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Research Article

Target genes expression biomarkers in Egyptian bladder cancer patients

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ABSTRACT

Introduction: Bladder cancer (BC) is a common urologic cancer. Early diagnosis of bladder cancer is critical to improving patient outcomes. Researchers have begun a search for an appropriate marker suitable for the screening of the bladder cancer, which should be specific, sensitive, reproducible, non-invasive and at an acceptable cost. To find the genes that are expressed differently in BC is one of the best ways to create new biomarkers and therapeutic targets.**Aim**: The aim of this study was to use a reverse-transcriptase polymerase chain (RT-PCR) assay for the detection of the genes that are overexpressed in the peripheral blood and tissue samples of bladder cancer and compared to normal bladder tissues and blood. **Subjects and Methods:**One hundred sixty individuals were included in this study and divided into two groups; group I : included eighty patients with bladder cancer were studied in both bladder tissue biopsies and blood sample,80 non-tumor tissue biopsies were taken from those 80 cases as a control and group II: include eightyhealthy control. A quantitative real-time (RT-PCR) assay was used to detect several genes expression which include cytokeratin 19 (CK19), cytokeratin 20 (CK20), survivin, epidermal growth factor receptor (EGFR) and human telomerase reverse transcriptase (hTERT) and Humanin (H) in the peripheral blood and tissue samples of bladder cancer and compared to normal bladder tissues.**Results**: For all parameters, the malignant group was significantly different from the control groups. There is a significant positive correlation between mRNA level expression of CK20, CK19 survivin, hTERT and H 6 in blood and tissue of the same patient. There is a significant increase in each of Ck19, CK20, Survivin, EGFR and H6 expression in blood and tissue with advancing tumors [grade G3] as compared to low-grade cancers [G1 and G2] ($P \leq 0.05$). The expression levels of the Ck19, CK20, Survivin, EGFR and H6 in blood and tissue were significantly lower in the non-invasive tumors [pTa/pTI] than in the invasive tumors [pT2/pT3] ($P \leq 0.05$). Concerning the mRNA of H10 expression in blood was higher in the invasive group than in the non-invasive group ($P \leq 0.05$).

Conclusion: The present study identified some genes that can be used as molecular biomarkers or target genes in Egyptian bladder cancer patients.

Keywords: Bladder carcinoma; real-time RT-PCR, biomarkers, Cytokeratin 19, cytokeratin 20, Telomerase activity, Disease progression, Sensitivity; Specificity, Apoptosis, diagnosis, and Humanin.

1- INTRODUCTION

Bladder cancer is the most commonly diagnosed cancers and a significant source of morbidity and mortality throughout the world **(Abdul-Maksoud et al., 2016).** Moreover, it is responsible for about 7% of all malignant diseases among Egyptians and the incidence rate of cancer bladder that ranked next to liver cancer in Egyptian males is also high (10.7%)

(Ibrahim et al.,2014). Standard procedure for the diagnosis of bladder cancer includes cystoscopy combined with the cytological examination. Cystoscopy is high- cost method, and is considered the gold standard for detection and monitoring of bladder tumors, and with a sensitivity of 70%, while the cytology is aa low-sensitive way. Because bladder cancer has a high rate of recurrence and long-term monitoring is a necessity, there is a need for abetter diagnostic tool **(Mbeutcha et al., 2016).** The principle for using Real-time polymerase chain reaction (RT-PCR) assays for detection of circulating tumor cells are based on that malignant cells are detached from the primary tumor and distributed to hematopoietic or lymphatic tissue**(Alix-Panabieres and Pantel, 2013).**Cytokeratin (CKs) are proteins of the cytoskeleton of theepithelial cell. Different types of CK can be over- expressed in different epithelium, so we can find specific CKs in patients with bladder cancer **(Huang et al., 2015).** They can be detected by ELISA or by RT-PCR. Their usefulness is under additional studies. Another protein currently being researched in the detection of bladder cancer is survivin, a member of the inhibitor of apoptosis protein family. Survivin inhibits apoptosis by directly inhibiting caspase-3 and caspase-7 **(Huang et al., 2015).** Survivin is expressed during embryonic and fetal development is undetectable in normal adult tissue and is abundantly expressed in a variety of human carcinomas **(Mull et al.,2014)**. Over-expression of survivin in bladder tissue is associated with aggressive disease, resistance to therapy, and poor clinical outcome in patients **(Zhang et al., 2016).** Telomeres are repetitive sequences that cap the terminal ends of eukaryotic chromosomes. During each cell division, telomeres decrease in size. This leads to chromosomal instability and, eventually, to cell death. Telomerase is ribonucleoprotein enzyme that helps assemble telomeres, thus preserving the ends of chromosomes **(O'Sullivan and Karlseder, 2010).** Epidermal growth factor family gene expression can reliably be studied at the mRNA level, and based on a number of studies the expression is likely to reflect the presence of the corresponding protein **(Arfaoui al., 2016).**Humanin (HN) is an identified endogenous peptide that has anti-apoptotic activity and suppresses apoptosis caused by various stimuli, e.g., serum deprivation and UV irradiation **(Yen et al., 2013).** The mechanisms for cytoprotective effects of HN through its antiapoptotic, metabolic (improvement of the mitochondrial bioactivity) and anti-inflammatory effects. Moreover, consider the effects of antiapoptotic of HN through its binding to Bax, a Bcl-2 family pro-apoptotic protein; HN can hide pro-apoptotic effects of chemotherapy agents **(Mottaghi-Dastjerdin et al., 2014).**

2. Subjects and Method

2.1. Study subjects: One hundred sixtyindividuals were included in this study and divided into two groups; group I : included 80 patients with bladder cancer were studied in both bladder tissue biopsies and blood sample and 80 normal mucosa specimens were taken from those 80 cases as a control and group II: include 80 healthy control. Patients were treated and followed up at TantaHospital of the University, Egypt during the periodfrom January2015 to September 2016. Written, informed consent was obtained from each patient before enrollment into the study. Tissue samples were obtained by transurethral resection of the bladder tumors whichcollected and dissected under stringent sterile conditions to prevent RNA contamination and immediately frozen in liquid nitrogen.

2.2. Patients inclusion criteria: Patients that participated in the study fulfilled the inclusion criteria included: age more than 18 years, bladder biopsy showing histological evidence of bladder cancerusing the TNM staging system by the seventh edition of the American Joint Committee on Cancer (AJCC) **(Edge S.B et al., 2010)**, and accordinglystandard treatment protocols and follow-up schedulesbased on published guidelines were selected**(NCCN V.2. 2016)**. Toxicity was gradedaccording to the National Cancer Institute CommonTerminology Criteria for Adverse Events**(CTC, 2006)**

2.3. Patients exclusion criteria: The presences of any uncontrolled chronic systemic illness were excluded from this study.

2.4. RNA Extraction and detection: For all the specimens collected from all groups, the following was carried out at thefaculty of pharmacy (girls), Al-Azhar University:

a. Extraction of mRNA: Total RNA was extracted from frozen (−80°C) bladder specimens normal and tumor cells and also from blood samples using a commercial kit (RNeasy MiniKit; Qiagen Austin, Texas USA) (Cat. nos. 74104 and 74106), according to the manufacturer's protocol. RNA was quantified spectrophotometrically at 260 nm and its quality was checked by electrophoresis through denaturing 1.5% agarose gels.

b. cDNA synthesis.: Reverse Transcription and PCR were carried out by a single-tube, single step Ready-to-Go RT-PCR Beads (Qiagen Sciences, Maryland, U.S.A.), which utilize Moloney Murine Leukemia Virus (MMuLV) reverse transcriptase to synthesize first strand cDNA from RNA. For each of the cDNAs to be amplified: keratin 19 telomerase, keratin 20, survivin, EGFR, and Humanin; a pair of primers were used as forward and reverse primers, their sequences are shown in Table (1). The sequences of the nine primer pairs were tested on NCBI Nucleotide Blast (http://blast.ncbi.nlm.nih.gov/blast) to confirm that they were specific for each target.Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to evaluate the performance of cDNA synthesis (housekeeping gene).

2.4.1. Tissue/blood quantitative PCR conditions: Amplifications were performed by using a 25 µL total reaction volume contains 25 pmol of each primer, 3 µL of cDNA template, 12.5 µL 2× SYBR Green Rox Master Mix (Qiagen Sciences, Maryland, USA) and adjusted volume by nuclease-free water. The plate was inserted in real-time thermal cycler (Step One Plus™ Instrument, Applied Biosystem, California, and USA). For each sample, the procedure was carried out in triplicate. Each amplification was performed for 40 cycles; a cycle profile consisted of denaturation at 94 °C for 20 s, annealing at 58 °C for 30 s, extension at 60 °C for 30 s. The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ method **(Livak and Schmittgen, 2001).**

2.4.2. Electrophoresis of PCR products. RT-PCR products were separated by electrophoresis on 2% agarose gel (ethidium bromide stained) prepared in Tris- Acetate EDTA electrophoresis buffer.

Table (1). Sequences of the assay primers.

Gene	Gene name	Product size	Sequence
CK19	keratin 19	159 bp	F:GAGCATGAAAGCTGCCTTGG
			R:GCTCACTATCAGCTCGCACA
CK20	keratin 20	253 bp	F:ACACGGTGAACTATGGGAGC
			R:GCGTTGGTTTCGTACCACTG
EGFR	epidermal growth factor receptor	480 bp	F:AACCCCGAGGGCAAATACAG
			R:GAGATCGCCACTGATGGAGG
Survivin	Baculoviral inhibitor of apoptosis	258 bp	F:AGACTTGGCCCAGTGGGTTT
			R:TGACAGAAAGGAAAGCGCAAC
hTERT	Telomerase reverse transcriptase	145 bp	F:CAAGCTGTTTGCGGGGATTC
			R:GGGCATAGCTGAGGAAGGTT
			R: AGGTTTCCTCTGGTCCTGGT
H1	Humanin 1	206□bp	F:CGCAGGCCCTAAACTACCAG
			R:TGCTACTGTCGATGTGGACC
H3	Humanin 3	164□bp	F:GGTGATAGCTGGCTGGCTTA
			R:ATTAGTGGCTGCTTTTGGGC
H6	Humanin 6	247□bp	F: TTTACCCAGGCGCAGTGGAC
			R:GGCTCAGTAGGCTTATCACCAC

H10	**Humanin 10**	**137□bp**	**F:CGAGAAGACCCTATGTATGGAGC**
			R: AGGTTGCTCGGAGGTTGAAT
			R: GATTCCTGGAAGGTGGTCAA
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**	**471 bp**	**F: CCA TGG AGA AGG CTG GGG**
			R: CAA AGT TGT CAT GGA TGA CC

2.5. Statistical analysis: All data were analyzed using Statistical Package for the Social Sciences software (SPSS v 15, USA). The results were expressed as the mean ± S.D. Differences between values were statistically analyzed using a Student's *t*-test. Categorical variables were compared by Pearson correlation analysis. The statistical significance was set at P□<□0.05.

1. RESULTS

3.1. Patient characteristics**:** The mean ages of the patients were55.3±9.1 years (range, 43-65 years) in Group-1 and 54.1±6.9 years (range, 44-66 years) in Group-2 (p = 0.24).Group-1 was composed of 16 females (20%) and 64 males (80%), and Group-2 was composed of 10females (12.5%) and 70males (87.5%) (p = 0.35). The history of the smoking variable was significantly higher in patients with bladder cancer (p = 0.01). The frequencies of bilharzia in patients with bladder cancer were (42.3%). Histopathologic examination of bladder carcinoma tissues revealed: transitional cell carcinoma (TCC) in 54 cases (67.5%) and squamous cell carcinoma (SCC) in 26 cases (32.5%). Regarding the tumor stage, the frequencies of non-muscle (Ta-T1) - and muscle-invasive tumors (T2-T3) were 35% and 65%, respectively. In terms of tumor grade, the frequencies of G1, G2, and G3 were 26.6%, 28.4%, and 45.0%, respectively.The non-muscle invasive patients received intravesical BCG. Onlytwenty four patients underwent radical cystectomy and the remaining twenty eight cases prefer bladder- preserving approaches.

3.2 Quantitative RT-PCR analysis of candidate bladder cancer markers in the blood which represent in figure 1 and in tissue which represents in figure 2.

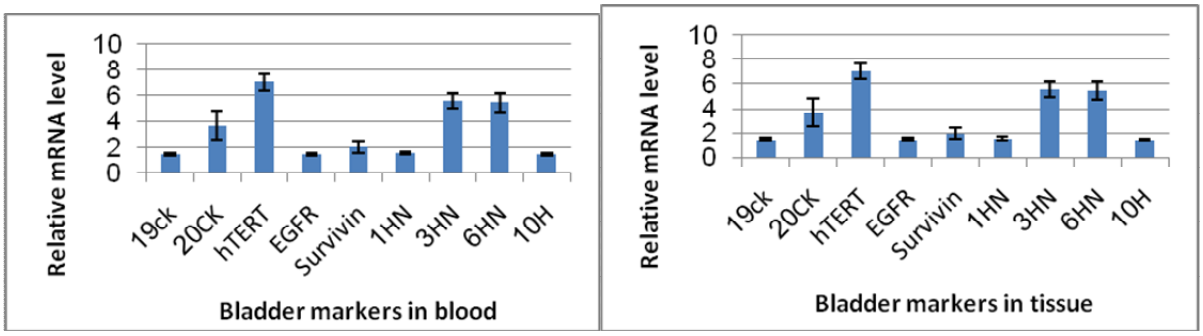


Fig. 1: represent relative mRNA expressions ofcandidate bladder cancer markers in blood

Fig. (2): represent relative mRNA expressions ofcandidate bladder cancer markers in tissue

Table 2. The expression level of candidate bladder cancer markers in relation to Gender

Biomarker	**the expression level of candidate markers in tissue**			**the expression level of candidate markers in Blood**		
	Male	**Female**	**p**	**Male**	**Female**	**P**
K19	1.69±0.22	1.66±0.25	0.70	1.51±0.08	1.49±0.08	0.49
K20	7.86±1.81	6.70±2.48	0.17	3.72±1.25	3.54±0.69	0.708
hTERT	3.9±0.43	3.77±0.37	0.41	7.16±0.66	6.87±0.63	0.294
EGFR	3.33±0.80	2.94±0.68	0.22	1.50±0.10	1.47±0.13	0.607
Survivin	3.27±0.76	2.82±0.54	0.13	2.04±0.48	1.99±0.42	0.785
H 1	1.96±0.44	2.1±0.24	0.40	1.61±0.15	1.56±0.05	-0.507
H 3	1.44±0.14	1.49±0.08	0.32	6.09±1.03	6.18±0.55	0.815
H 6	5.39±1.53	5.07±1.36	0.61	5.50±0.86	5.48±0.25	0.943
H 10	1.48±0.12	1.52±0.06	0.34	1.48±0.10	1.45±0.11	0.425

3.3 The expression level of candidate bladder cancer markers in relation to different clinicopathological features: We found a significant increase in each of Ck19, CK20, Survivin, EGFR

and H6 expression in blood and tissue with advancing tumors [grade G3] as compared to low-grade cancers [G1 and G2] ($P \leq 0.05$). The expression levels of the Ck19, CK20, Survivin, EGFR and H6 in blood and tissue were significantly lower in the non-invasive tumors [pTa/pTI] than in the invasive tumors [pT2/pT3] ($P \leq 0.05$). Concerning the mRNA of H10 expression in blood was higher in the invasive group than in the non-invasive group ($P \leq 0.05$). Also, there was a non-significant increase of H10 expression in blood with advancing tumor grades as compared to low-grade cancer ($P > 0.05$). Lastly, we found no significant differences between expressions of candidate bladder cancer markers and the tumor pathology or the gender of patients (Table 3, 4,and5).Overexpression of genes was detected in all malignant tissues and blood samples of patients with bladder cancer.

Table 3. The expression level of candidate bladder cancer markers in relation to grade.

Biomarker	**Expression level of candidate markers in tissue**			**Expression level of candidate markers in Blood**		
	G1/G2	G3	p	G1/G2	G3	P
K19	1.52 ± 0.08	1.85 ± 0.21	0.00*	1.45±0.045	1.60±0.12	0.00*
K20	6.70 ±1.20	8.40 ± 2.32	0.01*	3.18±0.48	4.66±2.21	0.017*
hTERT	3.88 ± 0.33	3.88 ± 0.50	0.98	6.84±0.51	7.23±0.96	0.178
EGFR	2.83 ± 0.48	3.62 ± 0.83	0.004*	1.44±0.08	1.54±0.11	0.013*
Survivin	2.83 ±0.48	3.47 ± 0.80	0.014*	1.92±0.37	2.37±0.73	0.044*
H 1	1.93 ±0.28	2.06 ± 0.48	0.4	1.53±0.05	1.67±0.15	0.003*
H 3	1.43 ±0.13	1.47 ± 0.13	0.31	5.87±0.71	6.027±1.70	0.762
H 6	4.39 ±0.86	6.21 ± 1.40	0.00*	5.18±0.71	5.84±0.62	0.013*
H 10	1.48±0.10	1.49 ± 0.12	0.88	1.48±0.06	1.47±0.13	0.766

Table 4. The expression level of candidate bladder cancer markers in relation to stage

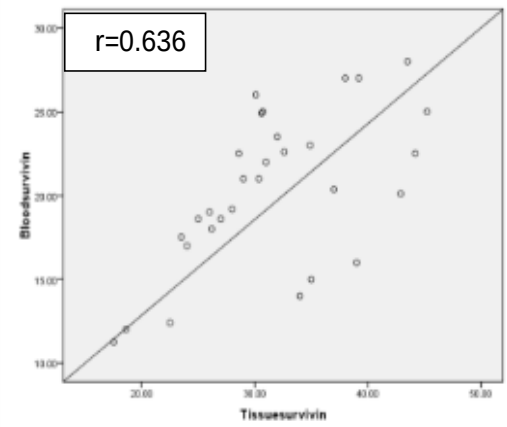
Biomarker	**Expression level of candidate markers in tissue**			**Expression level of candidate markers in Blood**		
	Ta/T1	T2/T3	P	Ta/T1	T2/T3	P
K19	1.44±0.02	1.73±0.22	0.007**	1.41±0.03	1.52±0.08	0.004**
K20	5.82±0.26	7.90±0.17	0.03*	2.41 ±0.26	4.22±1.76	0.03*
hTERT	3.96±0.04	3.86±0.45	0.64	6.72±0.17	7.10±0.84	0.32
EGFR	2.12±0.29	3.45±0.64	0.00***	1.40±0.11	1.51±0.10	0.04*
Survivin	2.12±0.29	3.36±0.6	0.00***	1.55±0.30	2.26±0.59	0.01**
H 1	1.67±0.23	2.06±0.39	0.04*	1.58±0.04	1.60±0.15	0.74
H 3	1.39±0.15	1.46±0.12	0.25	6.12±5.49	5.91±1.39	0.75
H 6	3.60±0.4	5.64±1.36	0.003**	4.56±0.74	5.68±0.60	0.001***
H 10	1.54±0.04	1.48±0.12	0.28	1.39±0.02	1.49±0.10	0.05*

Table 5. The expression level of candidate bladder cancer markers in relation to tumor pathology

Biomarker	**Expression level of candidate markers in tissue**			**Expression level of candidate markers in blood**		
	SCC	TCC	P	SCC	TCC	P
K19	1.71±0.24	1.67±0.22	0.95	1.49±0.09	1.51±0.08	0.63
K20	7.52±2.37	7.57±1.93	0.68	3.63±0.73	3.70±1.27	0.88
hTERT	3.68±0.44	3.96±0.38	0.09	7.11±0.56	7.07±0.70	0.89
EGFR	3.08±0.75	3.29±0.80	0.50	1.50±0.13	1.49±0.10	0.77
Survivin	2.99±0.71	3.22±0.74	0.45	1.95±0.55	2.06±0.42	0.54
H 1	2.11±0.42	1.95±0.38	0.30	1.58±0.08	1.61±0.15	0.60
H 3	1.49±0.10	1.43±0.13	0.26	6.13±0.95	6.10±0.93	0.93
H 6	5.41±1.25	5.26±1.58	0.79	5.57±0.54	5.46±0.82	0.71
H 10	1.54±0.06	1.46±0.12	0.09	1.47±0.13	1.47±0.09	0.92

3.3 Correlation between expression of candidate bladder cancer markers in blood and tissues:A significant association was found between the expression of CK20, CK19, survivin, EGFR, telomerase and H6 in both types of samples ($p \leq 0.05$).No statistically significant association was found between the expression of H1, H3, and H10 in both types of samples ($p > 0.05$). While it is important to point out that all of the patients who showed mRNA expression in the peripheral blood also showed simultaneous mRNA expression in the tissue which summarizes in figures3,4,5,6,7and 8.Figure 3 showed Pearson highly significant correlations between the expression levels of survivin gene in blood and tissue of

bladder cancer (r=0.636) at (p<0.001). Figure 4, showed Pearson significant correlations between the expression levels of EGFR gene in blood and tissue in bladder cancer (r=0.5) at (p=0.004). Figure 5: Pearson significant correlations between the expression levels of TERT gene in blood and tissue in bladder cancer (r=0.435) at (p=0.016). Figure 6: Pearson highly significant correlations between the expression levels of CK19 gene in blood and tissue in bladder cancer (r=0.816) at (p<0.001). Figure 7: Pearson correlations between the expression levels of CK20 gene in blood and tissue in bladder cancer (r=0.432) at (p<0.05). Figure 8: Pearson significant correlations between the expression levels of H6 gene in blood and tissue in bladder cancer (r=0.392) at (p<0.05).



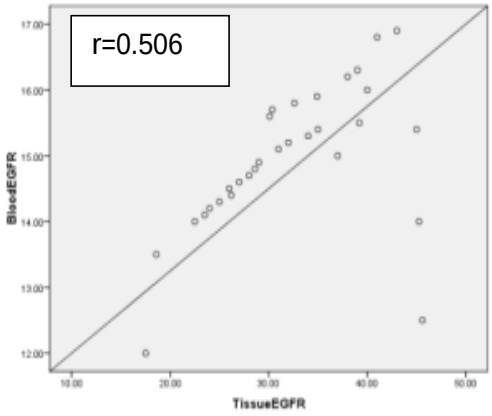
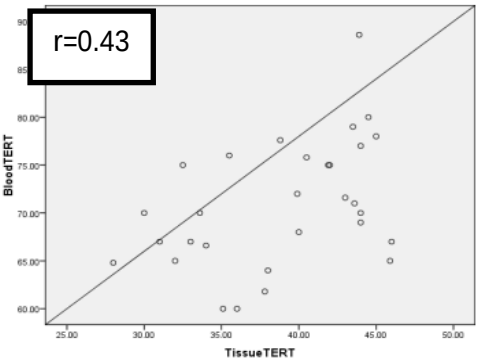


Fig. (3): Correlation between survivin in blood and tissue. Fig. (4): Correlation between EGFR in blood and tissue



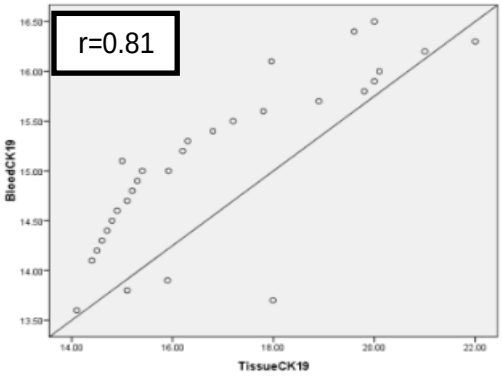
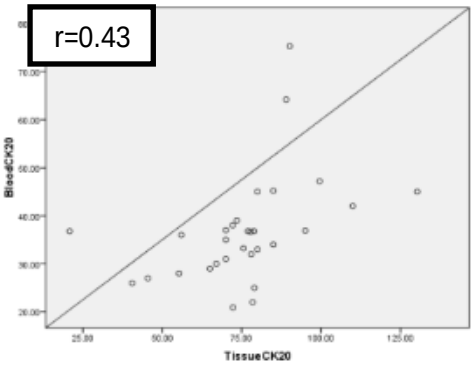


Fig. (5): Correlation between TERT in blood and tissue Fig. (6): Correlation between CK19 in blood and tissue



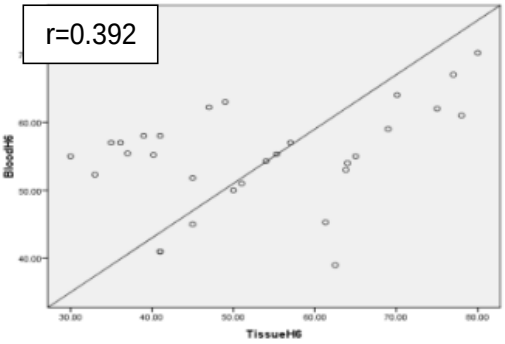


Fig. (7): Correlation between CK20 in blood and tissue Fig. (8): Correlation between H6 in blood and tissue

4. DISSCUSION

Bladder cancer has always received great attention in Egypt because of its high prevalence, with high mortality rates. Bladder cancer has displayed clinical biomarker is non-specific and difficult to diagnose**(Darwiche et al., 2015).**There is still a need for superior biomarker specific and reliable test for early diagnosis of bladder cancer.Detection of RNA

tumor markers in the blood and tissue samples represents another attractive option diagnosis. RNA isolation procedures and the stability of the polymerase chain reaction (PCR) amplicons as well as the quantity and especially the quality of the RNA of the factors that may affect the results of gene expression studies **(Romero et al., 2014).**Development of general standard operating protocol is an important one in order to compare gene expression data across studies. No biomarker-based blood tests currently in clinical practice to detect BC or surveillance. It may be the analysis of biomarkers based on blood more useful in the prognosis of bladder disease or inform therapeutic decisions **(Goodison et al., 2013).**The current study was conducted on 160 subjects (group 1 consists of 80 patients suffering from bladder cancer, Group 2 consists of 80 subjects properly matching and took the same age and socio-economic situation as a control with an average age of (55.3±9.1and 54.1±6.9years respectively). In group 1, males constituted (80%), while the percentage of females (20%) and the predominance of men in this study reflects the difference in the lifestyle of both genders in Egypt, with men more exposed to schistosomal infection, smoking, and occupational hazards known to cause bladder cancer (i.e., industries related to rubber, dye, paints, benzidine, and tobacco). Males consistently show a higher incidence of bladder cancer than females throughout the world **(Torre et al., 2015).**Included in the present study it was associated patients (36.7%) of bladder cancer with schistosomiasis. In the developing world, especially Africa and West Asia, chronic infection associated with Schistosoma hematobium (a parasitic worm that causes urinary schistosomiasis) with an increased risk of bladder cancer **(Torre et al., 2015).** In this study, the number of smokers was higher in the group of patients who suffer from bladder cancer compared with the control group. Although smoking is known to increase the risk of bladder cancer increased by 3 times, it was revealed there is no relationship between smoking and the expression levels of bladder cancer markers candidate in this study. It is estimated that smoking causes about 31 % of deaths from bladder cancer among men and 14% of deaths among women worldwide **(Freedman et al., 2011).** In malignant group, it was 35% of subjects had a non-muscle invasive disease, and 65% of the subjects had muscle invasive disease. In line with this result **(Rosser et al., 2009).**To detect the presence of tumors and assessing their importance prognostic, the present study analyzed cytokeratin 19 (CK19), cytokeratin 20 (CK20), survivin, epidermal growth factor receptor (EGFR) and human telomerase reverse transcriptase (hTERT), H 1, H 3, H 6, H 10 mRNA levels in peripheral blood and tissue samples of patients with bladder cancer using reverse transcriptase polymerase chain (RT-PCR) to identify potential roles in tumor incidence, development and prognosis of patients with bladder cancer. In this study, we measured the amount of mRNA expression level of all bladder cancer candidate markers in the blood and also in malignant and non-malignant bladder tissue from each patient. The expression is higher in malignant blood and tissues of patients who suffer from bladder cancer than in the controls. In this study, that there is a statistically significant positive correlation between the mRNA expression level of CK20, CK19 survivin, hTERT, and H 6 in the blood and tissues of the same patient ($p < 0.05$). Levels of CK20 mRNA extracted directly from the primary tumor tissues were two-fold higher compared to those from the blood. While levels of hTERT and H3 mRNA extracted from blood were 2-fold higher compared to those from tumor tissues. Moreover, because the tissue level up-regulation correlates significantly with the blood level of CK20, CK19, survivin, hTERT and H 6 expression, it indicates the prognostic utility of them as a biomarker in bladder disease predisposition, and as an important clinical target. The present study attempted to investigate whether the level of studied bladder cancer markers expression aids in defining the stage or predicting the prognosis of bladder cancer. Our findings showed that expressions of Ck19, CK20, Survivin, EGFR and H6 in both blood and tissue were significantly higher in invasive compared to that in non-invasive cancers.**CYTOKERATIN**

20:The present study showed that the highest expression of CK20 was in samples of bladder cancer was significantly associated with advanced tumor stage and grade. We can see that CK20 is the best of all the parameters assayed mRNA expression by RT-PCR with the greatest level of expression. According to previous reports, **Abdul-Maksoud et al.,** it also reported an increase in CK20 levels in biopsies **(Abdul-Maksoud et al., 2016),**while LEOTSAKOS and others found increased expression of CK20 in the blood **(LEOTSAKOS et al., 2014).**The results of this study were consistent with **Chu et al. (2000) and Bassily et al. (2000),** which showed increased expression of CK20 in the urothelium cancer. **Christophe and others. (2004)** analyzed the tissue and urine from bladder cancer patients using real-time RT-PCR and found that CK20 mRNA expressed in all tissues and urine. **Schmidt and others. (2016)** compared the relative CK20 transcript levels in tumor tissue and tumor-free and found a significant overexpression. Extensive studies found that quantitative PCR detection of CK20 was an important biomarker that can be used to determineBC in urine cytology smears (**Inoue et al., 2001; Retz et al., 2003; Morsi et al., 2006; Guo et al., 2009).**Recently, the use of cytokeratin 20 markers confirmed bladder cancer (**Schmidt et al., 2016; Poli et al., 2013). Mai et al. (2013)** reported a correlation of CK20 with the grade in TCC. **Pu et al. (2008) and van Oers etal. (2007)** confirmed the association of CK20 with advanced stage and grade. **Rajcani et al., (2013)** found that CK20 was present in all invasive tumors. Moreover, **Bertz et al. (2014)** suggested that the CK20 was a reliable indicator of biologic aggressiveness and may contribute to decision making on therapeutic strategy for pT1 bladder carcinomas. In the study of Morsi and co-workers **(Morsi et al., 2006),** CK20 expression was significantly correlated to grade in SCC however; they showed no correlation with the grade of the disease in bladder TCC. Also,**Parker et al. (2003)** confirmed the use of CK20 as a tumor marker for screening and diagnosing bladder cancer rather than determining patients' prognosis. On the contrary, **Ghaini et al. (2012); Hammam et al. (2014)** found a reverse relationship between CK20 and stage or grade of bladder cancer.CK20 gives the highest sensitivity and specificity by the ROC curve. The sensitivityof CK-19 mRNA level was 87.5% and specificity was 100%.The obtained sensitivity agrees with the study of Hassanien,Nossier [29] that showed a sensitivity of CK-19 of 31%, however the specificity was higher (100%). Other studies haveshown sensitivities ranging from 43% to 79% and specificitiesranging from 68% to 88% for CK-19 mRNA and protein [30,31].**CYTOKERATIN 19:** In this study, we quantified CK19 mRNA expression in blood and also in tumor tissue of bladder cancer patients and in normal reference tissue. The present study recorded that all of the patients who showed higher CK19 expression in the peripheral blood showed, at the same time, higher CK19 expression in tissue samples. We demonstrated a statistically significant correlation between CK19 expression with the clinicopathologic features in blood and also in tumor tissue of bladder cancer (tumor stage and grade) ($p \leq 0.05$). In accordance with previous reports, **Leotsakos** et al. found an increase of CK19 expression in the blood **(Leotsakos et al., 2014).** The present results indicate that CK19 mRNA expression could be regarded as a potential marker for bladder cancer. The present data for the first time indicates a good correlation between Ck19 expression levels in blood samples and in tumors. From a clinical point of view, more attention should be given to the significance of quantitative detection of CK20 and CK19 mRNA: (1) the detection helps to monitor the occurrence of metastasis, recurrence, and therapeutic outcome. The change in CK20 and CK19 mRNA level could reflect the presence of metastasis or recurrence **(Ren et al., 2011).Survivin:** Using RT-PCR to measure survivin mRNA is more accurate because survivin concentrations are largely controlled at the level of gene transcription **(Ryan et al., 2006).** Real-time RT-PCR has the advantages of being more quantitative than classical one **(Lv et al., 2014).** In accordance with previous reports, the present study recorded higher survivin levels

both in blood and tissue samples from patients with bladder cancer. **Chen** et al. also reported an increase in survivin levels in biopsies (**Chen et al., 2014),** while **Leotsakos** et al. found an increase of survivin expression in the blood (**Leotsakos et al., 2014**).**Gazzaniga et al.,** demonstrated using RT-PCR that Survivin mRNA is expressed in 30% of bladder tumors **(Gazzaniga et al., 2003).**Schultz et al found Survivin mRNA expression in 100% of bladder tumors **(Schultz et al., 2004).**Furthermore, the present results showed that survivin overexpression was significantly correlated with the advanced tumor stage and grade.To date, survivin expression has been reported as a novel prognostic factor in several human malignancies and survivin expression in tumors was correlated with more aggressive behavior and poor prognosis **(Chen et al., 2014).** Taken together, the results of the present study suggested that a high survivin expression level was a potential predictive biomarker of bladder cancer progression and poor prognosis. The detection of survivin mRNA levels especially in blood samples may play an important role in the assessment of bladder cancer, especially in early disease stages, monitoring the disease, or in selecting and modifying therapeutic strategies (**Mull et al., 2014)**. As a result, survivin could be an attractive marker of cancer and a useful tool to evaluate severity, progression, and relapse of the disease. Further research is required to reach to firm conclusions and investigate the possibilities for the clinical application of survivin as a novel cancer biomarker. The present study, which confirms the prognostic role of survivin expression at the tumor level, suggests that detecting survivin-expressing circulating tumor cells(CTCs) might add prognostic information in T1G3 bladder tumors, and thus help the urologist in the difficult decision of how best to treat the patient.**EGFR:** To verify the correlation between EGFR expression in peripheral blood and in primary bladder tumors, we analyzed by RT-PCR the expression of EGFR in tumoral tissues and in peripheral blood from 30 bladder cancer patients. According to our expectations, we found that all patients who expressed EGFR mRNA in peripheral blood were also expressing EGFR in primary tumors. In accordance with previous reports, **Carlsson et al.** also reported an increase in EGFR levels in biopsies **(Carlsson et al., 2015),** while **Leotsakos** et al. found an increase in EGFRexpression in the blood **(Leotsakos et al., 2014**). EGFR mRNA values increased gradually with higher tumor grade and stage. Overexpression of EGFR in bladder cancer has been widely reported and several studies have shown EGFR positivity to be associated with high tumor stage, tumor progression, and poor clinical outcome. The mechanism by which EGFR expression is associated with poor prognosis is not entirely clear, although there is some evidence linking EGFR stimulated activation of activator protein-1 transcription factor with induction of matrix metalloproteinase activity **(Arfaoui et al., 2016).** Previous studies have confirmed that systemic administration of anti-EGFR monoclonal antibody is able to inhibit the growth of TCC in nude mice; such effect is mediated also by a block of angiogenesis attributable to normalization of such angiogenesisrelated markers as basic fibroblast growth factor and vascular endothelial growth factor **(Martinelli et al., 2009).** Thus, the identification of bladder cancer patients with circulating EGFR-expressing cells may be useful in selecting patients who may benefit from EGFR targeted therapies.**Humanin:** Humanin (HN) is a novel, 24-amino acid mitochondria-associated peptide. Since its initial discovery over a decade ago, a role for HN has been reported in many biological processes such as apoptosis, cell survival, substrate metabolism, inflammatory response, and response to stressors such as oxidative stress, ischemia, and starvation **(Mottaghi-Dastjerdin et al., 2014).** In the present study, the overexpression of HN 1, HN 3, HN 6, and HN 10 isoforms was identified for the first time in bladder cancer cells. The present data for the first time seems to indicate a significant correlation between H6 expression levels in blood samples and in tumors. The present report is the first to quantify H3 mRNA expression in both blood and corresponding tumor tissues. In the present

study, no correlation could be demonstrated between tissue and blood H3 levels. Surprisingly, however, blood had an approximately 3-fold higher relative H3 expression than tumor tissues. Since HN was reported to be overexpressed in gastric cancer cells and considering that HN has antiapoptotic activity in these cells, it was suggested that HN upregulation could be an important molecular event in tumorigenesis**(Mottaghi-Dastjerdin et al., 2014).**Full elucidation of HN antiapoptotic action and its role in the physiology and pathology of the bladder will contribute to a better understanding of the basic mechanisms possibly involved in the pathogenesis of BC. Furthermore, upregulation of identified HN isoforms was confirmed using qRT-PCR. The importance of this study is the specific isoforms of HN identified as the overexpressed genes in BC (HN isoforms 1, 3, 6 and 10). Due to the high similarities between HN isoforms and lack of isoform-specific antibodies, detection of HN isoforms at the protein level by western blotting or IHC was not provided in this study.The present results showed that mRNA expression of HN3, with a (5.6±0.60 –folds) increase in BC tissues, is the dominant isoform. HN isoforms have a unique coding sequence for the HN peptide. Various isoforms of the HN gene with different 5′-UTR and 3′-UTR might have probable roles in the stability of its peptide. Peptides with the highest stability, with increased residence time in cancer cells, could also have more influence in tumorigenesis and chemoresistance. The detection of serum/tissue H6 by RT-PCR seems to be a promising assay to detect both newly diagnosed and recurrent bladder cancer. Increased H6 expression in cancer patients is an unfavorable prognostic marker.H6 overexpression may be a predictive factor to determine response to chemotherapy and radiotherapy in patients with bladder cancer. Recently, studies have shown that HN is specifically bound to BAX, tBID, and BimEL and executes its anti-apoptotic activity through selective attachment to BAX and translocation inhibition of BAX to the mitochondria (**Yen et al., 2013).** Considering the fundamental role of anti-apoptosis in the chemoresistance of cancer cells and the high expression level of HN in BC, further studies are needed to evaluate the role of HN isoforms and chemoresistance. In addition, since overexpression of HN isoforms could lead to chemoresistance in BC this gene could be a candidate in drug discovery investigations for targeting chemoresistance in this cancer.**Telomerase:**In the study, telomerase was expressed in a high percent of the malignant group. To confirm the finding shown in blood samples, hTERT expression study was carried out in tissue levels also and correlated with blood level expression. The results showed similar up-regulation of hTERT mRNA expression at the blood levels in the bladder cancer disease cases, highest being in blood levels (7.08 ±0.66folds); and the blood level hTERT mRNA expression correlated significantly with the tissue level expressions, thereby highlighting the prognostic significance of hTERT expression in bladder anomalies. The present data for the first time indicates a significant correlation between hTERT expression levels in blood samples and in tumors. In normal somatic tissue, cells do not produce telomerase.Similar to our results, RT-PCR based detection of the mRNA for hTERT has been used as prognostic diagnostic marker for cancer diagnosis in urogenital organs **(Ito etal., 1998; Fukui et al., 2001)** in breast cancer **(Hiyama et al., 1996),** cervical cancer **(Takakura et. al., 1998),** hepatocellular carcinoma **(Miura et al., 2005),** thyroid neoplasm **(Lerma et al., 2005**), pancreatic juices and cancer **(Ohuchida et al., 2005),** biliary tract and pancreatic cancer **(Kawahara et al., 2007),** colorectal cancer **(Baichoo et al., 2014)**and in gallbladder cancer **(Uchida et al., 2003 ; Deblakshmi et al., 2015).** Studies have revealed that the levels of hTERT mRNA, as well as, telomerase activity were high in hepatobiliary neoplasm **(Koyanagi et al., 2000)** which can also be used as adiagnostic marker. The clinical usefulness of hTERT mRNA, especially combined with EGFR mRNA **(Miura et al., 2006),** as a novel tumor marker in primary lung cancer, viewed in the light of early detection and diagnosis **(Fujita et al., 2003).** Malignant neoplasms, including bladder cancer,

have been shown to produce telomerase and thus to regenerate telomeres and prevent cell death. Human telomerase reverse transcriptase (hTERT) assay, measures the messenger RNA levels of the catalytic subunit of the telomerase by RT-PCR **(Liu et al., 2016).**We are aware that this study has several limitations. First, we tend to see more high-grade, high-stage disease, which is reflected in the present study cohort. To confirm the robustness of our signature, we must assess the subsequent large cohorts, which include subjects with low-grade, low-stage disease studies. Many of the biomarkers in this study have not been associated with cancer previously. Analysis of the role of these biomarkers in tumor cell biology may elucidate mechanisms in tumor initiation or progression and may reveal novel therapeutic targets.Clinically, accurate BC assays will have a clear impact on initial diagnostic performance, and on the clinical management of patients post-treatment. If reliable blood diagnostic biomarker assays can reduce the number of necessary invasive and uncomfortable cystoscopies, improvements in patient compliance and satisfaction will follow, and increased efficiency and cost-savings will benefit the healthcare system. The ultimate goal is to be able to detect BC in a timely fashion such that the patient can expect to improve the quality of life and overall outcome. In conclusion, using the method of RT-PCR, 1-Upregulation of all parameters of expression of mRNA assayed by RT-PCR in the tissues and blood of a patient with bladder cancer. 2- There was a significant correlation between Ck19, CK20, Survivin, EGFR and H6 with tumor grade and stage. So they are the best candidates in the examination of peripheral blood tests for the detection of bladder cancer.3- The level of mRNA expression of candidate bladder cancer markers is superior to their protein products as molecular detection markers because their presence seems to be an indication of disseminated tumor cells in circulation.4- On the other hand, RT-PCR carries the advantages of high sensitivity and reproducibility with deprivation of the high cost.

RECOMMENDATION: 1- Further studies on a larger number of patients along with follow-up of the patients for tumor recurrence and presence of residual disease will determine the exact value of these markers for BC monitoring.2- Using real-time RT-PCR based detection to facilitate quantifying therapy response and choosing the best treatment option.3- Moreover, further investigations should focus on optimizing strategies for detecting candidate markers in urine.

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