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Microsatellite instability profiling in Egyptian bladder cancer patients: A pilot study

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A B S T R A C T

Microsatellite alterations have been implicated in the pathogenesis of many cancers; however, they are still not well addressed in the bladder cancer (BC) of Egyptian population. We assessed microsatellite instability (MSI) profile and loss of heterozygosity (LOH) using 13 microsatellite markers in tumor tissue samples and urine sediments obtained from 30 Egyptian patients with BC. The concordance between MSI in tumor tissue and urine samples was determined, and correlated to relevant clinicopathologic features. We found that MSI was more frequent than LOH (100% and 46.7%, respectively). *D16S310*, *MBP*, and *IFN-α* showed the highest MSI frequency in urine samples (70%, 70%, and 66.67%, respectively), while *MBP*, *ACTBP2*, and *D9S171* (66.67%, 63.33%, and 60%, respectively) were the most frequently detected in tumor tissues. All assessed MSI markers correlated significantly with pathologic subtype (being more frequent in TCC) and with hematuria. The concordance between tissue and urine samples was statistically significant for *D16S476, D9S171, FGA*, and *ACTBP2* ($P = 0.04$, 0.015, 0.02, and 0.007, respectively). When we combined *D16S476* and *D9S171*, the sensitivity, specificity, positive predictive value, and negative predictive value for

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the diagnosis of BC were 80.0%, 75.0%, 82.8%, and 71.4%, respectively. Accordingly, we concluded that MSI in urine sediments could be a potential tool for the diagnosis of BC.

ARTICLE INFO

Keywords: Bladder cancer; Microsatellite instability (MSI); Loss of heterozygosity (LOH); *D16S476*; *ACTBP2*; *D9S171*; *FGA*

Introduction

Bladder cancer (BC) is one of the most common cancers worldwide, with approximately 400,000 new cases diagnosed annually.[1] The incidence rates are 3-4 times higher in men than in women.[2] The main risk factor for BC development is tobacco smoking.[3] In Egypt, BC represented 6.94% of all cancer cases during the period from 2008 to 2011, and BC was the second most common cancer in males after hepatocellular carcinoma.[4]

The vast majority of all diagnosed BC cases (98%) are epithelial in origin, mainly transitional cell carcinomas (TCCs),[5] and the diagnosis is based mainly on cystoscopy and urinary cytology. Although considered the gold standard, urethrocystoscopy can miss certain lesions, especially carcinoma in situ. The second gold standard for diagnosing BC is urine cytology, which can detect high-grade tumors and carcinoma in situ. However, urine cytology has a limited clinical value for the detection of low-grade tumours.[6] Therefore, identification of additional sensitive, specific, and noninvasive biomarkers for the early detection and prediction of BC are urgently required for better management of patients. These biomarkers can also decrease the need for frequent cystoscopy.

One of the most common genetic changes detected in BC is microsatellite instability (MSI). The DNA microsatellites are highly polymorphic repeats of 1-6 nucleotides that are abundant and repeated throughout the genome. These repeats are prone to frequent errors during DNA replication, and if this error is not corrected by the mismatch repair system, it will result in MSI, which is observed as a difference in the length of an allele's repetitive sequence in cancer cells compared to the original length in the normal cells of the same patient.[7]

Loss of heterozygosity (LOH) is another molecular change that affects these loci either with or without MSI. LOH occurs when one of the microsatellite alleles is present in the normal DNA but not in the paired tumor DNA.[7] LOH affecting chromosomes 9p and 9q was detected in BC patients regardless of tumor grade, tumor stage, or other pathologic variables, while LOH affecting other chromosomes is associated with a higher tumor grade and stage.[8]

In the current study, we sought to assess the profiling of MSI and/or LOH in BC patients using 13 microsatellite repeat markers (*D9S747, IFN-a, D9S171, D9S162, ACTBP2, D4S243, FGA, D16S310, D16S476, D18S51, MJD, MBP*, and *D21S1245*) that have been frequently reported in previous studies from Europe and the USA. These markers were assessed in the urine and tumor tissues of BC patients and were then correlated to relevant clinicopathologic features of the patients.

Patients and methods

Patients

This retrospective cohort study included 30 patients with histopathologically confirmed BC who were diagnosed and treated at the National Cancer Institute, Cairo University during the period from January 2013 to December 2016. Peripheral blood and urine samples were collected from the patients, and fresh tumor tissue samples were obtained by transurethral resection. The

diagnosis of BC was histologically confirmed in all tissue samples by examination of hematoxylin and eosin-stained slides from the representative tumor samples. The study protocol was approved by the Ethical Committee of the NCI, Egypt, which was in accordance with the 2011 Declaration of Helsinki. A signed informed consent form was obtained from all participants before enrolment in the study.

Sample preparation and DNA extraction

Urine samples (200 mL) from each patient and control subjects were collected in four sterile 50-mL plastic Falcon tubes, the tubes were centrifuged in a cooling centrifuge at 4°C (4000× g) for at least 15 minutes. The supernatant fluid was discarded, and the pellet was washed twice with PBS and stored at −80°C until DNA extraction.

For DNA extraction from the PBL samples, 3 mL of whole blood were treated with RBC lysis solution (8.26 g of ammonium chloride [NH4Cl], 1 g of potassium bicarbonate [KHCO3], and 0.037 g EDTA in 1 L of ddH2O) to isolate lymphocytes as a reference (germ-line DNA). In addition, fresh tumor tissue samples were obtained from each patient and immediately frozen in liquid nitrogen for 5 minutes and then stored at −80°C for subsequent DNA extraction. The DNA was extracted from blood and urine samples using the Genomic Prep™ blood isolation kit (GE Healthcare, UK), whereas DNA isolation from fresh-frozen tissue samples was performed using the ReliaPrep™ FFPE gDNA Miniprep System (Promega, USA) according to the manufacturer's protocol. The concentration and purity of the DNA were measured using a nanodrop instrument (Agilent Technologies, Cary60-UV-VIS) at 260/280 nm absorbance.

PCR amplification and MSI analysis

Patients and control samples were subjected to PCR amplification using 13 MSI primer sets mapped to chromosome: 9p (*D9S747, IFN -a, D9S171, D9S162*), 5 (*ACTBP2*), 4 (*D4S243, FGA*), 16 (*D16S310, D16S476*), 18 (*D18S51*), 14 (*MJD, MBP*), and 21 (*D21S1245*) in the PBL, urine, and tumor tissues to assess MSI and LOH (Table 1), using the MSI Analysis System from (Promega, USA). The PCR cycles were performed using a thermal cycler (GeneAmp System 9700, Applied Biosystems), and the data were analyzed using the ABI PRISM 3100 Genetic Analyser with Data Collection Software, version 2.0, according to manufacturer's protocols.

Statistical methods

Statistical analysis was performed using the chi-square test for the association between different variable frequencies and percentages. A likelihood test was used to detect sensitivity and specificity. The concordance between microsatellite alterations in tissues and urine samples was measured using Cohen's kappa test of agreement. All statistics were performed using SPSS, version 22 (IBM SPSS, Armonk, NY). A *P* value less than 0.05 was considered statistically significant.

Results

Clinicopathologic features of the patients

The age of the patients ranged from 38 to 81 years with a median age of 58.5 years. There were 22/30 males (73.3%) and 8/30 females (26.7%). Smokers represented 56.7% of all tested patients. Twenty-seven patients (90%) had TCC. Two patients (6.7%) had grade 1 (G1) tumors, 13 (43.3%) had G2 tumors, and 15 (50%) had G3 tumors. Twelve patients (40%) presented with pT3,

Table 1
Primer sequences and lengths of PCR amplified products for microsatellite makers.

Microsatellite Markers	Primer Sequence	Fragment Length (Base Pair)
D4S243 (F)	5′ TCAGTCTCTCTTTCTCCTTGCA 3′	173
D4S243 (R)	5′ TAGGAGCCTGTGGTCCTGTT 3′	
D9S747 (F)	5′ GCCATTATTGACTCTGGAAAAGAC 3′	182
D9S747 (R)	5′ CAGGCTCTCAAAATATGAACAAAAT 3′	
D16S310 (F)	5′ GGGCAACAAGGAGAGACTCT 3′	162
D16S310 (R)	5′ AAAAAAGGACCTGCCTTTATCC 3′	
D16S476 (F)	5′ TTGCACTCCACTCTGGGCA 3′	144-181
D16S476 (R)	5′ TTGCCTTGGCTTTCTGTTGG 3′	
D18S51 (F)	5′ GAGCCATGTTCATGCCACTG 3′	263
D18S51 (R)	5′ CAAACCCGACTACCAGCAAC 3′	
D21S1245 (F)	5′ GTCAGTATTACCCTGTTACCA 3′	289
D21S1245 (R)	5′ GTTGAGGATTTTTGCATCAGT 3′	
IFN-α (F)	5′ TGCGCGTTAAGTTAATTGGTT 3′	130-155
IFN-α (R)	5′ GTAAGGTGGAAACCCCCACT 3′	
D9S171 (F)	5′ AGCTAAGTGAACCTCATCTCTGTCT 3′	158-177
D9S171 (R)	5′ ACCCTAGCACTGATGGTATAGTCT 3′	
D9S162 (F)	5′ GCAATGACCAGTTAAGGTTC 3′	172-196
D9S162 (R)	5′ AATTCCCACAACAAATCTCC 3′	
FGA (F)	5′ GAAGACAGAGTGCTCCCA3′	172
FGA (R)	5′ AACAGTCCTTATGAGATTGAGAA3′	
MJD (F)	5′ CCAGTGTTCTGTGCTGCCTTTT 3′	154
MJD (R)	5′ GGAGTTGGTCAGCTTCGCAAT 3′	
ACTBP2 (F)	5′ AATCTGGGCGACAAGAGTGA3′	230-280
ACTBP2 (R)	5′ ACATCTCCCCTACCGCTATA 3′	
MBP (F)	5′ CTGTCCTTGGTCTGTTAAACTG 3′	117
MBP (R)	5′ TGATGGTAATTGCCTGTGGTG 3′	

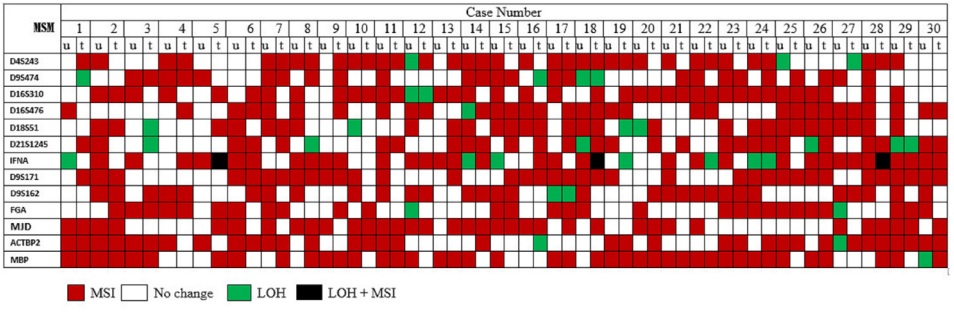

Fig. 1. Mapping of microsatellite alternations in 30 patients with BC.

6 (20%) with pT4, 10 (33%) with pT2, and only 2 patients had pT1 nonmuscle invasive disease (Table 2).

Frequency of MSI and/or LOH at the 13 microsatellite loci in urine and tissue samples

All tested BC patients showed a high frequency of MSI in both urine and tumor tissues (4-10 MSI repeat markers in tumor tissue samples and 3-11 MSI repeat markers in urine sediments, Fig 1 and Table 3). On the other hand, LOH was detected in 14/30 patients (46.7%) in urine and/or tumor tissue samples.

In urine samples, *D16S310, MBP*, and *IFN-α* showed the highest MSI frequency (70%, 70%, and 66.67%, respectively), whereas in tumor tissues, *MBP, ACTBP2*, and *D9S171* exhibited the most frequent MSI (66.67%, 63.33%, and 60%, respectively). Although there were no significant differ-

Table 2
Relevant clinicopathologic features of the bladder cancer patients.[21]

Variable	Number (%)
Gender	
Male	22 (73.3%)
Female	8 (26.7%)
Age (y)	
≤60	20 (66.67%)
>60	10 (33.33%)
Mean ± SD	60.7 ± 11.3
Smoking	
Smoker	17 (56.7%)
Nonsmoker	13 (43.3%)
Symptoms	
Hematuria	99 (51.5%)
Dysuria	93 (48.4%)
Pathologic subtype	
TCC	27 (90%)
SCC	3 (10%)
Grade	
G 1	2 (6.7%)
G 2	13 (43.3%)
G 3	15 (50%)
Pathologic T stage	
T1	2 (6.7%)
T2	10 (33.3%)
T3	12 (40%)
T4	6 (20%)

SCC, squamous cell carcinoma; TCC, transitional cell carcinoma.

Table 3
Frequency MS alterations (MSI and/or LOH) in the tissue of BC patients from Egypt and western countries.

Marker*	Egyptian Patients		Western Patients		Region
	MSI	LOH	MSI	LOH	
D18S51	43.33%	6.67%	17.14%	24.29%	Macedonia (18)
			–	4.2%	France (11)
			–	27.8%	Italy (17)
D4S243	53.33%	3.33%	–	0.0%	France (11)
			37.0%	14.0%	Italy (17)
D9S747	43.33%	10	14.0%	21.0%	Italy (17)
D16S310	53.33%	3.33%	–	10.5%	France (11)
			25.0%	20.0%	Italy (17)
D16S476	50%	0	–	37.9%	USA (13)
			18.0%	12.0%	Italy (17)
D21S1245	56.67%	10%	–	–	
IFN-α	50%	20%	–	23.5%	France (11)
			2.0%	10.0%	Italy (17)
D9S171	60%	0	–	20%	France (11)
D9S162	40%	3.33%	–	20%	France (11)
			43.0%	10.0%	Italy (17)
FGA	46.67%	0	–	0.0%	France (11)
MJD	56.67%	0	–	8%	France (11)
ACTBP2	63.33%	3.33%	–	11.4%	France (11)
			10.8%	–	UK (21)
MBP	66.67%	0	–	38.9%	Italy (17)

* Markers were assessed in the bladder tumor tissues. Regarding assessment of MSI and LOH in the urine, it was included in the discussion section.

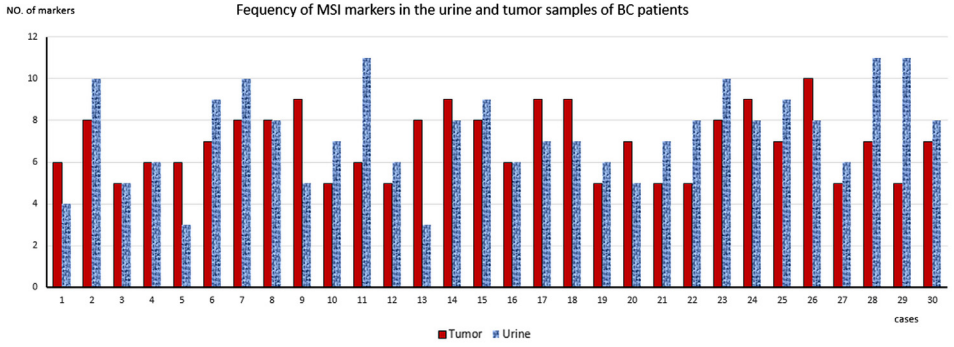

Fig. 2. Frequency of MSI markers in the urine and tumor tissue samples of BC patients.

ences among all tested MSI markers regarding their expression in urine or tissue samples ($P > 0.05$ for all), out of the 30 patients assessed, 16 (53.3%) showed higher MSI repeat markers in urine than in tumor tissues (Fig 2).

Regarding LOH, *IFN-α* showed the highest frequency in the urine samples of 4/30 patients (13.33%) and in the tumor tissues of 6/30 patients (20%). The difference in *IFN-α* expression between the urine and tissue samples was not statistically significant ($P = 0.731$). The markers that showed the highest frequency for both MSI and LOH in urine samples were *IFN-α, MBP, D16S310, and D4S243* (80%, 73.3%, 73.3%, and 70%, respectively), while *MBP, ACTBP2*, and *D21S1245* (66.67%, 66.67%, and 66.67%, respectively) were the highest in tumor tissues (Supp. 1, Figs 3 and 4).

Association between microsatellite alterations and relevant clinicopathologic features

All MSI and/or LOH repeat markers in tumor tissues and urine samples associated significantly with hematuria in all assessed patients ($P = 0.001$). Tumor stage associated significantly with *ACTBP2* (MSI and/or LOH) in tumor tissues ($P = 0.046$) and with *D16S476, D9S171, and MBP* markers in urine sediments ($P = 0.04$, 0.01, and 0.01, respectively). Tumor grade associated significantly with MSI and/or LOH of *D16S476* in tumor samples ($P = 0.02$) and urine sediments ($P = 0.03$). On the contrary, no significant association was found between any of the microsatellite alterations and smoking or patient age (Tables 4 and 5).

The concordance between microsatellite alterations in urine and tumor tissues

Significant concordance was detected between urine and tumor tissue samples regarding *D16S476, D9S171, FGA*, and *ACTBP2* alterations (MSI and/or LOH) ($P = 0.04$, 0.015, 0.02, and 0.007, respectively, Table 6).

Diagnostic accuracy of microsatellite alterations (MSI and LOH) in the urine of BC patients

Microsatellite alterations in *D9S171* and *D16S476* markers in the urine of BC patients showed the same values for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of 60.0%, 80.0%, 81.8%, 57.1%, and 68.0%; respectively ($P = 0.004$). *ACTBP2* showed sensitivity, specificity, PPV, NPV, and accuracy of 53.3%, 80.0%, 80.0%, 53.3%, and 64.0%, respectively ($P = 0.016$). However, *FGA* showed the lowest diagnostic accuracy ($P = 0.33$),

Table 4
Clinicopathologic features in association with MS alterations (MSI and/or LOH) in tissues of BC patients.

Features	Total No	Microsatellite Alterations (MSI and/or LOH) (%)											
		D4S243 (n = 17)	*D9S747* (n = 16)	*D16S310* (n = 17)	*D16S476* (n = 15)	*D18S51* (n = 15)	*D21S1245* (n = 20)	*IFN-α* (n = 18)	*D9S171* (n = 18)	*D9S162* (n = 13)	*FGA* (n = 14)	*MJD* (n = 17)	*ACTBP2* (n = 20)	*MBP* (n = 20)
Sex														
Male	22	13 (76.5)	13 (81.3)	12 (70.6)	11 (73.3)	12 (80.0)	14 (70.0)	11 (61.1)	12 (66.7)	11 (84.6)	11 (78.6)	12 (70.6)	14 (70.0)	16 (80.0)
Female	8	4 (23.5)	3 (18.7)	5 (29.4)	4 (26.7)	3 (20.0)	6 (30.0)	7 (38.9)	6 (33.3)	2 (15.4)	11 (21.4)	5 (29.4)	6 (30.0)	4 (20.0)
P* value		**0.03**†	**0.01**†	0.09	0.07	**0.02**†	0.07	0.35	0.16	**0.013**†	**0.03**†	0.09	0.07	**0.02**†
Age														
<60	20	9 (52.9)	9 (50.0)	10 (58.8)	7 (46.7)	8 (53.3)	7 (35.0)	6 (33.3)	8 (44.4)	7 (53.9)	8 (57.1)	7 (41.2)	8 (40.0)	11 (55.0)
≥60	10	8 (47.1)	9 (50.0)	7 (41.2)	8 (53.3)	7 (46.7)	13 (65.0)	12 (66.7)	10 (55.6)	6 (46.1)	6 (42.9)	10 (58.8)	12 (60.0)	9 (45.0)
P* value		0.81	1.0	0.47	0.79	0.79	0.18	0.16	0.64	0.78	0.6	0.47	0.37	0.65
Smoking														
Yes	17	9 (52.9)	11 (68.8)	11 (64.7)	9 (60.0)	9 (60.0)	12 (60.0)	8 (44.4)	9 (50.0)	8 (61.5)	8 (57.1)	10 (58.8)	10 (50.0)	13 (65.0)
No	13	8 (47.1)	5 (31.2)	6 (35.3)	6 (40.0)	6 (40.0)	8 (40.0)	10 (55.6)	9 (50.0)	5 (38.5)	6 (42.9)	7 (41.2)	10 (50.0)	7 (35.0)
P* value		0.81	0.13	0.23	0.44	0.44	0.44	0.64	1.00	0.41	0.6	0.47	1.0	0.18
Hematuria														
Yes	16	16 (94.1)	13 (81.3)	14 (82.4)	14 (93.3)	13 (86.7)	17 (85.0)	16 (88.9)	16 (88.9)	12 (92.3)	12 (84.6)	17 (100.0)	89.6	17 (85.0)
No	14	1 (5.9)	3 (18.7)	3 (17.6)	1 (6.7)	2 (13.3)	3 (15.0)	2 (11.1)	2 (11.8)	1 (7.7)	2 (5.4)	0 (0.0)	10.4	3 (15.0)
P* value		**<0.001**†	**0.02**†	**0.008**†	**<0.001**†	**0.008**†	**0.003**†	**0.001**†	**0.002**†	**0.002**†	**0.01**†	NA	**<0.001**†	**0.003**†
Dysuria														
Yes	14	5 (29.4)	4 (25.0)	5 (29.4)	2 (13.3)	4 (26.7)	6 (30.0)	3 (16.7)	4 (22.2)	4 (30.8)	3 (21.4)	4 (23.5)	4 (20.0)	26.3
No	16	12 (70.6)	12 (75.0)	12 (70.6)	13 (86.7)	11 (73.3)	14 (70.0)	15 (83.3)	14 (77.8)	9 (69.2)	11 (78.6)	13 (76.5)	16 (80.0)	73.7
P* value		**0.003**†	0.07	0.09	**0.005**†	0.11	0.07	**0.005**†	**0.03**†	0.17	**0.03**†	**0.03**†	**0.02**†	**0.04**†
Stage														
T1	2	0 (0.0)	1 (6.25)	1 (5.9)	1 (6.7)	2 (13.3)	1 (5.0)	0 (0.0)	2 (11.1)	2 (15.4)	2 (14.3)	2 (11.8)	1 (5.0)	1 (5.0)
T2	10	7 (41.2)	6 (37.5)	4 (23.5)	6 (40.0)	6 (40.0)	8 (40.0)	7 (38.9)	6 (33.3)	4 (30.8)	3 (21.4)	7 (41.2)	7 (35.0)	6 (30.0)
T3	12	6 (35.3)	7 (43.8)	8 (47.1)	6 (40.0)	5 (33.4)	7 (35.0)	7 (38.9)	7 (38.9)	3 (23.0)	7 (50.0)	4 (23.5)	9 (45.0)	9 (45.0)
T4	16	4 (23.5)	2 (12.5)	4 (23.5)	2 (13.3)	2 (13.3)	4 (20.0)	4 (22.2)	3 (16.7)	4 (30.8)	2 (14.3)	4 (23.5)	3 (15.0)	4 (20.0)
P* value		0.66	0.07	0.12	0.14	0.33	0.11	0.61	0.29	0.84	0.18	0.39	**0.046**†	0.08

(*continued on next page*)

Table 4 (*continued*)

Features	Total No	Microsatellite Alterations (MSI and/or LOH) (%)												
		D4S243 (n = 17)	*D9S747* (n = 16)	*D16S310* (n = 17)	*D16S476* (n = 15)	*D18S51* (n = 15)	*D21S1245* (n = 20)	*IFN-α* (n = 18)	*D9S171* (n = 18)	*D9S162* (n = 13)	*FGA* (n = 14)	*MJD* (n = 17)	*ACTBP2* (n = 20)	*MBP* (n = 20)
Grade														
G1-G2	15	10 (58.8)	9 (56.3)	8 (47.1)	12 (80.0)	10 (67.7)	9 (45.0)	10 (55.6)	10 (55.6)	8 (61.5)	6 (42.9)	11 (52.9)	11 (55.0)	9 (45.0)
G3	15	7 (41.2)	7 (43.7)	9 (52.9)	3 (20.0)	5 (33.3)	11 (55.0)	8 (44.4)	8 (44.4)	5 (38.5)	8 (57.1)	8 (47.1)	9 (45.0)	11 (55.0)
P* value		0.47	0.63	0.80	**0.02**†	0.16	0.65	0.64	0.64	0.41	0.07	0.81	0.65	0.65
Pathology														
TCC	27	16 (94.1)	15 (93.8)	15 (88.2)	13 (86.7)	12 (80.0)	19 (95.0)	17 (94.4)	17 (94.4)	11 (84.6)	13 (92.9)	16 (94.1)	17 (85.0)	19 (95.0)
SCC	3	1 (5.9)	1 (6.2)	2 (11.8)	2 (13.3)	3 (20.0)	1 (5.0)	1 (5.6)	1 (5.6)	2 (15.4)	1 (7.1)	1 (5.9)	3 (15.0)	1 (5.0)
P* value		**<0.001**†	**<0.001**†	**0.002**†	**0.005**†	**0.02**†	**<0.001**†	**<0.001**†	**<0.001**†	**0.01**†	**0.001**†	**<0.001**†	**0.002**†	**<0.001**†

* Association of MSI marker with clinicopathologic features using chi-square test.
† Significance at $P < 0.05$.

Table 5
Clinicopathologic features in association with MS alterations (MSI and/or LOH) in urine of BC patients

Features	Total No	Microsatellite Alterations (MSI and/or LOH) (%)											
		D4S243 (n = 21)	*D9S747* (n = 13)	*D16S310* (n = 22)	*D16S476* (n = 18)	*D18S51* (n = 18)	*D21S1245* (n = 13)	*IFN-α* (n = 24)	*D9S171* (n = 18)	*D9S162* (n = 19)	*FGA* (n = 16)	*MJD* (n = 20)	*ACTBP2* (n = 16)	*MBP* (n = 22)
Sex														
Male	22	17 (81.0)	12 (92.3)	16 (72.7)	6 (66.7)	13 (72.2)	10 (76.9)	18 (75.0)	13 (72.2)	16 (84.2)	13 (81.8)	14 (70.0)	12 (75.0)	15 (68.2)
Female	8	4 (19.0)	1 (7.7)	6 (27.3)	12 (33.3)	5 (27.8)	3 (23.1)	6 (25.0)	5 (27.8)	3 (15.8)	3 (18.3)	6 (30.0)	4 (25.0)	7 (31.8)
P* value		**0.005**†	**0.002**†	0.06	0.16	0.06	0.052	**0.01**†	0.06	**0.005**†	**0.01**†	0.07	0.045†	0.08
Age														
<60	20	11 (52.4)	6 (46.2)	11 (50.0)	5 (27.8)	7 (38.9)	5 (38.5)	10 (41.7)	8 (44.4)	8 (42.1)	7 (43.7)	7 (35.0)	7 (43.8)	11 (50.0)
≥60	10	10 (47.6)	7 (53.8)	11 (50.0)	13 (72.2)	11 (61.1)	8 (61.5)	14 (58.3)	10 (55.6)	11 (57.9)	9 (56.3)	13 (65.0)	9 (56.2)	11 (50.0)
P* value		0.83	0.78	1.0	0.06	0.35	0.41	0.41	0.64	0.64	0.62	0.18	0.62	1.0
Smoking														
Yes	17	13 (61.9)	8 (61.5)	10 (45.5)	9 (50.0)	9 (50.0)	8 (61.5)	15 (62.5)	10 (55.6)	11 (57.9)	10 (62.5)	10 (50.0)	7 (43.8)	14 (65.6)
No	13	8 (38.1)	5 (38.5)	12 (54.5)	9 (50.0)	9 (50.0)	5 (38.5)	9 (37.5)	8 (44.4)	8 (42.1)	6 (37.5)	10 (50.0)	9 (56.2)	8 (36.4)
P* value		0.40	0.41	0.62	1.0	1.0	0.41	0.32	0.64	0.64	0.32	1.0	0.62	0.20
Hematuria														
Yes	16	19 (90.5)	13 (100.0)	19 (86.4)	17 (94.4)	16 (88.9)	11 (84.6)	21 (87.5)	15 (83.3)	18 (94.7)	14 (87.5)	19 (95.0)	16 (100)	19 (86.4)
No	14	2 (9.5)	0 (0.0)	3 (13.6)	1 (5.6)	2 (11.1)	2 (15.4)	3 (22.5)	3 (16.7)	1 (5.3)	2 (12.5)	1 (5.0)	0 (0.0)	3 (13.6)
P* value		**0.003**†	NA	**0.001**†	**<0.001**†	**0.002**†	**0.01**†	**<0.001**†	**0.008**†	**<0.001**†	**0.005**†	**<0.001**†	NA	**<0.001**†
Dysuria														
Yes	14	16 (76.2)	2 (15.4)	7 (31.8)	3 (16.7)	5 (27.8)	2 (15.4)	6 (25.0)	4 (22.2)	3 (15.8)	4 (25.0)	5 (25.0)	2 (12.5)	5 (22.7)
No	16	5 (23.8)	11 (84.6)	15 (68.2)	15 (83.3)	13 (72.2)	11 (84.6)	18 (75.0)	14 (77.8)	16 (84.2)	12 (75.0)	15 (75.0)	14 (87.5)	17 (77.3)
P* value		**0.025**†	**0.02**†	0.13	**0.008**†	0.09	**0.01**†	**0.001**†	**0.03**†	**0.008**†	0.07	**0.04**†	**0.005**†	**0.01**†
Stage														
T1	2	2 (9.5)	1 (7.7)	2 (9.0)	1 (5.6)	2 (11.1)	1 (7.7)	2 (8.3)	1 (5.6)	2 (10.5)	1 (6.3)	2 (10.0)	1 (6.2)	2 (9.1)
T2	10	7 (33.3)	7 (53.9)	6 (27.3)	8 (44.4)	7 (38.9)	4 (30.8)	8 (33.3)	7 (38.8)	7 (36.8)	5 (31.2)	8 (40.0)	5 (31.2)	4 (18.2)
T3	12	7 (33.3)	2 (15.4)	8 (36.4)	7 (38.9)	5 (27.8)	6 (46.1)	10 (41.7)	9 (50.0)	6 (31.6)	6 (37.5)	7 (35.0)	7 (43.8)	12 (54.5)
T4	16	5 (23.9)	3 (23.1)	6 (27.3)	2 (11.1)	4 (22.2)	2 (15.4)	4 (16.7)	1 (5.6)	4 (21.1)	4 (25.0)	3 (15.0)	3 (18.8)	4 (18.2)
P* value		0.36	0.09	0.33	**0.04**†	0.41	0.21	0.08	**0.01**†	0.41	0.32	0.16	0.18	**0.01**†

(*continued on next page*)

Table 5 (*continued*)

Features	Total No	Microsatellite Alterations (MSI and/or LOH) (%)											
		D4S243 (n = 21)	*D9S747* (n = 13)	*D16S310* (n = 22)	*D16S476* (n = 18)	*D18S51* (n = 18)	*D21S1245* (n = 13)	*IFN-α* (n = 24)	*D9S171* (n = 18)	*D9S162* (n = 19)	*FGA* (n = 16)	*MJD* (n = 20)	*ACTBP2* (n = 16)	*MBP* (n = 22)
Grade														
G1-G2	15	13 (61.9)	8 (61.5)	12 (54.5)	14 (77.8)	12 (66.7)	8 (61.5)	13 (54.2)	11 (61.1)	12 (63.2)	9 (56.2)	12 (60.0)	6 (37.5)	10 (45.5)
G3	15	8 (38.1)	5 (38.5)	10 (45.5)	4 (22.2)	6 (33.3)	5 (38.5)	11 (45.8)	7 (38.9)	7 (36.8)	7 (43.8)	8 (40.0)	10 (62.5)	12 (54.5)
P* value		0.40	0.41	0.60	**0.03**†	0.23	0.41	0.65	0.47	0.35	0.79	0.58	0.32	0.63
Pathology														
TCC	27	19 (90.5)	12 (92.3)	20 (90.9)	16 (88.9)	15 (83.3)	12 (92.3)	22 (91.7)	17 (94.4)	18 (94.7)	13 (81.3)	18 (90.0)	14 (87.5)	20 (90.9)
SCC	3	2 (9.5)	1 (7.7)	2 (9.1)	2 (11.1)	3 (16.7)	1 (7.7)	2 (8.3)	1 (5.6)	1 (5.3)	3 (18.7)	2 (10.0)	2 (12.5)	2 (9.1)
P* value		**<0.001**†	**<0.001**†	**<0.001**†	**0.005**†	**<0.001**†	**<0.001**†	**<0.001**†	**<0.001**†	**<0.001**†	**0.01**†	**<0.001**†	**0.003**†	**<0.001**†

* Association of MSI marker with clinicopathologic features using chi-square test.
† Significance at $P < 0.05$.

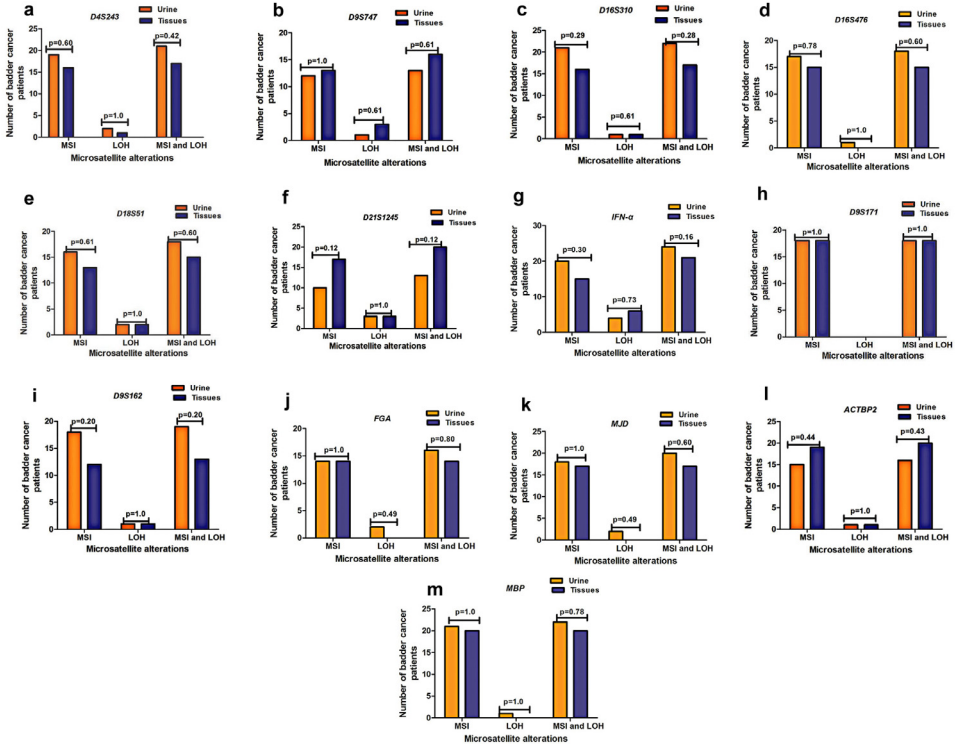

Fig. 3. Frequency of MSI and/or LOH at 13 microsatellite loci in both urine and tumor tissue samples of bladder cancer patients.

with sensitivity, specificity, PPV, NPV, and accuracy of 53.3%, 55.0%, 64.0%, 44.0%, and 54.0%, respectively (Table 7).

Sensitivity and specificity using more than one MSI and LOH marker for the diagnosis of BC

The combination of *D16S476* and *D9S171* had 80% sensitivity, 75% specificity, 82.8% PPV, 71.4% NPV, and 78% accuracy ($P < 0.001$). The combination of *D16S476, D9S171*, and *ACTBP2* showed 93.3% sensitivity, 55% specificity, 75.7% PPV, 84.6% NPV, and 78% accuracy ($P < 0.001$). However, by adding *FGA* to *D16S476, D9S171*, and *ACTBP2*, this combination exhibited 96.7% sensitivity, 30.0% specificity, 67.4% PPV, 85.7% NPV, and 70.0% accuracy ($P = 0.007$, Table 8).

Discussion

Microsatellite instability has an important role in the pathogenesis of many cancers, and it was recently found to be predictive for the response to immunotherapy irrespective of tumor histology.[9,10] Although MSI in BC had been well studied in literature, yet we do not have well-defined microsatellite alterations profiling for our Egyptian patients.

The current study was designed to assess the frequency and patterns of MSI and LOH in Egyptian patients with BC, using 13 microsatellite repeat markers distributed on 7 different chromosomal arms. MSI and LOH were assessed in tumor tissues and urine sediments of the patients compared to normal control. We also correlated the results of MSI and LOH to relevant

Table 6
Concordance between microsatellite markers alterations in urine and tumor tissue samples of bladder cancer patients.

Marker	κ	*P* Value*
D4S243	0.2	0.24
D9S747	0.23	0.14
D16S310	0.02	0.9
D16S476	**0.36**†	**0.04**†
D18S51	0.15	0.26
D21S1245	0.18	0.17
IFN-α	0.12	0.31
D9S171	**0.44**†	**0.015**†
D9S162	0.27	0.08
FGA	**0.38**†	**0.02**†
MJD	0.25	0.18
ACTBP2	**0.43** †	**0.007** †
MBP	0.03	0.88

κ, Cohen's kappa.
* Concordance between markers in tissue and urine was tested using Cohen's kappa measure of agreement.
† Significant at $P < 0.05$.

Table 7
Sensitivity, specificity, PPV and NPV according to microsatellite alterations in urine samples of bladder cancer patients compared to healthy controls.

Marker	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %	Likelihood ratio	*P* Value*
D4S243	70.0	80.0	60.0	80.0	76.0	15.7	<0.001†
D9S474	43.3	50.0	56.5	37.0	46.0	0.22	0.64
D16S310	73.3	85.0	87.5	65.4	76.0	14.4	<0.001†
D16S476	**60.0**	**80.0**	**81.8**	**57.1**	**68.0**	**8.2**	**0.004**†
D18S51	60.0	50.0	64.3	45.5	56.0	2.4	0.31
D21S1245	43.3	75.0	72.2	46.9	56.0	1.8	0.18
IFN-α	80.0	85.0	88.9	73.9	82.0	22.1	<0.001†
D9S171	**60.0**	**80.0**	**81.8**	**57.1**	**68.0**	**8.2**	**0.004**†
D9S162	63.3	85.0	86.4	60.7	72.0	12.3	<0.001†
FGA	53.3	55.0	64.0	44.0	54.0	2.2	0.33
MJD	60.0	75.0	78.3	55.6	66.0	6.1	0.013†
ACTBP2	**53.3**	**80.0**	**80.0**	**53.3**	**64.0**	**5.18**	**0.016**†
MBP	73.3	85.0	88.0	68.0	78.0	17.6	<0.001†

PPV, positive predictive value; NPV, negative predictive value.
* Likelihood test.
† Significant at $P < 0.001$.

Table 8
Combined sensitivity and specificity of the microsatellite markers for diagnosis of bladder cancer patients.

Marker	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %	Likelihood Ratio	*P* Value
D16S476 + D9S171	**80.0**	**75.0**	**82.8**	**71.4**	**78.0**	**15.5**	**<0.001***
D16S476 + FGA	80.0	45.0	68.6	60.0	66.0	3.5	0.06
D16S476 + ACTBP2	80.0	65.0	77.4	68.4	74.0	10.5	**0.001***
D9S171 + FGA	83.3	45.0	69.4	64.3	68.0	4.7	**0.03***
D9S171 + ACTBP2	80.0	70.0	80.0	70.0	76.0	12.8	**<0.001***
FGA + ACTBP2	76.7	40.0	65.7	53.3	62.0	1.6	0.21
D16S476 + D9S171 + FGA	93.3	30.0	66.7	75.0	72.0	4.8	**0.028***
D16S476 + D9S171 + ACTBP2	**93.3**	**55.0**	**75.7**	**84.6**	**78.0**	**15.1**	**<0.001***
D16S476 + FGA + ACTBP2	90.0	30.0	65.6	66.7	66.0	3.2	0.07
D9S171 + FGA + ACTBP2	90.0	35.0	67.5	70.0	68.0	4.6	**0.031***
D16S476 + D9S171 + FGA + ACTBP2	**96.7**	**30.0**	**67.4**	**85.7**	**70.0**	**7.3**	**0.007***

* Significant at $P < 0.05$.

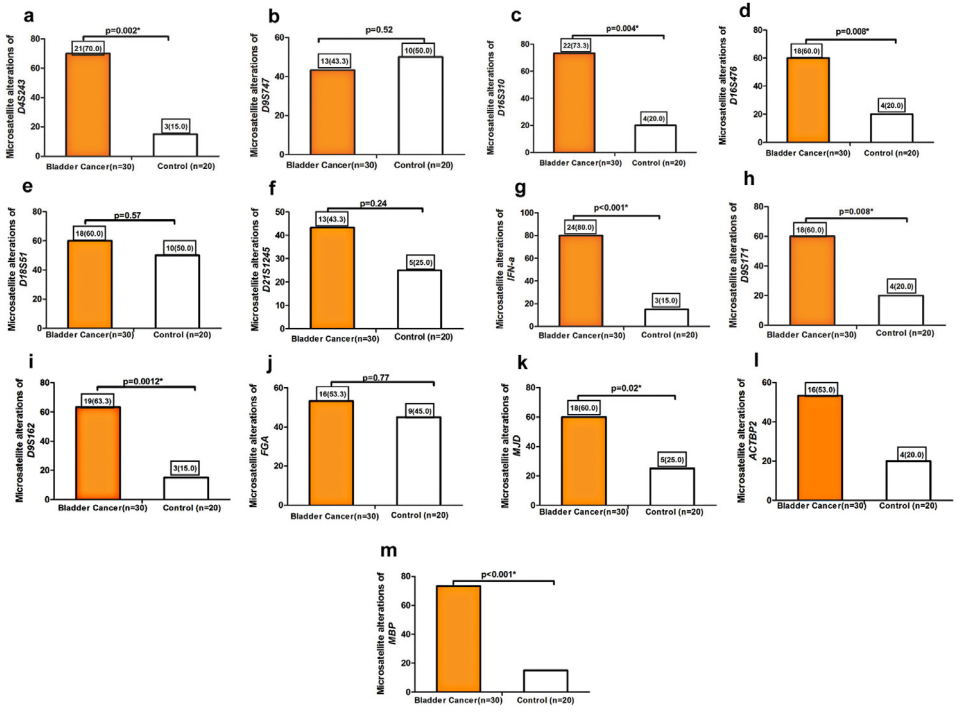

Fig. 4. Frequency of microsatellite alterations at the studied 13 microsatellite loci in the urine of bladder cancer patients.

clinicopathologic features of the patients with respect to the sensitivity and specificity of the microsatellite alterations.

The prevalence of LOH in the current study is relatively low compared to the values previously published by other groups,[11-13] who found that LOH ranged from 30% to 50% of the assessed BC patients. Despite the relatively lower prevalence of LOH in all tested markers in our data, the highest rate was reported in *IFN-a* (9p22), reaching up to 20% in tumor tissues and 13.33% in urine samples. This difference could be partially attributed to the variability in population characteristics, ethnic variation, environmental factors, or differences in the techniques used for the detection of MSI (conventional PCR, commercially available kits, immunohistochemistry, etc.). However, our data are comparable to earlier studies done by Legrand et al[11] and Szarvas et al[14] who found that the highest rate of LOH usually affects chromosome 9, and it occurs at an early stage in the process of urothelial carcinogenesis.[11,13,14]

Although MSI and/or LOH of all tested markers were detected in all assessed BC patients, *IFN-α, MBP, D16S310*, *and D4S243* showed the highest frequency for both MSI and LOH alterations in urine samples, whereas *MBP, ACTBP2*, and *D21S1245* showed the highest frequency in tumor tissue samples. However, the markers that showed a statistically significant concordance between urine and tumor tissue samples were *D16S476, D9S171, FGA*, *and ACTBP2*. Based on these data, assessment of microsatellite abnormalities in urine sediments could represent an easy, accessible, and sensitive technique for the detection of MSI using these markers.

Our data revealed that alterations in all microsatellite loci were strongly associated with the TCC subtype. However, no significant relationship was found between MSI and age or smoking habit. This lack of a significant relationship between MSI and smoking can limit its use as a noninvasive technique for screening high-risk smokers. Our data in this context are consistent with Legrand et al[11]; however, Saletta et al[15] provided an evidence in their study that exposure to some components of tobacco smoke, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), induces MSI in all treated clones (12/12) of the BC cell line (RT112).

In our study, we also assessed the relationship between relevant clinicopathologic features of BC patients and the presence of MSI and/or LOH. We found a statistically significant association between advanced disease stage and the presence of *D9S171, MBP*, and *D16S476* MSI in urine, as well as between advanced disease stage and the presence of *ACTBP2* alterations in tumor samples. We also found significant association between *D16S476* MSI in urine as well as in tumor tissues and high tumor grade. These data are in concordance with those previously published by Legrand et al,[11] who found that MSI of *D9S171, MBP, ACTBP2* associated significantly with younger age of the patients, advanced disease stage, and high tumor grade in BC patients. However, Zhang et al and Migaldi et al[16,17] did not find any relationship between alterations in their tested panel of microsatellite markers and age, sex, tumor type, stage, or grade. This discrepancy in the results of different studies could be attributed, at least in part, to ethnic variability or environmental factors that affect the genetic and molecular makeup of the tested population.

Our data regarding the lack of any correlation between *D18S51* MSI in tumor tissue samples and advanced disease stage or grade are comparable to those of Cai et al,[18] but not with the results of Saidi et al,[19] who found a significant correlation between *D18S51* MSI in bladder tumor samples and histologic grade but not with the pathologic stage, recurrence, metastasis, or cancer-related death within 2-year follow-up period.

The *D16S476* is one of the MSI markers that were significantly altered in urine, indicating that loss of the chromosomal region 16q24 is important in bladder carcinogenesis in Egypt. *D16S476* loss showed a strong correlation with tumor stage and grade. Our data regarding tumor stage are in agreement with those of Szarvas et al[14]; however, they did not find any correlation with tumor grade, and they concluded that *D16S476* may be used as a prognostic marker for BC.

An interesting and alarming finding in the current study is the high number of cases showing MSI (30/30) and more importantly, that all the assessed MSI markers showed positive results. Therefore, by studying each case individually, we found a large number of aberrations reaching up to 11 MSI repeat markers per patient in the urine and up to 10 markers in tumor tissue samples. Another important finding is that some patients exhibited high expression of MSI in their urine (53.3%) compared to their tumor samples, whereas in the other patients, the expression was nearly the same between the urine and tumor samples. This discrepancy may be attributed to the underlying etiological factor(s), because the main risk factor for BC in Egypt at the time of diagnosis of these cases was urinary schistosomiasis, which was more frequent in Upper Egypt, and its prevalence decreased when going north.[20] Despite control of schistosomiasis, its effect on BC needs time to be resolved.[5] Though this study was done on 30 patients only, however, the data were confirmed by assessing it in blood, urine, and tumor tissue of each patient. These results confirm our hypothesis that urine can represent a source for detection of MSI, and accordingly for early detection of BC. Therefore, it supports the role of liquid biopsy in the management of BC cases.

A previous study from Hungary[14] showed that *IFN-a* represents a sensitive and specific biomarker that can help in the diagnosis of BC through assessment of *IFN-a* MSI, and/or LOH in the urine. *IFN-a* LOH was present in 46% of the cases and achieved sensitivity 57% when combined with *D4S243* (4q32). The diagnostic rate could be increased to 64% if *D16S476* (16q24) was added. Our results in this context showed no concordance between urine and tissue samples regarding *IFN-a*, and thus it may not be useful (should not be recommended) for diagnosing BC in Egyptian patients.

Regarding the diagnostic power of our tested MSI markers, we found that out of the 13 tested markers, only *D16S476, D9S171, FGA, and ACTBP2* showed significant concordance between urine and tumor tissue samples. *D9S171* and *D16S476* each showed 60.0% sensitivity, 80.0% specificity, 81.8% PPV, 57.1% NPV, and 68.0% accuracy. Additionally, the sensitivity, specificity, PPV, and NPV are 80.0%, 75.0%, 82.8%, and 71.4%, respectively, when we combine *D16S476 + D9S171,* and if we add *FGA* and *ACTBP2,* this could increase the sensitivity to 96.7%; however, the specificity would decrease to 30%. Therefore, we recommend *D16S476 + D9S171* to be used as a reliable and non-invasive microsatellite marker combination for the diagnosis of BC in patients' urine; however, this needed to be validated in further studies including higher numbers of patients.

Conclusion

Microsatellite repeat markers *D16S476, MJD, ACTBP2*, *and D9S171* could be used to assess patients with BC using a noninvasive technique. This technique has a high sensitivity and specificity for the early detection and diagnosis of BC in urine as well as in tissue samples. Additionally, these markers could also be used as predictive biomarkers for BC patients, although this needs to be confirmed in a validation set with a greater number of patients.

Compliance with ethical standards

Authors declare that there is no conflict of interest regarding this manuscript.

The study was approved by the ethical committees of the National Cancer Institute, Cairo University which was in accordance with 2011 Declaration of Helsinki. A written informed consent was obtained from each patient before enrollment in the study.

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