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RESEARCH ARTICLE

Peripheral blood expression of matrix metalloproteinases -2, -9 and tissue inhibitor of matrix metalloproteinase -2 as molecular markers for diagnosis and staging of bladder cancer in Egypt.

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Abstract

Some of the best suggested biological markers in bladder cancer are the MMPs, which promote invasion, immune escape and metastasis. We investigated the expression of MMP-2, -9 and TIMP-2 in peripheral blood cells of BC patients to evaluate their role in diagnosis and molecular staging of the disease. Total RNA was extracted from circulating blood cells in 70 subjects (35 healthy controls, 35 patients with bladder cancer in different stages) and expression of MMP-9, MMP-2 and TIMP-2 by real-time RT-PCR was performed. Our results revealed that the relative expression levels of MMP-2 and MMP-9 in BC patients were higher and TIMP-2 was lower than matched controls. The sensitivity of MMP-2, MMP-9 and TIMP-2 in diagnosis of BC was 94.29%, 77.14% and 82.86% respectively. Combining of MMP-2 and MMP-9 resulted in improving the sensitivity. There were increasing trends of the relative expression of MMP-2 and MMP-9 with advancement of grades and stages. In conclusion the single bladder cancer diagnostics marker with the highest sensitivity is MMP-2 and the best two markers combination is MMP-2 and MMP-9. The MMP-2 expression level is the best molecular marker in bladder cancer grading and staging. TIMP-2 is an indicator of advanced BC grade and/ or stage.

INTRODUCTION

Bladder cancer (BC) is ranking as the fourth most common cancer worldwide and the eighth leading cause of cancer death among men ***(Kang et al., 2014)***. In Egypt, it is the first site of incidence for males accounting for 17% of all cancer cases; while in females it accounts for 5%, ranking first among male cancer with a male/female ratio of 3.5:1 ***(El-Sharkawi et al., 2014).*** BC is considered a chronic disease as it has high recurrence rate, propensity to progress and metastasize, so requiring long-term follow-up and treatment ***(van Rhijn et al., 2009).***

Egypt showed changing trends of BC incidence due to the control of schistosomal infection, with an increase in median age of diagnosis from 47.4 years to 60.5 years and with a decrease in squamous cell carcinoma. Transitional cell carcinoma (TCC) is the commonest form of cancer in low eggs positivity condition ***(Gouda et al., 2007).***

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that have a similar structure and have the capacity to degrade virtually every component of the extracellular matrix ***(Sbardella et al., 2012).*** MMPs are regulated at the levels of gene transcription, post-translational modifications ***(Fontana et al.,***

2012). Also MMPs are inhibited by a group of structurally related, naturally occurred inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) ***(Rachel & Sirisha, 2014).***

In neoplastic diseases, the imbalance between MMPs and their inhibitors, resulting in increased their degradative activity, which is assumed to be linked to the invasive character of tumor cells ***(Łukaszewicz-Zajac et al., 2014)***. The expression of MMP-9 and MMP-2 has been implicated in the development and progression of many cancers ***(Reis et al., 2012).***

We investigated the expression of MMP-2, -9 and TIMP-2 in peripheral blood cells of BC patients to evaluate their role in diagnosis and molecular staging of the disease.

Subjects and methods

Study design and sample selection

This is a case control study carried out in Clinical Pathology and Urology Departments, Zagazig University Hospitals. The study included 70 subjects; 35 apparently healthy subjects as a control group and 35 patients newly diagnosed as TCC of bladder by cystoscopy and histopathological examination of the biopsy. Both groups are matched in age and sex. Patients with BC on follow up, with cancer from other origins or with liver disease as well as non Egyptian patients were excluded from the study. The study protocol was approved by the institutional ethics committee and written informed consent was obtained from all participants.

All patients were graded according to the World Health Organization (WHO) ***(Mostofi et al, 1973)*** and were staged according to the American Joint Committee on Cancer (AJCC) criteria (TNM staging system; primary tumor, nodes, and distant metastasis) ***(Edge et al., 2010)***. The demographic and clinical characteristics of the studied groups are described in table 1.

Table 1. Demographic and clinical characteristics of the studied groups

Parameter	**Control (No. = 35)**	**Bladder cancer (No. = 35)**	***p***
Age (years) [#]	64.6 ± 8.4	67 ± 10.2	0.26
Sex Male/female	30/5 (85.7/14.3)	27/8 (77.1/22.9)	0.36
Smoking history	14 (40 %)	26 (74.3%)	0.008*
Bilharziasis history	2(5.7%)	3 (8.6 %)	0.39
Family history of carcinoma	2 (5.7%)	4 (11.4%)	0.16
Tumor grade (G)**:**			
G1	—	10 (28.6%)	
G2	—	15 (42.8%)	
G3	—	10 (28.6%)	
TNM Staging:			
Stage 0a	—	8 (22.9%)	
Stage 1	—	8 (22.9%)	
Stage 2	—	6 (17.1%)	
Stage 3	—	4 (11.4%)	
Stage 4	—	9 (25.7%)	

No.: number of subjects

Data are presented as No. (%) or mean ± SD[#]

** Significant*

RNA isolation, cDNA preparation:

Total RNA was extracted from EDTA peripheral blood samples by using the Total RNA Purification kit (Jena Bioscience, Germany) following the manufacturer's protocol. The reverse transcription (RT) was done using the SCRIPT Reverse Transcriptase kit (Jena Bioscience, Germany). RNA was reverse-transcribed to first strand cDNA by using Oligo (dT) primer and M-MLV RT enzyme. On ice 1.5 µl RNase-free water, 10 µl RNA template and 1 µl Oligo-(dT) primer were mixed together, then 4 µl SCRIPT RT buffer, 1 µl dNTP Mix, 1 µl RNase inhibitor, 1 µl Dithiothreitol stock solution and 0.5 µl SCRIPT reverse transcriptase were added and incubated at 30°C for 10 min and 50°C for 60 min. The cDNA was stored at -20°C till analysis.

Quantitative Real-time RT-PCR

Quantitative real-time RT-PCR for MMP-2, MMP-9 and TIMP-2 were performed on a Stratagene Mx3005P qPCR System (Agilent Technologies, Germany) using the qPCR GreenMaster (Jena Bioscience, Germany). PCR reaction with 20 µl final volume was prepared by adding 10 µl qPCR Green Master**,** 0.5 µl forward primer **(**10 µM)**,** 0.5 µl reverse primer (10 µM) (Table 2)**,** 5 µl template cDNA and 4 µl PCR grade water into real-time PCR wells. The cycling conditions were 95 º C for 10 min, 40 cycles (95 º C for 15 sec, 58 º C for 1 min). The transcription levels of target genes were normalized to those of ribosomal protein S18 (RPS18) which used as reference gene. The normalized quantity of the target gene was obtained by subtracting cycle threshold (CT) for RPS18 from the CT for the target gene (ΔCT sample). The same calculation was performed with controls (ΔCT control). Then ΔΔCT was calculated as the difference of these values (ΔΔCT = ΔCT sample – ΔCT control). Finally, the relative expression was expressed as fold change by $2^{-\Delta\Delta CT}$ relative to control ***(Livak & Schmittgen, 2001).***

Table 2: Primers Sequences

	Forward primer	**Reverse primer**
MMP-2	5'-CCGCAGTGACGGAAAGATGT-3'	5'-CACTTGCGGTCGTCATCGTA-3'
MMP-9	5'-GGACGATGCCTGCAACGT-3'	5'-CAAATACAGCTGGTTCCCAATCT-3'
TIMP-2	5'-AGCATTTGACCCAGAGTGGAA-3'	5'-CCAAAGGAAAGACCTGAAGGA-3'
RPS18	5'- TAGCCTTTGCCATCACTGCC-3'	5'-CATGAGCATATCTTCGGCCC-3'

Obtained from the BLAST program (http://www.ncbi.nlm.nih.gov)

Statistical analysis

The sample size was calculated with 80% statistical power and 95% confidence interval (CI). Shapiro-Wilk test was used to verify the normality of distribution of continuous variables. Data are presented as mean ± SD or mean ± SEM for continuous variables, frequency and percentage for categorical ones. Comparisons between quantitative variables were done using independent sample t test or Mann Whitney U test when appropriate. For comparing categorical data chi-squared test was performed. Receiver operating characteristic (ROC) analysis, area under curve (AUC) and 95% CI were used to determine the optimum cutoff value (ΔCT) for the studied diagnostic markers and their diagnostic efficiencies. The ROC-AUC is used as a measure of diagnostic accuracy such that values from 0.5 to 0.7 indicate low accuracy; values from 0.7 to 0.9 indicate moderate accuracy and high accuracy indicated with values more than 0.9 ***(Swets, 1988)***. Analyses of data were performed using SPSS 17.0 (SPSS, Chicago, IL, USA) and statistical significance was considered at $p < 0.05$.

Results

In the peripheral blood of BC patients the relative expression of MMP-2 and MMP-9 were significantly higher in cancer patients than controls *(p<0.001)*, but the level of TIMP-2 were significantly lower in BC patients than controls *(p<0.001)* (figure1).

Figure 2 showed ROC-AUC of MMP-2, MMP-9 and TIMP-2 in diagnosis of BC. MMP-2 ROC-AUC had high accuracy, while MMP-9 and TIMP-2 both had moderate accuracy. The diagnostic performance criteria of individual molecular marker were demonstrated in table 3. The best two markers combination was MMP-2 and MMP-9 which gave 100% sensitivity, 88.6% specificity, 83.3% positive predictive value (PPV) and 100% negative predictive value (NPV) in detection of BC.

The association between the relative expression of MMP-2, MMP-9 and TIMP-2 and the histopathological grading of BC are showed in figure 3. There were increasing trends of the relative expression of MMP-2 and MMP-9 with advancement of grades, while TIMP-2 relative expression was significantly decreased in all grades compared to control and significantly increased in G3 compared to G2.

Regarding TNM staging system, the relative expression level of MMP-2 and MMP-9 showed an increasing trend of expression in the more advanced stages of cancer. However, the relative expression level of TIMP-2 significantly decreased in all stages of cancer when compared with controls, and there was a significant increase in its relative expression in stage 4 when compared to stage 3 (figure 4).

Table 3. The diagnostic performance of the MMP-2, MMP-9 and TIMP-2 in bladder cancer

Markers	Cutoff (ΔCT)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MMP-2	< 4.08	94.29	94.29	94.29	94.29
MMP-9	< 6.69	77.14	94.29	93.1	80.49
TIMP-2	> 5.85	82.86	85.71	85.29	83.33

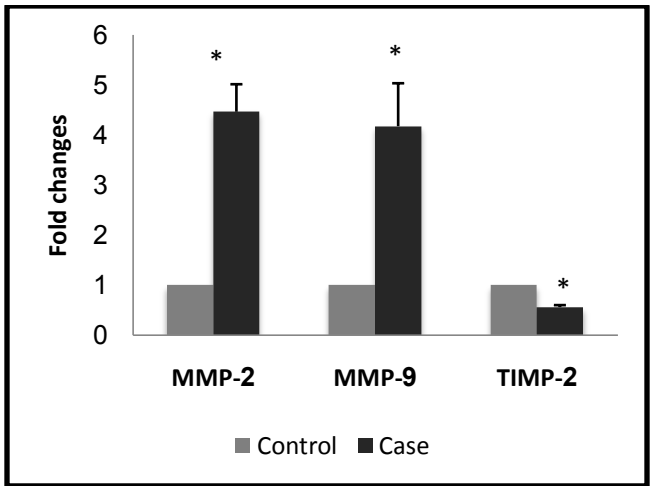


Figure 1. Relative expression of MMP-2, MMP-9 and TIMP-2 (mean ±SEM) in BC patients compared to controls. * Significant when compared to control.

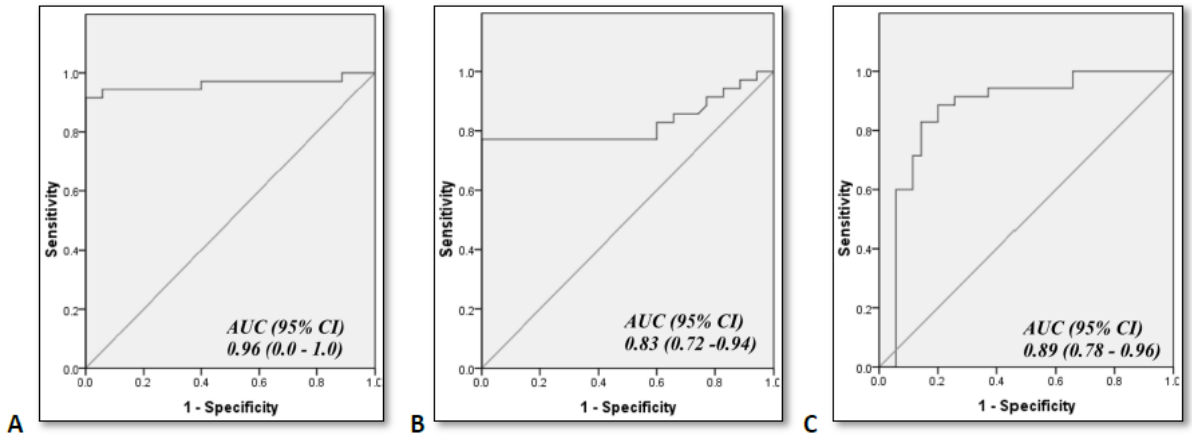
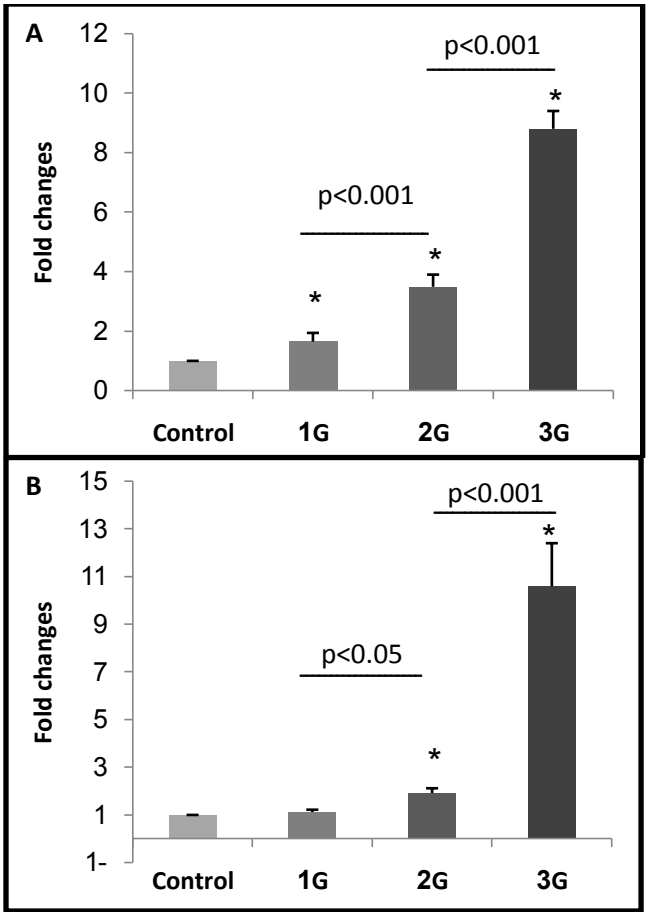


Figure 2. ROC curves of (A) MMP-2, (B) MMP-9 and (C) TIMP-2 expression



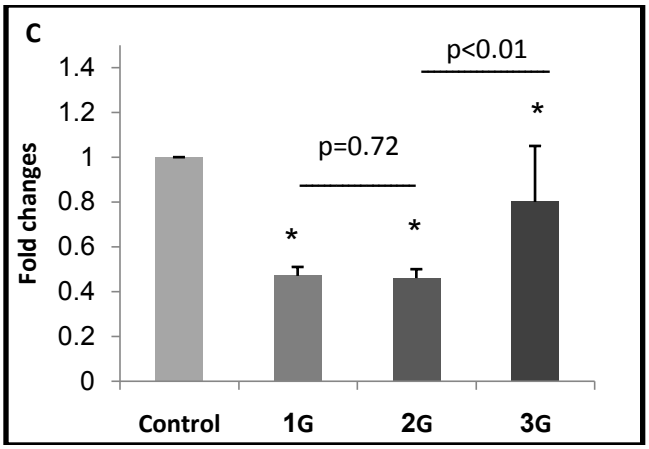
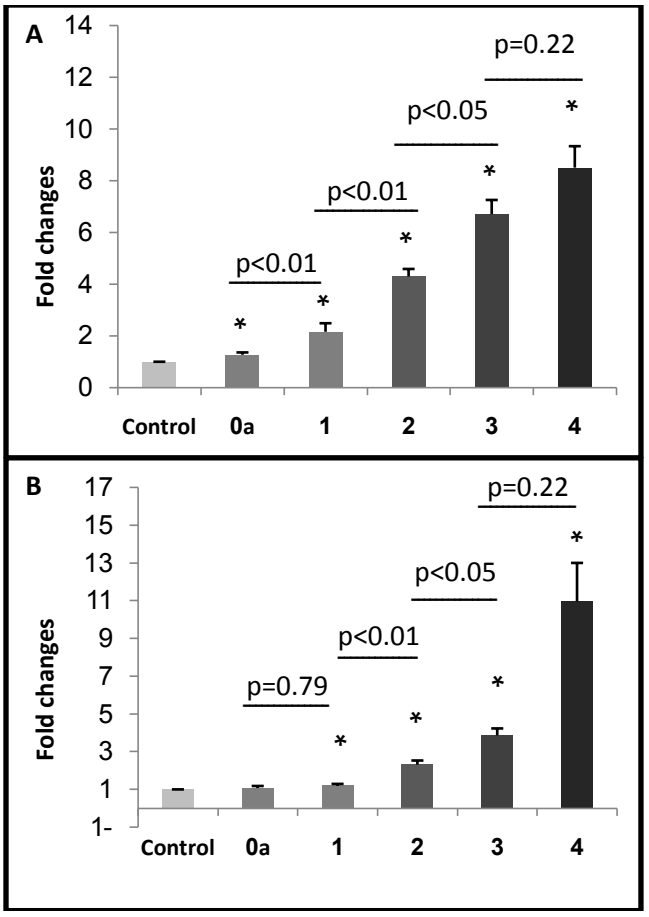


Figure 3. Relative expression of (A) MMP-2, (B) MMP-9 and (C) TIMP-2 (mean ±SEM) in BC patients with different grades.
** Significant when compared to control.*



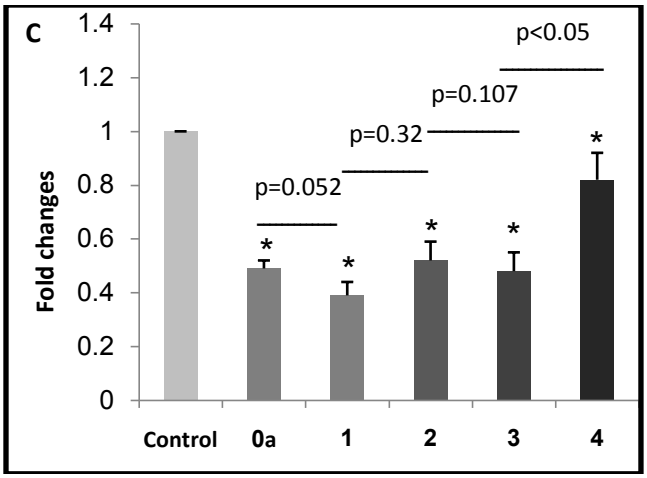


Figure 4.Relative expression of (A) MMP-2, (B) MMP-9 and (C) TIMP-2 (mean ±SEM) in BC patients with different TNM stages.
** Significant when compared to control.*

Discussion

Cystoscopic evaluation forms the basis of BC diagnosis and staging. But cystoscopy still has some limitations; for example, it is invasive, time-consuming, and expensive, requires sedation or anesthesia, and sometimes leads to iatrogenic injury. All this makes cystoscopy unsuitable for screening ***(Chen et al., 2014).*** This calls for searching of markers for screening of bladder cancer. This marker should be sensitive, specific, non invasive and with acceptable cost.

A balance between MMPs and TIMPs activities is a prerequisite for the normal function of many physiological processes. Disruption of the MMP-TIMP balance can result in a number of pathogenic processes including tumor invasion, metastasis and angiogenesis ***(Chirco et al., 2006)***. Despite the pathogenetic impact of MMPs and TIMPs in BC, only a limited number of studies are available. MMP-2, MMP-9, and TIMP-2 are the most frequently investigated MMPs and inhibitors in BC ***(Staack et al., 2006).***

In the present study, the expression levels of MMP-2 and MMP-9 in the peripheral blood were significantly higher in the BC patients than healthy controls. This result confirms the previous result of ***Angulo et al. (2011)***. Over expression of MMP-2 and MMP-9 in the BC tissue was also reported ***(Wallard et al., 2006)***. While, ***Reis et al. (2012)*** reported, in BC tissue, that MMP-2 was underexpressed, but MMP-9 was overexpressed. Other investigators examined their protein levels in the serum by ELISA. They reported that MMP-2 ***(Gohji et al., 1996*** and ***El Baz et al., 2009)*** and MMP-9 ***(Guan et al., 2003; Gunes et al., 2013*** and ***Ramón de Fata et al., 2013)*** significantly increased in BC when compared to healthy controls.

Our results revealed that the expression level of TIMP-2 in the peripheral blood was significantly lower in the BC patients when compared with healthy controls. ***Angulo et al. (2011)*** reported no significant difference in the expression level of TIMP-2 in the peripheral blood between the BC patients and the healthy controls. There are controversial findings regarding the tissue expression of TIMP-2 in BC. One study reported its underexpression ***(Reis et al., 2012)*** while others reported its overexpression ***(Wallard et al., 2006*** and ***Wang et al., 2011)***. At the protein level, TIMP-2 was reported to be significantly decreased in BC patients ***(Staack et al., 2006; Vasala & Turpeenniemi-Hujanen, 2007*** and ***El Baz et al., 2009).*** While ***Ramón de Fata et al. (2013)*** reported it was significantly increased in BC patients.

By studying the diagnostic performance of the three molecular markers in BC, the best single indicator with the highest sensitivity was MMP-2. Combining of MMP-2 and MMP-9 resulted in improving the sensitivity from 94.3% to 100%. Others examined the diagnostic performance of MMPs and TIMPs proteins in the serum, ***Staack et al., (2006)*** found the single indicators with the highest sensitivity and specificity was MMP-2, but the best two-

marker combination included MMP-9 and TIMP-1which results in improving both the sensitivity and the specificity. While ***Ramón de Fata et al. (2013)*** reported the single indicators with the highest sensitivity and specificity was MMP-9, they recommended the use of MMP-9/TIMP-2 ratio to improve the sensitivity.

We investigated the relationship between the studied molecular markers and tumor grade. The expression levels of MMP-2 and MMP-9 showed significantly increasing trend with grades. Similar results were previously reported for MMP-2 (higher expression in G3 and G2 compared to G1) but not for MMP-9 ***(Angulo et al., 2011)***. In BC tissue, the same results were reported for MMP-2 expression (***Wallard et al., 2006*** and ***Wang et al., 2011)*** but not for MMP-9 expression ***(Wang et al., 2011)***. TIMP-2 expression level was significantly higher in G3 when compared with G2 with no significant difference between G1 and G2. In contrast ***Angulo et al. (2011)*** found that no significant difference in TIMP-2 expression with different grades. Studies on BC tissue expression of TIMP-2 revealed that it was higher in G1 when compared with G2-3 ***(Wang et al., 2011)***, or not correlated with grades ***(Wallard et al., 2006).***

To our knowledge, this is the first study examining the association between peripheral blood expression of MMP-2, MMP-9, TIMP-2 and TNM staging of cancer bladder. The best marker for TNM molecular staging was MMP-2. MMP-2 was significantly increased with TNM stages except in stage 4. MMP-9 was not significantly higher in the early 0a stage than control or in the late stage 4 than stage 3, so it had a limited value in BC staging. Although TIMP-2 expression was significantly lower in different BC stages than control, it was only significantly higher in stage 4 than stage 3. Elevated TIMP levels are reported in association with cancer progression and identified as poor prognostic indicators in several human tumor types, as TIMPs have anti apoptotic and growth stimulatory effect ***(Jiang et al., 2002). Angulo et al., (2011)***, the only report on the peripheral blood expression of the same three molecular markers in BC, studied their association with clinical staging using T and M. They reported an increasing trend of MMP-2 but not MMP-9 or TIMP-2 with increased clinical staging.

Conclusion

Our results support the role of MMP-2, MMP-9 and TIMP-2 as non invasive screening tests in the diagnosis of BC. The best single indicator with the highest sensitivity was MMP-2; combining of MMP-2 and MMP-9 resulted in improving the sensitivity. The MMP-2 expression level is the best marker in BC grading and staging. TIMP-2 is an indicator of advanced BC grade and/ or stage.

Further studies on a large number of participants are recommended to confirm their role in early diagnosis and to evaluate its possible value in grading and staging of bladder cancer patients.

Conflict of interest

The authors have no conflict of interest to declare.

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