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Prognostic role of CHEK2, FASN and Her2/neu Expression in Stage Ta-T1 Non-Muscle-Invasive Bladder Cancer (NMIBC)

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ABSTRACT

Background: Though linked with a favorable overall survival rate, the heterogeneity of NMIBC (non-muscle invasive bladder cancer) has an effect on patients' rates of recurrence. The decision making is depended on the established pathological and clinical parameters for each individual. Therefore, to improve clinical outcome, life quality and survival rates of oncological patients, more parameters are needed to support the decision.

Objectives: Analysis the prognostic potential of CHEK2, FASN and Her2/neu immunohistochemical expression in fifty cases of non-muscle invasive bladder cancer who underwent TURBT as well as adjuvant intravesical BCG with a focus on prognostication of recurrence as well as progression among NMIBC.

Materials and Methods: Our study included fifty cases who had primary papillary superficial (noninvasive) TCC " transitional cell carcinoma"(Stage Ta-T1; with or without CIS of the bladder. Only the tumors containing proper detrusor muscle in biopsy were involved in this study. Between January 2016 and December 2019, the Urology department performed surgical resection.

Results: Decrease or loss of CHE2 expression was reported among (56%) of the patients, which was significantly linked with tumor size, smoking, grade, multifocality and concomitant Cis. High FASN expression was demonstrated among (68%) NMIBC cases, expression was significantly linked with tumor size, smoking, grade, multifocality as well concomitant Cis. Positive Her2/neu was found in of (42%) the cases, and the expression was significantly linked with tumor size, smoking, grade, multifocality and concomitant carcinoma in situ. The overall disease-free survival was significantly correlated with low expression of CHEK2 and positive Her2neu.

Conclusion: Loss of CHEK2, high FASN and positive Her2/neu in the biopsies of noninvasive "superficial" urothelial carcinomas is an independent predictor of tumor progression.

Keywords: CHEK2, FASN and Her2/neu expression, Stage Ta-T1 Non-Muscle-Invasive, Bladder Cancer.

INTRODUCTION

The incidence of bladder cancer ranks 12th among all cancers [1]. In Egypt, the peak incidence rate of carcinoma of bladder is recorded being one of the most frequent malignancies in Egyptian male population (37.1 per 100,000 males) [2]. Nearly three-quarters of all cases of urothelial carcinoma begin as non-invasive bladder cancer (NMIBC). Its recurrence rates up to 60% during the first year of diagnosis. It has accepted that; T1 bladder cancer patients are at risk for under-treatment when BCG is used despite recurrence, and for over-treatment when early radical cystectomy is performed [3].

The main process in the beginning and progression of carcinogenesis is the tumor metabolism. Aggressive cancer cells rely on glucose and glutamine to fuel their proliferation. One of the fundamental components of the DNA damage response (DDR) is cell cycle checkpoint kinase 2 (ChEk2), which controls glucose flux and thus cellular energy generation [4].

Low kinase function has been linked with poor outcome in many tumors as breast cancer, prostate cancer and gastric carcinomas [5]. Fatty acid synthase (FASN) is a chief enzyme in the synthesis of fatty acids and also its activation is a universal hallmark of majority of human carcinomas. FASN increases the activation of Her2neuas well its downstream related to PI3K/AKT/mTOR and MAPK signaling pathways [6].

Her2 neu functions as an epidermal growth factor receptor (EGFR) to stimulate the development, migration, survival, and invasion of cancer cells in many different malignancies [7]. Reports of Her2-neu expressions in NMIBC are under investigate [8]. This fact has forced us for assessment its prognostic value in NMIBC.

It is a need to study the parameters of tumor that is involved in cancer cell metabolism. This leads to improve the life quality and clinical outcome of NMIBC patients.

MATERIALS AND METHODS

Our study included fifty cases who had primary papillary superficial (noninvasive) TCC " transitional cell carcinoma"(Stage Ta-T1; with or without CIS of the bladder. Only the tumors containing proper detrusor muscle in biopsy were involved in this study. Between January 2016 and December 2019, the Urology department performed surgical resection. All patients undergoing a TURBT for NMIBC must also undergo the normal BCG regimen. Tissue samples were obtained from Zagazig University's Pathology department and preserved in formalin/paraffin. paraffin sections of five-micron thickness, review of H&E-stained. Slides was followed by staging according to the 8th edition of the American Joint Committee on Cancer

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Staging System and grading according to the 2016 WHO tumor classification [9].

Age, sex, diagnosis date, multifocality, treatment method, and cystoscopic follow-up were some of the patient characteristics described in clinical oncology and urology. Patients were not included if they had a recurring tumor, a single Tis, inconclusive follow-up data, or missing clinical information. Our research was sanctioned by the ethics board of Zagazig University Hospitals. Patients were monitored with upper tract and abdominopelvic imaging. Upper tract imaging is recommended at 12 months and every 1-2 years for the next 10 years for people at high risk. During the follow-up period, recurrence was defined as a new tumor incidence confirmed by biopsy after 3 months of the initial TURBT, and progression was defined as a recurrence confirmed by biopsy showing muscle invasion or distant metastasis.

Immunohistochemistry method

The streptavidin-biotin immunoperoxidase technique was used for immunohistochemical staining. Formalin-fixed paraffin embedded blocks were cut for sections of four-to-five-micron thickness. Then deparaffinization in xylene was done and as well they were rehydrated in graded alcohol. Microwave treatment in 10 mmol/l citrate buffer, pH 6 was used for antigen retrieval. Washing of these sections were done 3times with cold 0.01 mol/l PBS. 3% Hydrogen peroxide for 20 min was used to block endogenous peroxidase. Then the used primary antibodies; mouse monoclonal Chk2 antibody 1C12 nd mouse monoclonal Her2/neu antibody as well as the detection kit were purchased from the Thermo Scientific Lab Vision, USA).

Immunohistochemistry assay

CHEK2 scoring

Tumor cells were tested for nuclear CHEK2 expression. Nuclear staining intensity was evaluated as follows: negative (CHEK2 staining in less than 10% of cells) or strong (CHEK2 staining in 10% or more of cells) [10].

FASN scoring

The semiquantitative method was used for evaluation and scoring the cytoplasmic FASN expression in tumor cells. The intensity was scored as: zero “negative", one “weak", two “moderate" and three “strong". The scoring of extent of stain was as: zero “no staining", one “less than 10%", two"10 to50%" and three “more than 50%". The summation of both intensity scores and the extent was the final score (zero–six). Scores less than or equal to three were identified as low expression. Scores more than or equal to four were considered as a high expression [11].

Her2/neu scoring

Membranous staining of Her2/neu was regarded as a positive expression. The staining intensity was assessed using following semi-quantitative method; None (no staining), 1 (weakly stained; less than ten percent of cells stained), 2 (moderately stained; more than ten percent of cells stained), 3 (intensely stained; more than ninety percent of cells stained) (strong staining in more or equal to 10 percent of cells).So the tissue sections were considered to be positive forHer-2/neu for score 2 or 3 staining [12].

Ethical approval:

This experiment was ethically approved by the Faculty of Medicine, Zagazig University's. After being fully informed, all participants provided written consent. The study was conducted out in line with the Helsinki Declaration.

Statistical analysis

Categorical variables were also expressed numerically, while continuous variables were given measures such as mean, standard deviation, and median. If you want to know if your continuous variables have a normal distribution, you can apply the Shapiro-Wilk test. If the variables were normally distributed, then the Kruskal-Wallis H test was employed to compare three or more groups. The Chi-square test was used to compare the frequencies of categorical variables. P value < 0.05 was considered significant.

RESULTS

Clinical and pathological characters among studied NMIBC patients:

Table 1 summarizes the clinical and pathological criteria for fifty NMIBC patients. The average time of observation was 45 months. Twenty-two patients (44 percent) experienced tumors recurrence, and 14 individuals experienced tumors progression (28 percent).

Table (1) Demonstrate clinic pathological data of the patients:

Parameters	N=50	%
Sex:		
Male	34	68%
Female	16	32%
Age group:		
≤50 years	15	30%
>50 years	35	70%
Smoking:		
Absent	18	36%
Present	32	64%
Size:		
<2 cm	19	38%
>2 cm	31	62%
T stage:		
Ta	20	40%
T1	30	60%
Grade:		
Low grade	24	48%
High grade	26	52%
CIS:		
Absent	34	68%
Present	16	32%
Multiplicity:		
Absent	35	70%
Present	15	30%
Chek2:		
Low	28	56%
High	22	44%
FASN:		
Negative	16	32%
Positive	34	68%
Her2-neu expression:	29	58%
Negative	21	42%
Positive		
Progression to muscle invasive:	36	72%
Negative	14	28%
Positive		
Recurrence:		
Negative	28	56%
Positive	22	44%

CHEK2, FASN and Her2/neuexpression in the examined NMIBC cases (Table 2, 3):

Immunoexpression of CHEK2 was low in 56 % of NMIBC patients (Fig 1.), with a significant correlation with tumor size, smoking, grade, multifocality and concomitant Cis.

Expression of FASN was high in 34 cases of examined NMIBC cases (68%) (Fig 2). FASN expression was significantly up-regulated in relation to tumor grade, tumor size, smoking, multi-site disease, and co-occurrence of Cisplatin.

Her2/neu positivity was found in 21/50 (42%) of NMIBC cases (Fig 3.). Her2/neu expression is significantly correlated with tumor grade, smoking status, tumor multilocularity, and the presence of concurrent Cisplatin.

The expression of CHEK2 correlated negatively with that of FASN and Her2/neu in the studied NMIBC cases.

Prognostic value of CHEK2, FASN and Her2/neu expression in among NMIBC cases (Table 2, 4)

NMIBC recurred in 22 patients over the observation period. Tumor recurrence was confirmed to be associated with low CHEK2 expression, high FASN levels, positive Her2 status, and statistical significance ($p<0.001$).

However, despite therapy with BCG, 14 instances (28%) develop to MIBC. In addition, low CHEK2 expression, high FASN, and positive Her2 were all strongly associated with tumor growth.

Kaplan-Meier plots were used to graphically represent the distribution of disease-free survival rates by subgroup (CHEK2, FASN, and Her2/neu expression). (Figure 4). Kaplan-Meier curve showed that low CHEK2 and positive Her2 were associated with shorter DFS .Mean survival in high CHEK2 was 47.29 month versus 46.75 month in low CHEK2.Furthermore, mean survival in negative Her2/neu was 47.57 month versus 46.5 month in Her2/Neu positive .On the other hand, non- significant association was detected between high FASN expression and DFS where the mean survival in high FASN was 48 month versus 46.79 month in low FASN.

Table (2): Show the relation between CHEK2, FASN and Her2/neu expression levels in the studied patients and disease-specific characteristics:

	Total	CHEK2			FASN expression			Her2/neu expression:		
		High	Low	p#	Low	High	p	Negative	Positive	p#
		N=28 (%)	N=22 (%)		N=16 (%)	N=34(%)		N=29 (%)	N=21 (%)	
Age group:										
≤50 years	15	12(80)	3 (20)	0.025*	5 (33.3)	10 (66.7)	0.89	6 (40)	9 (60)	0.091
>50 years	35	16(45.7)	19 (54.3)		11 (31.3)	24 (68.7)	5	23 (65.7)	12 (34.3)	
Sex:										
Male	34	15 (44.1)	19 (55.9)	0.014*	8 (23.5)	26 (76.5)	0.06	21 (61.8)	13 (38.2)	0.432
Female	16	13 (81.3)	3 (18.9)		8 (50)	8 (50)		8 (43.8)	8 (56.2)	
Size:										
<2 cm	19	18 (94.7)	1 (5.3)	<0.001*	10 (52.6)	9 (47.4)	0.014*	16 (84.2)	3 (15.8)	0.003*
>2 cm	31	10 (32.3)	21 (67.7)		6 (19.4)	25 (80.6)		13 (41.9)	18 (58.1)	
Grade:										
Low grade	24	23 (95.8)	1 (4.2)	<0.001*	4 (16.7)	20 (83.3)	0.035*	20 (83.3)	4 (16.7)	<0.001*
High grade	26	5 (19.2)	21 (80.8)		12 (46.2)	14 (53.8)		9 (34.6)	17 (65.4)	
T stage:										
Ta	20	19 (95)	1 (5)		11 (55)	9 (45)	0.004*	6 (30)	14 (70)	0.001*
T1	30	9 (30)	21 (70)	<0.001*	5 (16.7)	25 (83.3)		23 (76.7)	7 (23.3)	
Smoking:										
No	18	5 (27.8)	13 (72.2)	0.003*	11 (61.1)	7 (38.9)	0.001*	14 (77.8)	4 (22.2)	0.034*
Yes	32	23 (71.9)	9 (28.1)		5 (15.6)	27 (84.4)		15 (46.9)	17 (53.1)	
CIS:										
Negative	34	13 (38.2)	21 (61.8)	<0.001*	14 (41.2)	20 (58.8)	0.043*	23 (67.7)	11 (32.3)	0.04*
Positive	16	15 (93.8)	1 (6.2)		2 (12.5)	14 (87.5)		6 (37.5)	10 (62.5)	
Multiplicity:										
Negative	35	23 (65.7)	12 (34.3)	0.035*	15 (42.9)	20 (57.1)	0.012*	26 (74.3)	9 (25.7)	0.001*
Positive	15	5 (33.3)	10 (66.7)		1 (6.8)	14 (93.2)		3 (20)	12 (80)	
Non-muscular invasive recurrence:										
Negative	15	3 (20)	12 (80)	0.001*	8 (53.3)	7 (46.7)	**0.034***	14 (93.3)	1 (6.7)	**0.001***
Positive	35	25 (71.4)	10 (28.6)		8 (22.9)	27 (77.1)		15 (42.9)	20 (57.1)	
Progression to muscular invasive:										
Absent	36	16 (44.4)	20 (55.6)	0.008*	8 (22.2)	28 (77.8)	**0.018***	27 (75)	9 (25)	0.001*
Present	14	12 (85.7)	2 (14.3)		8 (57.1)	6 (42.9)		2 (14.3)	12 (85.7)	
Relapse:										
Negative	28	9 (32.1)	19 (68.9)	<0.001*	15 (53.6)	13 (46.4)	**<0.001***	23 (82.1)	5 (17.9)	<0.001*
Positive	22	19 (86.4)	3 (13.6)		1 (4.5)	21 (95.5)		6 (27.3)	16 (72.7)	

#Chi square test

Table (3): Demonstrate the correlation between the three markers.

	CHEK2		Her2/neu		FASN	
	Phi	**p**	**Phi**	**p**	**Phi**	**p**
CHEK2			-0.591	<0.001*	-0.342	0.016*
Her2/neu	-0.591	<0.001*			0.323	0.022*
FASN	-0.342	0.016*	0.323	0.022*		

Table (4): Shows the relation between disease free survival time and markers expressions

		Total N	N of Events	Censored		Survival time, Months		P
				N	%	Mean		
						Estimate's	95% CI	
CHEK2	High	28	19	9	32.1%	47.29 ± 0.72	45..87 — 48.7	0.037*
	Low	22	3	19	86.4%	46.75 ± 1.01	44.77 – 48.74	
FASN	Low	16	1	15	93.8%	48.0±0	48.0 – 48.0	0.148
	High	34	21	13	38.2%	46.79 ± 0.73	45.36 – 48.22	
Her2/neu	Negative	29	6	23	79.3%	47.57 ± 0.47	46.65 – 48.48	0.009*
	Positive	21	16	5	23.8%	46.5 ±1.1	44.34 – 48.66	
Overall		50	22	28	56.0%	47.1 ± 0.54	46.04 – 48.16	

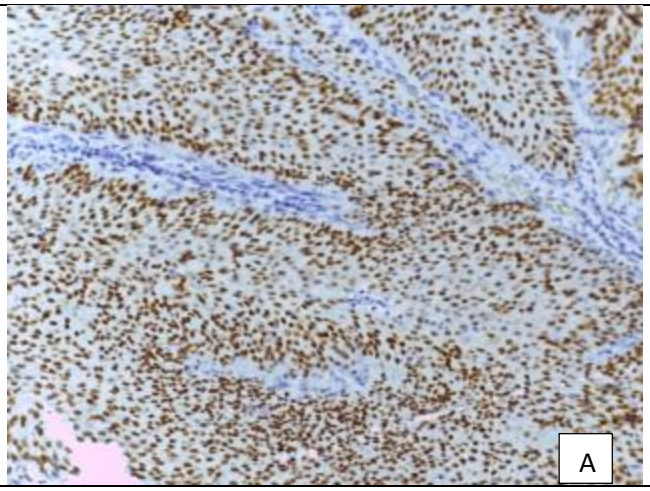

Figure 1A: photomicrograph of section of low-grade non-muscle invasive bladder cancer (NMIBC) that shows strong CHECK2 expression. (X:200)

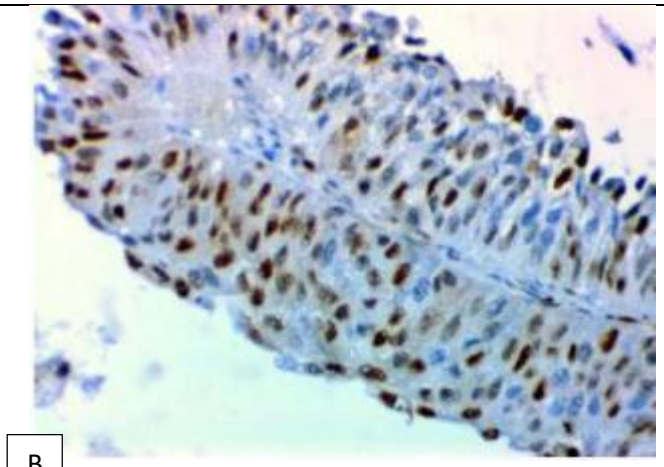

Figure 1B: photomicrograph of section of low-grade non-muscle invasive bladder cancer (NMIBC) that shows: strong CHECK2 expression.(X:200)

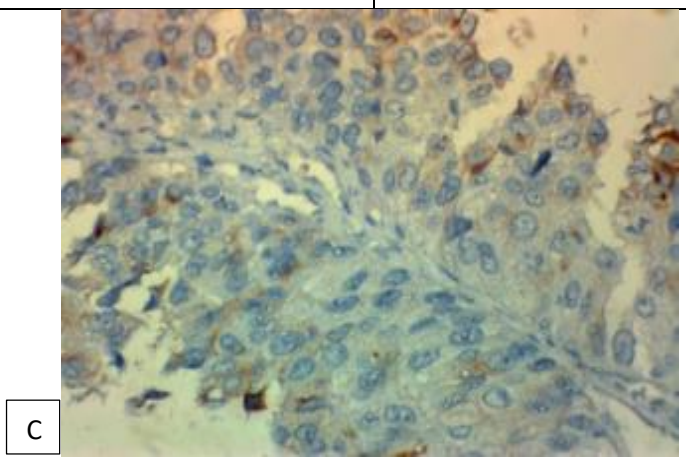

Figure1C: photomicrograph of section of high-grade non-muscle invasive bladder cancer (NMIBC) that shows: negative CHECK2 expression.(X:200)

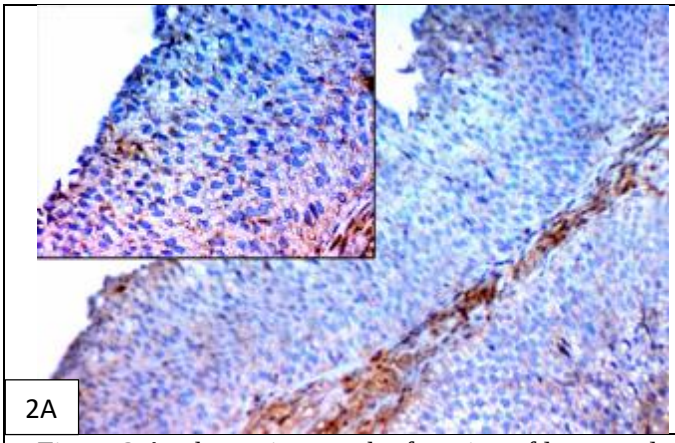

Figure 2 A: photomicrograph of section of low -grade NMIBC showing: negative cytoplasmic FASN (IHC, X:100), the same case with high magnification.

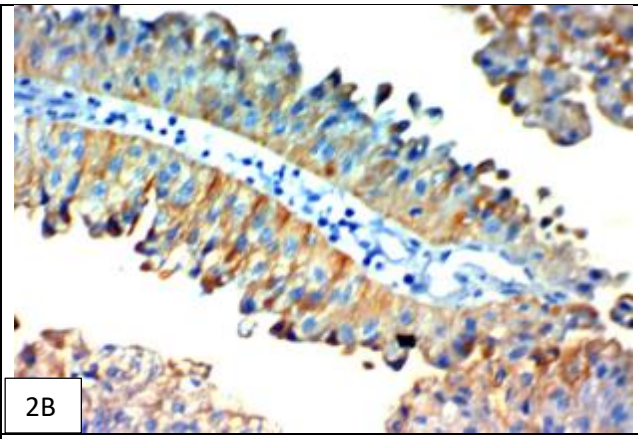

Figure 2 B: photomicrograph of section of low-grade NMIBC showing: moderate cytoplasmic FASN. (X200).

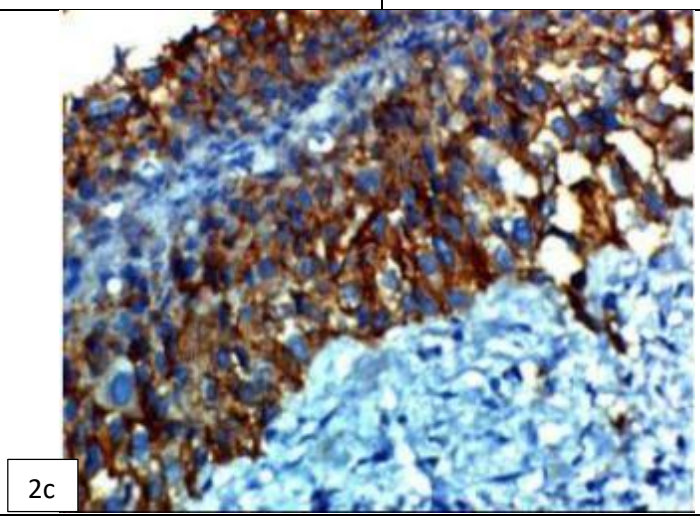

Figure 2C: photomicrograph of section of high-grade non-muscle invasive bladder cancer (NMIBC) showing: strong cytoplasmic FASN (X: 200).

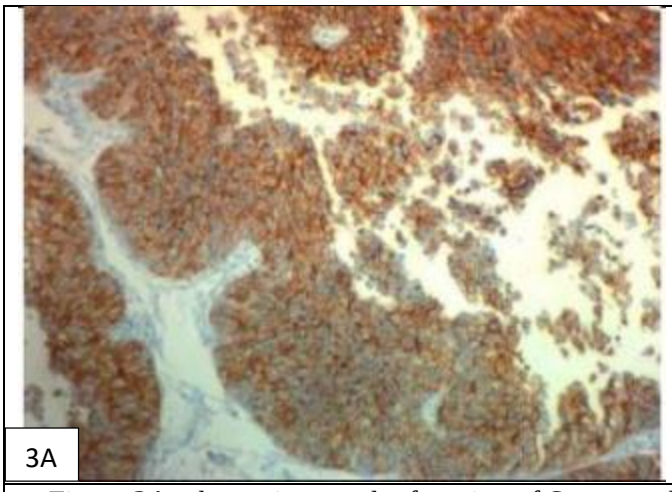

Figure 3A: photomicrograph of section of Strong and diffuse membranous Her2/neu expression (+3) in high-grade non-muscle-invasive bladder cancer (NMIBC).(X:200)

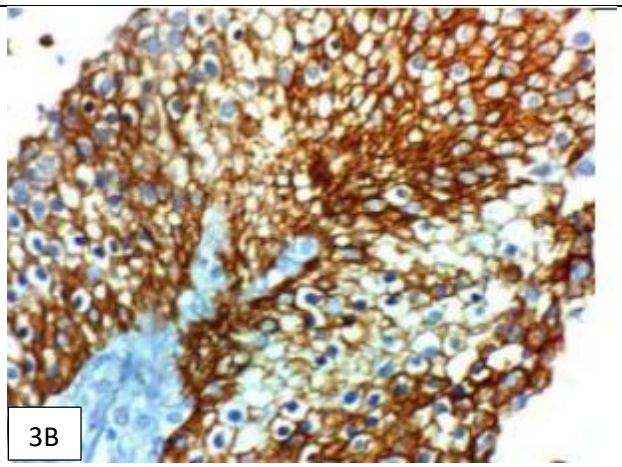

Figure 3B: photomicrograph of section of strong and diffuse membranous Her2/neu expression +3 in high-grade, non-muscle invasive bladder cancer (NMIBC). (X:200)

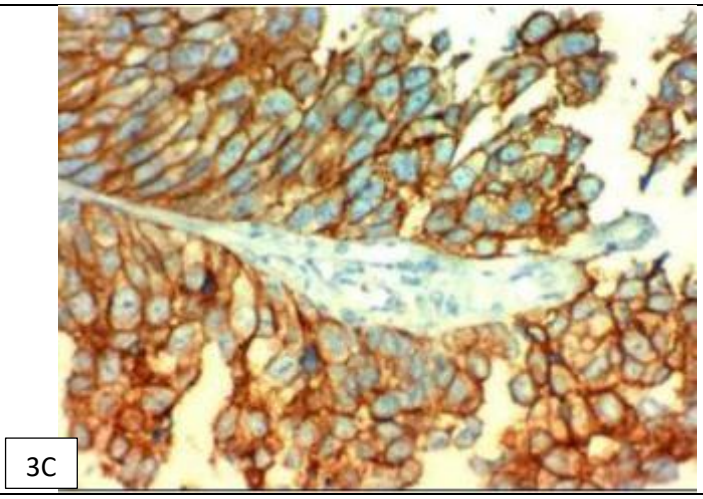

Figure 3 C: photomicrograph of section of Strong membranous Her2/neu expression (+3) in high-grade non-muscle invasive bladder cancer (NMIBC).(X:200)

Figure 4 Kaplan Meier curves of disease-free survival (DFS) stratified according to (A) CHEK 2 expression, (B) FASN expression, and (C) Her2 neu expression.

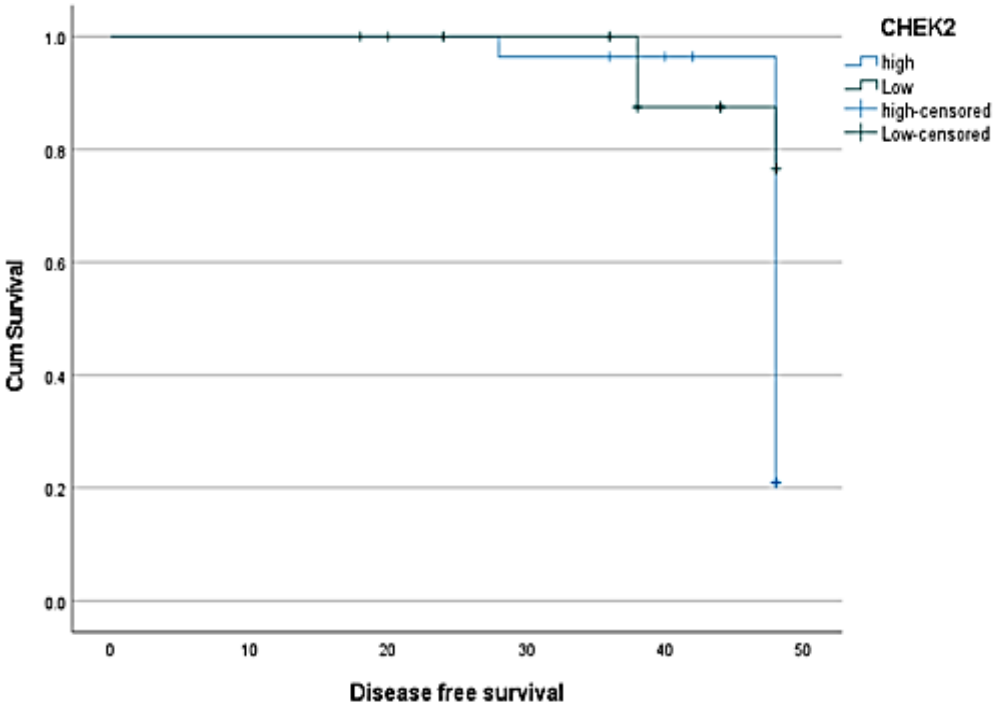

Figure 4 (A): Kaplan Meier plot showing relation between disease free survival and CHEK2.

Figure 4 (A): Kaplan Meier plot showing relation between disease free survival and CHEK2 (mean survival in high CHEK2 was 47.29 month versus 46.75 month in low CHEK2) ($p<0.05$).

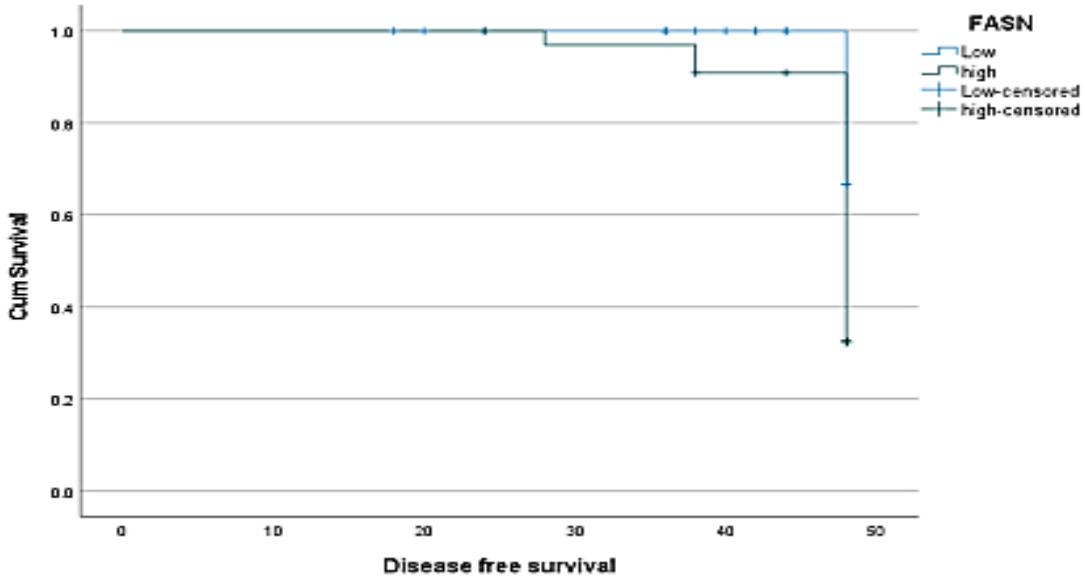

Figure 4 (B): Kaplan Meier plot showing relation between disease free survival and FASN.

Figure 4 (B): Kaplan Meier plot showing relation between disease free survival and FASN (mean survival in positive FASN was 48 month versus 46.79 month in FASN low) (p>0.05).

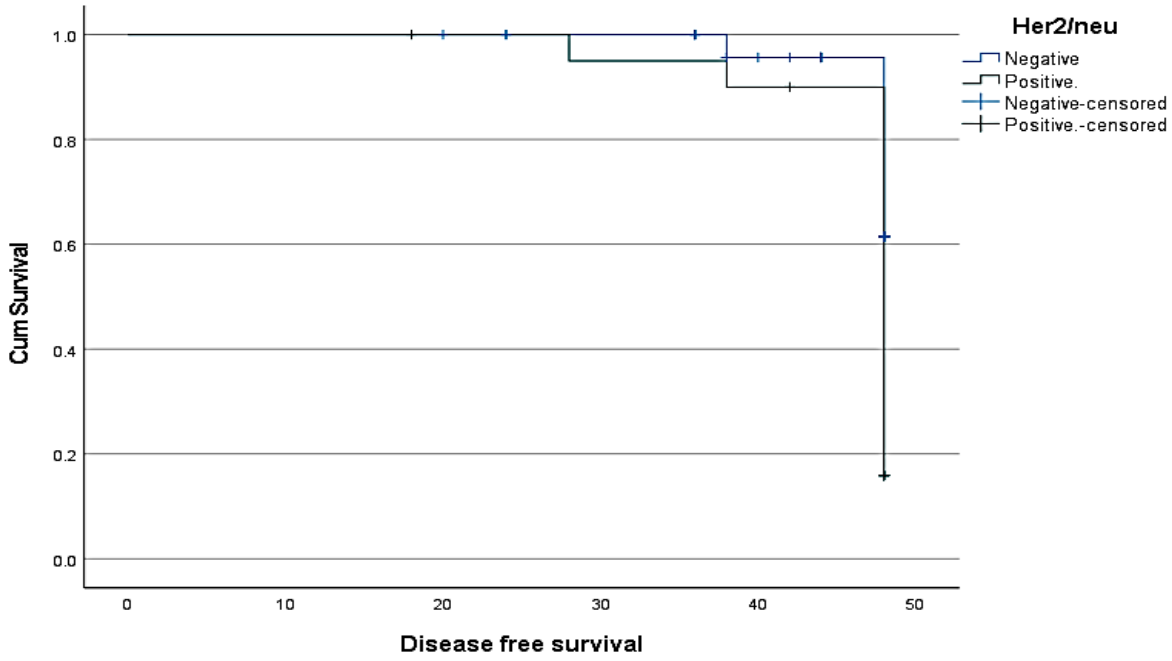

Figure 4 (C): Kaplan Meier plot showing relation between disease free survival and Her2/neu expression.

Fig. 4 (C) Kaplan Meier plot showing relation between disease free survival and Her2/neu expression (mean survival in negative Her2/neu was 47.57 month versus 46.5 month in Her2/Neu positive) (p<0.05).

DISCUSSION

We sought to review the evidence of immunohistochemistry expression of CHEK2, FASN and her2neu for the prediction of recurrence and progression in NMIBC.

To date, few studies proved the prognostic effect of the expression ofCHEK2 in MIBC but only one study that explained its role in NMIBC progression [10].

Low CHEK2 expression was associated with the adverse outcome in gastric [13] and in ovarian cancer[14]. This was contradictory to previous results that high CHEK2 expression correlated with a shorter survival of patients [15, 16]. In our study, low expression of CHEK2 was found in 56% of the studied cases. **Spachmann *et al.*** [10], in their study reported that loss of CHE2 expression was in 11 samples (8.7%) of studied cases. Previous studies reported that loss or low CHEK2 detected a subgroup of patients who were with worse disease free-survival rate in the high-risk groups with multifocal tumors, concomitant carcinoma in situ, high stage and tumor G3 [10,17]. Consistent with these findings, our results confirmed that the previous observation of the CHEK2 loss role in tumor progression.

Furthermore, multivariate analysis indicated that decreased CHEK2 expression in our examined cases was significantly associated with NMIBC progression and shorter disease free - survival rate. Previous reports

were verified by our research [10, 17]. During the course of our study's follow-up period, 28% of NMIBC patients developed MIBC, and 44% experienced a recurrence. Meanwhile, **Spachmann *et al.*** [10] observed progression of tumor in (15.1%) and recurrence in (35.7 %) in their studied cases of NMIBC

Smoking down regulates CHEK2 in normal urothelium, possibly linked with an early step in carcinogenesis of bladder carcinoma. Damage of DNAvia prolonged exposure of tobacco impairs the healthy stem cell defenses which involve CHEK2 via decreasing the CHEK2 activity [18]. Similar results were reported in other study [10].

FASN is a necessary enzyme in the de novo lipogenesis pathway that can also hinder the intrinsic pathway of apoptosis. It has been found to be overexpressed in various cancers [19].

We found that elevated FASN expression was present in 68% of the NMIBC patients we examined. Consistent with earlier findings, we found a strong correlation between FASN expression and tumor stage, tumor grade, tumor recurrence, and the development of MIBC [20, 21]. Those findings back up the hypothesis that FASN expression plays a critical role in tumour growth and development via the fatty acid synthetic pathway. In light of these results, it is clear that FASN inhibitors like Orlistat possess anticancer efficacy [22].

One of the best therapeutic targets in oncology is her2-neu. It has been accepted as a potential biomarker in a variety of cancer types particularly breast, gastric carcinomas and bladder cancer [23, 24]. Its reported overexpression rate ranges from less than 10% to greater than 80%. Its prognostic significance is also unclear due to conflicting findings [25]. Although NMIB has received some focus, most research has focused on MIBC.

A subset of high-grade NMIBCs contained Her-2 neu amplification was linked with markedly aggressive behavior as it was reported by **Chen *et al.*** [26]**.** Unlike; Non significant relationship between Her-2neu prognosis in 285 patients with NIMBCas it was reported by **Olsson *et al.*** [27].

This variability might be owing to heterogeneity in grade , stage , fixation issues as well use of dissimilar antibodies and also use of tissue sections rather than tissue microarray as well as of given treatment [23].

In our study Her2/neu was expressed in 43.3 % of our studied cases with a significant link with tumor grade and size. Near results were reported by **Abdou Hassan *et al.*** [28] in their study who observed HER2 expressions in 49% of their studied cases of NMIBC. Her2/neu was positively expressed with a significant association with concomitant carcinoma in situ and smokers in our study. These results were in line with. **Hurle *et al.*** [29] who suggested that the carcinoma in situ was a significant predictor of progression.

As previously observed by **Chen *et al.*** [26]**,** high Her2 expression in NMIBC cases was substantially connected to the tumor recurrence and its development to MIBC with short disease free- survival rate. In a study of 83 patients with T1G3 BC, **Bongiovanni *et al.*** [30] found that HER-2 expression was not a reliable predictor of tumor recurrence or progression.

It is well accepted that Her2/neu over-expression is linked with poor prognosis in breast cancer. Trastuzumab is a monoclonal antibody that specifically targets HER2 protein via binding to an extra-cellular protein receptor directly and it enhanced the overall patients' survival in the primary and metastatic breast cancer together [31, 32]. In our study, Her2 is expressed in patients who were with highest risk group; within pT1G3 tumors, multifocal tumors and concomitant carcinoma in situ who could be candidates for anti-HER2-targeted therapy so they could avoid tumor recurrence or progression. Our results confirmed its adverse prognostic role on tumor recurrence and also the progression that might occur in NMIBC. So, careful follow up should be attained for those patients.

The expression of FASN could be increased if Her2/neu overexpressed in NMIBC as reported by **Abdelrahman *et al.*** [20]**.** In our study, a strong association between FASN and Her2/ neu expression was found. These findings indicate the important role for those together molecules in the occurrence of tumor invasiveness.

The most important risk factors in NMICB have been found across a wide range of clinical and pathological criteria, including tumor grade, stage, focality, tumor size, CIS, and number of recurrences. Therefore, the treatment decision between a bladder-sparing approach and radical cystectomy may be aided by the addition of CHEK2, FASN, and Her2neu expression, especially in individuals with those risk characteristics.

CONCLUSION

Loss of CHEK2, high FASN and positive Her2-neu in biopsies of NMIBC is an independent predictor of tumor progression. Therefore, they could supply us with significant prognostic information for patients with NMIBC and could help in identifying patients who were with an unfavorable prognosis so they could benefit from an early and aggressive therapy after TURBT.

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