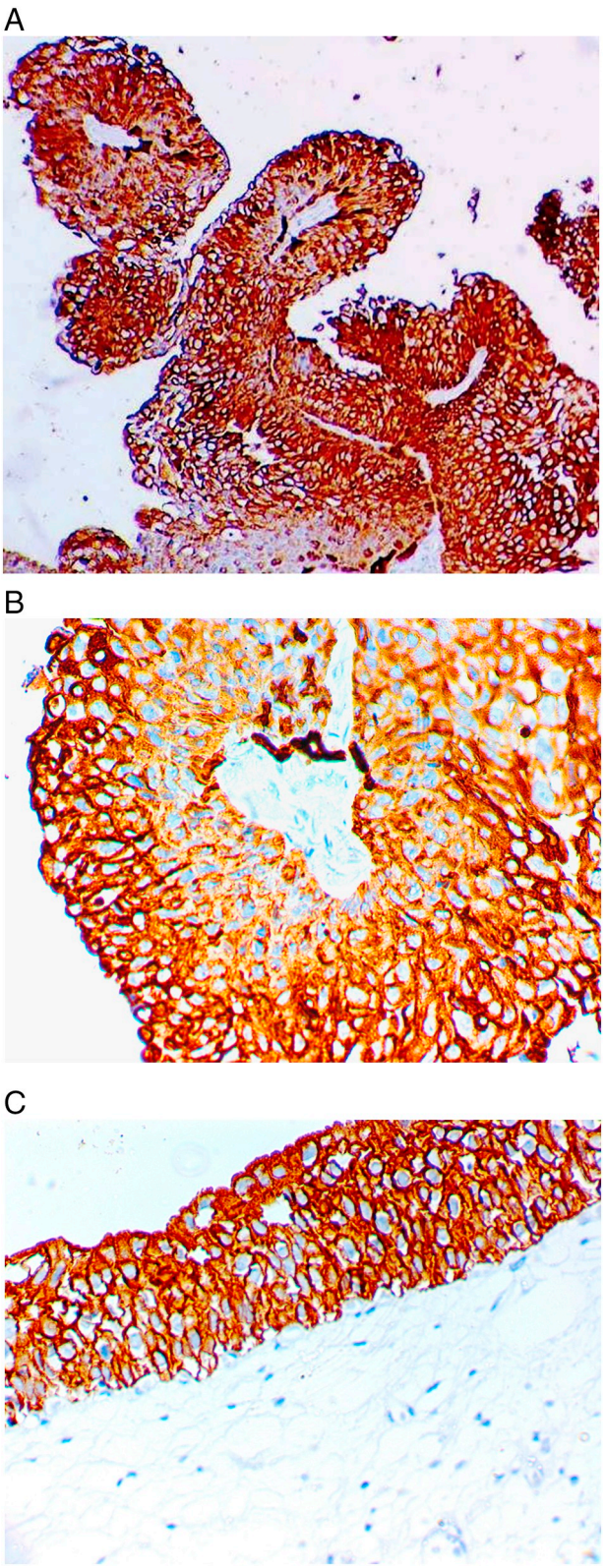

Fig. 3. (a) A case of high-grade non-muscle invasive bladder cancer (NMIBC) showing strong and diffuse membranous Her2/neu expression (+3) (IHC ×100 magnification), (b) a case of high-grade NMIBC showing strong and diffuse membranous Her2/neu expression (+3) (IHC ×400 magnification), (c) a case of carcinoma in-situ (CIS) showing strong and diffuse membranous Her2/neu expression (+3) (IHC ×400 magnification).

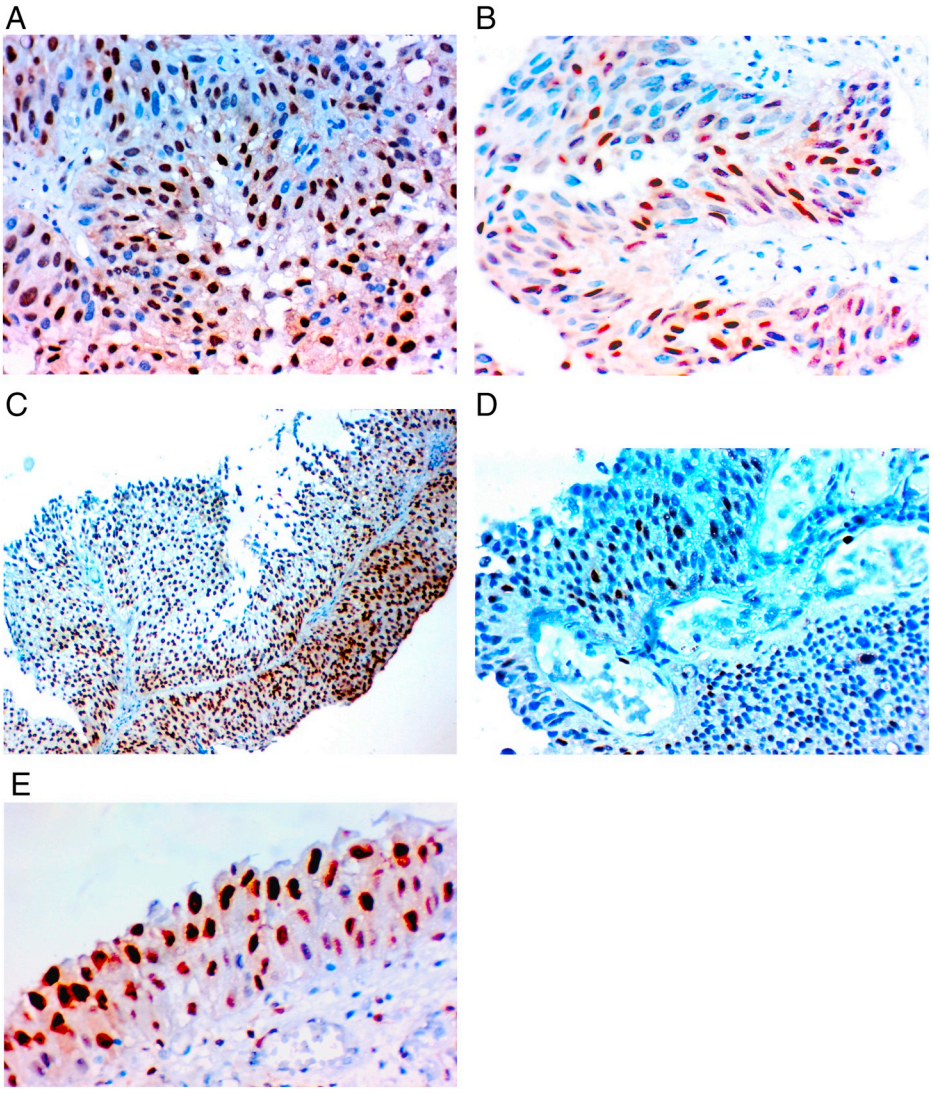

Fig. 4. (a) A case of high-grade non-muscle invasive bladder cancer (NMIBC) showing high nuclear E2F1 expression (IHC ×400 magnification), (b) a case of high-grade NMIBC showing low nuclear E2F1 expression (IHC ×400 magnification), (c) a case of low-grade NMIBC showing high nuclear E2F1 expression (IHC ×100 magnification), (d) a case of low-grade NMIBC showing low nuclear E2F1 expression (IHC ×400 magnification), (e) A case of carcinoma in-situ showing high nuclear E2F1 expression (IHC ×400 magnification).

3.3. Prognostic relevance of FASN, Her2/neu, and E2F1 expression in the studied NMIBC cases (Table 3)

During the follow-up period, 24 patients (40%) suffered a recurrent NMIBC. There was a significant association between the high expression of FASN and E2F1 and occurrence of recurrent tumor ($p = 0.0006$, $p = 0.004$ respectively). In contrast, Her2/neu expression exhibited a non-significant relation ($p = 0.741$) with the tumor recurrence. Kaplan-Meier plot curves of RFS were stratified according to FASN, Her2/neu, and E2F1 expression and are illustrated in Fig. 5. Kaplan-Meier curve with log-rank test showed that high FASN and high E2F1 were associated with shorter RFS.

On the other hand, during the follow-up 15 cases (25%) progress to MIBC. About 44.1% of high FASN had progressed while none of low FASN progressed ($p < 0.001$). Furthermore, there was a significant association of Her2/neu protein expression and E2F1 expression with the tumor progression ($p = 0.021$, $p = 0.15$ respectively). Kaplan-Meier plot curves of PFS were stratified according to FASN, Her2/neu, and E2F1 expression and are illustrated in Fig. 6. Kaplan-Meier curve with log-rank test showed that high FASN, positive Her2/neu and high E2F1 were associated with shorter PFS. Multivariate logistic regression showed that the tumor stage, FASN, and Her2/neu were independent predictive parameters to NMIBC progression ($p = 0.003$, 0.01, 0.03 respectively) (Table 4).

Table 3
Relation between immunohistochemical staining for FASN, HER2/neu and E2F1 and outcome in NMIBC (N = 60).

Characteristics	All (N = 60)	FASN		*p*-Value	HER2/neu		*p*-Value	E2F1		*p*-Value
		Low (N = 26)	High (N = 34)		Negative (N = 49)	Positive (N = 11)		Low (N = 36)	High (N = 24)	
	No. (%)	No. (%)	No. (%)		No. (%)	No. (%)		No. (%)	No. (%)	
Recurrence										
Absent	36 (60%)	22 (84.6%)	14 (41.1%)	0.0006[a]	30 (61.2%)	6 (54.5%)	0.741[a]	27 (75%)	9 (37.5%)	0.004[a]
Present	24 (40%)	4 (15.4%)	20 (58.9%)		19 (38.8%)	5 (45.5%)		9 (25%)	15 (62.5%)	
RFS										
Mean (months)	43.61 months	46.30 months	40.73 months	< 0.001[b]	44.44 months	40 months	0.119[b]	46.25 months	39.04 months	< 0.001[b]
(95%CI)	(41.59–45.63)	(44.01–48.60)	(37.69–43.77)		(42.42–46.47)	(34.27–45.72)		(44.53–47.96)	(35.02–43.05)	
1 year RFS	100%	100%	100%		100%	100%		100%	100%	
2 year RFS	91.3%	96.2%	87.3%		93.8%	80%		97.2%	81.3%	
3 year RFS	87.5%	96.2%	79.3%		93.8%	60%		97.2%	71.1%	
4 year RFS	50.6%	73.1%	22.4%		53.4%	40%		69.4%	17.8%	
Progression										
Absent	45 (75%)	26 (100%)	19 (55.9%)	< 0.001[a]	40 (81.6%)	5 (45.5%)	0.021[a]	31 (86.1%)	14 (58.3%)	0.015[a]
Present	15 (25%)	0 (0%)	15 (44.1%)		9 (18.4%)	6 (54.5%)		5 (13.9%)	10 (41.7%)	
PFS										
Mean (months)	43.98 months	46.31 months	35.21 months	< 0.001[b]	44.56 months	40.64 months	0.004[b]	46.02 months	40.92 months	0.002[b]
(95%CI)	(41.60–46.35)	(44.03–48.59)	(31.69–38.72)		(42.01–47.11)	(33.59–47.69)		(43.80–48.23)	(36.01–45.83)	
1 year PFS	98.3%	100%	97.1%		100%	90.9%		100%	95.8%	
2 year PFS	88%	100%	78.9%		87.3%	90.9%		94.3%	78.6%	
3 year PFS	82.4%	100%	68.2%		85.1%	68.2%		91.2%	68.8%	
4 year PFS	68.8%	100%	21%		78.5%	0%		84.2%	34.4%	

Continuous variables were expressed as mean (95%CI); categorical variables were expressed as number (percentage); $p < 0.05$ is significant.

[a] Chi-square test.

[b] Log rank test.

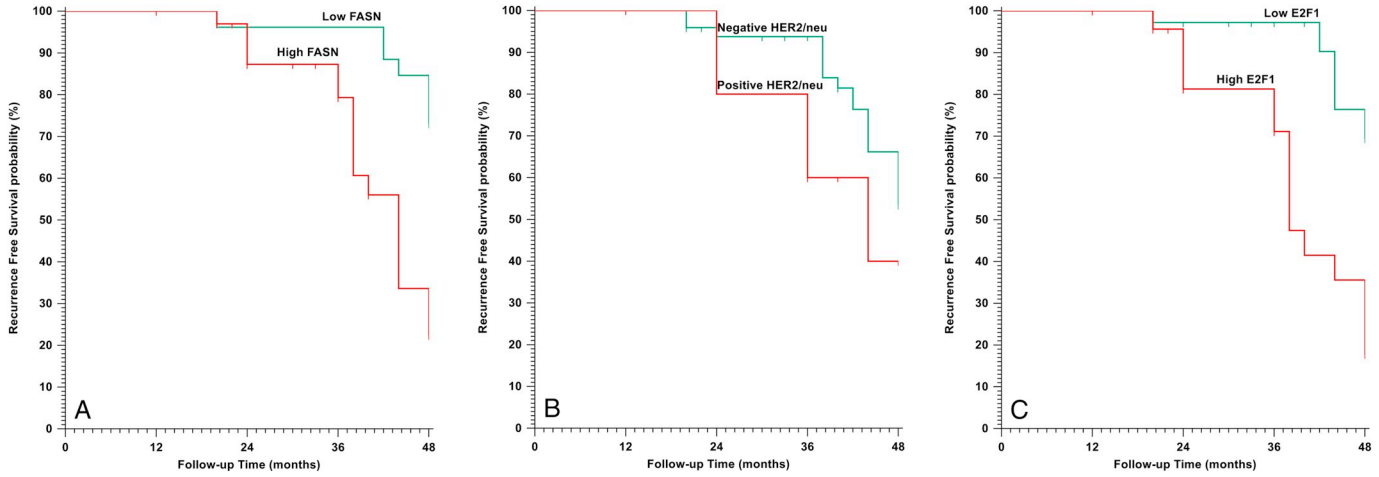

Fig. 5. Kaplan Meier curves of recurrence-free survival (RFS) stratified according to (a) FASN expression, (b) Her2/neu expression, and (c) E2F1 expression.

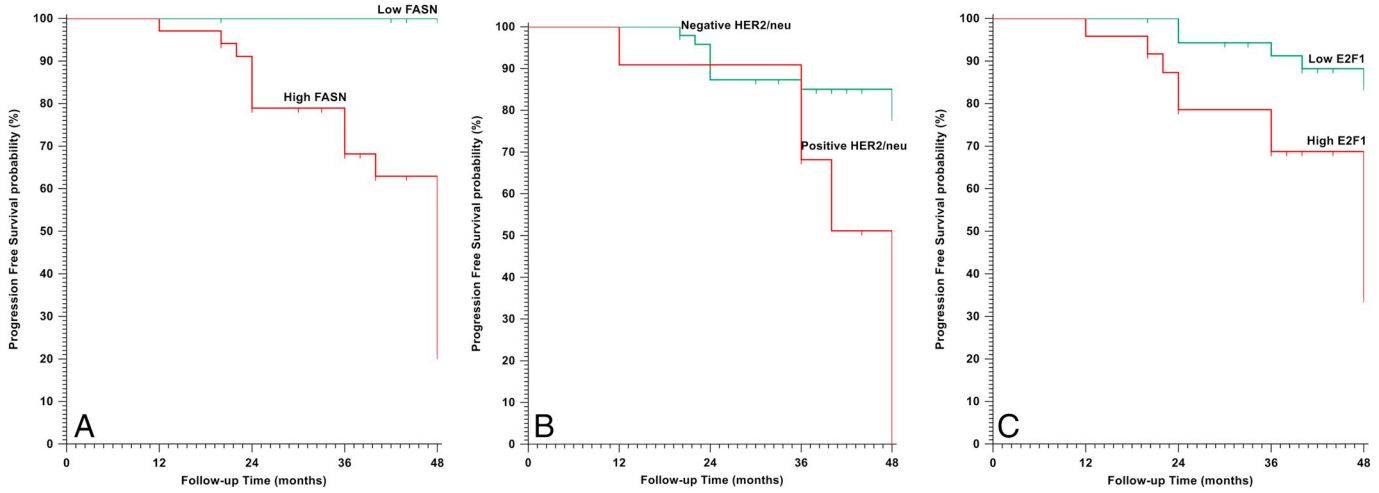

Fig. 6. Kaplan Meier curves of progression-free survival (PFS) stratified according to (a) FASN expression, (b) Her2/neu expression, and (c) E2F1 expression.

Table 4
Multivariate analysis to predictive value of all parameters to NMIBC progression:

Variable	B	SE	*p*-Value	Odds ratio (CI 95%)
size	0.4	0.13	0.4	0.4 (0.15–0.7)
stage	0.3	0.18	0.003*	0.3 (−0.06–0.6)
grade	−0.09	0.19	0.09	−0.1 (−0.4–0.2)
CIS	0.04	0.1	0.6	0.04 (−0.16–0.25)
multiplicity	0.2	0.09	0.6	0.23 (0.04–0.4)
FASN	0.2	0.12	0.01*	0.29 (0.008–0.5)
Her2/neu	0.01	0.12	0.04*	0.01 (−0.2–0.2)
E2F1	0.08	0.11	0.9	0.09 (−0.14–0.3)

Null model-2 log likelihood = 23.5; full model-2 log likelihood = 11.3; overall model fit: chi square = 49.6, d.f. = 2, $p < 0.05$ is significant.
β: regression coefficient; SE: standard error; CI: confidence interval.

4. Discussion

Non-muscle-invasive bladder cancer accounts for about 70% of BC and is a heterogeneous disease with variable clinical course and outcomes. Among these tumors, > 50% will recur within one year and nearly 30% will progress to MIBC despite adequate TURBT and adjuvant BCG therapy. These patients have a poorer prognosis after radical cystectomy (RC) than patients treated with RC for primary MIBC [25]. Therefore, there is a real need to find novel prognostic biomarkers of tumor recurrence and progression paving the way to individualized treatment.

Tumor metabolism is a chief process in the initiation and progression of carcinogenesis. Several studies have verified that glucose metabolism is necessary for cancer growth and invasion. However, a few studies have focused on the role of lipid metabolism in carcinogenesis and progression. The previous studies have reported that FASN overexpression enhances tumor proliferation, invasion, and metastasis in different cancers [10,26]. The mechanisms by which FASN is upregulated in cancer cells have been explained in many epithelial malignancies [14]. The Ras/Raf/MAPK & PI3K/Akt pathways and Her2/neu have been described to play a role in upregulating FASN expression in breast cancer [27] and thyroid papillary carcinoma [28]. Furthermore, the “Her2/neu–PI3K/Akt–FASN axis” is involved in the regulation of the malignant phenotype in colorectal cancer cells [29]. It is verified that a cross-talk between ErbB and FASN mediates ovarian cancer cell proliferation. Various evidences indicate that FASN gene network and Her2/neu oncogene system have a synergetic effect on tumorigenesis [13].

In the present study, high FASN expression was noted in 56.7% of the studied NMIBC cases. FASN expression was significantly associated with tumor size, histologic grade and tumor stage ($p < 0.05$) which was similar to previous reports [30,31]; confirming the past observation of the crucial role of FASN expression in tumor progression [32], supposed that FASN overexpression can provide the material and

energy for the rapid proliferation of tumor cells as previously concluded in ovarian cancer [33]. These results led to the hypothesis that the fatty acid synthetic pathway may contribute to tumorigenesis and FASN may be a useful anti-cancer target [34]. In support of this finding, a FASN inhibitor and an FDA-approved anti-obesity medicine, Orlistat, was reported to have antitumor activity [36]. Particularly, Orlistat has exhibited a potent antiproliferative and proapoptotic effects in prostate, colon, stomach, breast, and ovarian cancer cells, with no significant effects on normal cells [36].

Though tumor progression in NMIBC ranges widely from 1% to 45%; Kim et al. observed tumor progression in 16.3% of their studied cases of NMIBC [15]. On the other hand, in the current study during the follow-up period, 25% of NMIBC progressed to MIBC. High FASN expression in our studied cases has been associated with tumor recurrence and progression to MIBC and confirmed by multivariate analysis. Furthermore, it was significantly associated with poor RFS and PFS as previously reported [30]. Formerly, it has been suggested that FASN overexpression correlates with a shorter survival in patients with ovarian cancer [34].

The prognostic significance of Her2/neu expression in BC is unclear due to the conflicting results. The previous reports observed a wide range of Her2/neu expression in BC (9.2%–85%) [37]. The first explanation for this variation is the different degree of tumor stages and histologic grades of BC used in these investigations. The second explanation is the technical limitations of IHC and the absence of obvious guidelines for estimating Her2/neu status in BC. In addition, various methods of estimation (gene amplification and protein overexpression), or using different techniques (FISH, and immunohistochemistry), or variant clones of primary antibodies used could be another explanation [38].

There are few reports that investigated Her2/neu status in NMIBC, and they have revealed controversial results. Olsson et al. reported that Her2/neu was overexpressed in 12.4% of pT1 BC, and there was a non-significant association with the prognosis, including tumor progression and recurrence [39]; where they used whole sections of pT1 tumors and analyzed Her2/neu-IHC according to ASCO/CAP guidelines presented for breast carcinoma. On the other hand, a parallel study had been conducted by Chen et al. that assessed Her2/neu status of NMIBC on TMA using IHC and FISH. They demonstrated that Her2/neu gene amplification was 9% and correlated with the aggressive outcome in high grade NMIBC, and suggested that Her2/neu status would be helpful for distinction of patients with NMIBC who need cautious observation [40].

Her2/neu in our cases was positively expressed in 18.3% and showed a significant relationship with tumor size, grade and stage as previously reported [15,41]. In contrast to Alexai et al. who showed a significant relationship between Her2/neu and tumor grade, not the stage [42]. Furthermore, Her2/neu expression in our studied cases was positively correlated with the progression to MIBC and shorter PFS which was confirmed by multivariate analysis. In contrast, it wasn't correlated to either the recurrence or RFS. Our results confirmed the previous reports which found a highly significant association between Her2/neu expression and progression of NMIBC; where Kim et al. observed that that all Her2/neu-IHC positive patients showed tumor progression [15].Therefore, we believed Her2/neu-IHC positive patients should receive more aggressive surveillance and monitoring with respect to personalized medicine.

Marked variation in the prognostic role of immunoexpression of Her2/neu in BC was found in the results of previous related researches. Thus, although numerous researches [43,44], have detected a negative prognostic significance and an aggressive role of Her2/neu expression in BC, others [42,45], have not found any poor prognostic association but these studies were on MIBC.

It has been hypothesized that there is a potential correlation between FASN and Her2/neu, with numerous studies confirmed this fact. For example: in many tumors including breast [46], ovary [13] or oral cancer [47], with Her2/neu and FASN upstream and downstream molecules of the PI3K and MAPK pathways were interrelated. In normal human tissues, this type of correlation has not been found between FASN and Her2/neu. Oliveras et al. research results showed that the expression of FASN could be elevated along with Her2/neu overexpression in BC, and vice versa. This interaction seemed to serve as a positive feedback pathway that can mutually regulate the expression of both FASN and Her2/neu [48]. FASN inhibition inhibits the correct localization and function of tyrosine kinases such as Her2/neu and EGFR by enhancing changes in the synthesis of membrane phospholipids and assembling of lipid rafts and so inhibits their downstream signals [35].

In the current study, a potent relationship between FASN and Her2/neu expression was detected and a potential value for predicting patient outcome was demonstrated in the studied NMIBC patients. These findings indicate an essential role for these two combined molecules in the tumor invasiveness, and the potential settlement for upcoming targeted therapy. Therefore, once the activity or expression of FASN was inhibited, there would not be enough fatty acids and phospholipids available to maintain the activity of membrane microdomain as platforms for cell signaling. This decreased level of membrane phospholipids might lead to the decreased levels of p-AKT after FASN inhibition [48].

The mechanisms implicated in the mutual regulation of Her2/neu and FASN have been discovered. Her2/neu stimulates the FASN gene promoter via the PI3K and MAPK signaling pathway, and lastly increases FASN expression. Furthermore, Her2/neu can directly stimulate FASN protein by its intracellular phosphorylation domain. Alternatively, Her2/neu gene and Her2/neu protein activity can be adjusted via the concentration changes of acetyl-CoA and malonyl-CoA that are synchronized by FASN. Moreover, as the chief enzyme of de-novo lipid synthesis, FASN can enhance the stability of Her2/neu by the creation of a domain known as a lipid raft, situated on the membranes. These mechanisms eventually assemble a positive feedback pathway between Her2/neu and FASN [48].

Obviously with the increased metabolism of the tumor microenvironment, tumor cells reveal a shift from exogenous uptake of fatty acids (FAs) to endogenous assembly of these molecules using FASN. Consequently, the rapidly-proliferating tumor cells become able to meet the elevated requirement for biosynthetic molecules desired for increasing the cytoplasmic membrane and for consequent division. Intracellular production of long-chain FAs also gives a source of energy through β-oxidation of lipids into metabolism precursor molecules, such as acetyl-CoA, NADH, and FADH2. Therefore, FASN plays a fundamental role in maintaining metabolism, division, and proliferation pathways of tumor cells [49].

E2F1 is a member of a family of eight transcription factors which control the cell cycle. The activation of E2F1 via its release from phosphorylated pRB complexes leads to transcription of > 1200 genes [50]. E2F1 regulates genes implicated in cell cycle regulation, DNA replication, nucleotide biosynthesis, and apoptotic targets as caspases and Apaf-1 [51]. E2F1 also induces genomic instability and enhances tumor progression, via stimulation of the mitotic regulator Mad2 [52].

In the current study, the high E2F1 expression was revealed in 40% of the cases with a significant association with tumor size, grade and stage confirming the significant role of E2F1 in tumor cell proliferation and enhancement of tumor progression as previously noted [15]. NMIBC cases with high E2F1 expression was significantly related to the tumor recurrence and progression to MIBC with poor RFS and PFS as previously reported by Lee et al. where they concluded that the progression of superficial tumors to invasive tumors was significantly higher in the E2F1-high group than in the E2F1-low group ($p = 0.012$ by log-rank test) [18]. On the other hand, it has been reported that the E2F1 + immunophenotype is a marker of poor prognosis (the worst DFS and OS) of patients with colon cancer treated with 5FU-based adjuvant therapy [24]. Consequently, we supposed that E2F1 expression is strong

predictor of the progression of superficial tumors to invasive tumors and can be used as a target for novel therapeutic drugs.

Tumors usually have high rates of lipid synthesis, which are used both for membrane assembly and as signaling molecules [53]. Lipogenesis is not only essential during proliferation; but it also contributes to the metastatic potential of tumor cells. Besides promoting lipogenesis in the liver [54], E2F1 enhances fatty acid synthase expression in response to Sonic hedgehog signaling as reported in medulloblastoma [55]. Using both the genetic and pharmacological approaches in Bhatia et al. study, they detected a necessity for E2F1 in FASN expression and for sustained suppression of fatty acid oxidation (FAO) in both normally proliferating cerebellar granule neuron precursors (CGNPs) and tumor cells. This relation between E2F1 and the lipogenic/lipolytic balance emphasizes a novel aspect of E2F1 task in cell cycle regulation and tumor cell growth [55]. This finding was confirmed in the current study where a significant relation between E2F1 and FASN expression was found.

Consequently, we believe that our results could be relevant when counseling patients with NMIBC about treatment options. Patients with low FASN, Her2/neu, and E2F1 expression could safely be treated with BCG. Conversely, patients with altered expression should be informed of their risk of recurrence and progression despite BCG treatment. These patients should be made aware that early cystectomy provides better survival than cystectomy delayed at the time of progression.

The major weakness of the present study is its small number of cases with data from a single-center. To confirm and verify the results a validation with larger number in a multi-center study will be necessary.

5. Conclusion

High FASN, Her2/neu and E2F1 are considered as adverse prognostic factors for tumor recurrence and progression in NMIBC and these patients should be monitored thoroughly for disease recurrence and progression. Therefore, we suggest that FASN, Her2/neu and E2F1 should be evaluated and considered during the selection of the appropriate management strategy for NMIBC patients. Indeed, our studied prognostic markers provide an advanced individualized risk assessment of progression facilitating the decision-making concerning early RC.

Conflict of interest

The authors declare that they have no conflict of interest.

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