RESEARCH ARTICLE

Differentially Expressed Genes in Metastatic Advanced Egyptian Bladder Cancer

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Abstract

Background: Bladder cancer is one of the most common cancers worldwide. Gene expression profiling using microarray technologies improves the understanding of cancer biology. The aim of this study was to determine the gene expression profile in Egyptian bladder cancer patients. Materials and Methods: Samples from 29 human bladder cancers and adjacent non-neoplastic tissues were analyzed by cDNA microarray, with hierarchical clustering and multidimensional analysis. Results: Five hundred and sixteen genes were differentially expressed of which *SOS1, HDAC2, PLXNC1, GTSE1, ULK2, IRS2, ABCA12, TOP3A, HES1*, and *SRP68* genes were involved in 33 different pathways. The most frequently detected genes were: *SOS1* in 20 different pathways; *HDAC2* in 5 different pathways; *IRS2* in 3 different pathways. There were 388 down-regulated genes. *PLCB2* was involved in 11 different pathways, *MDM2* in 9 pathways, *FZD4* in 5 pathways, *p15* and *FGF12* in 4 pathways, *POLE2* in 3 pathways, and *MCM4* and *POLR2E* in 2 pathways. Thirty genes showed significant differences between transitional cell cancer (TCC) and squamous cell cancer (SCC) samples. Unsupervised cluster analysis of DNA microarray data revealed a clear distinction between low and high grade tumors. In addition 26 genes showed significant differences between low and high tumor stages, including fragile histidine triad, Ras and sialyltransferase 8 (alpha) and 16 showed significant differences between low and high tumor grades, like methionine adenosyl transferase II, beta. Conclusions: The present study identified some genes, that can be used as molecular biomarkers or target genes in Egyptian bladder cancer patients.

Keywords: Human bladder cancer - gene expression - cDNA microarray - Egypt

Asian Pac J Cancer Prev, **16 (8)**, 3543-3549

Introduction

Bladder cancer is the fifth most common malignancies world-wide. It is a genetic disorder driven by progressive accumulation of multiple genetic and epigenetic changes. These genetic changes result in decreasing of the cell death, uncontrolled cell proliferation, invasion, and metastasis (Soloway et al., 2002; Shi et al., 2014). Bladder cancer frequently occurs as a multifocal disease involving several simultaneous tumors scattered over the bladder. More than 90% of bladder cancers are transitional-cell carcinomas and after endoscopic resection, the majority of bladder cancer patients develop cancer recurrences (Walton et al., 2009; Laishram et al., 2012).

In Egypt, Schistosoma-associated bladder cancer once represented the commonest malignancy in all diagnosed cancer cases according to National Cancer Institute registry, Cairo ('National Cancer Institute registry: the national cancer registry newsletter, Ministry of Health and Population' 2002). The patients with bladder cancer are monitored for cancer recurrence or progression by periodic cystoscopy and urine cytology. However, cystoscopic examination is associated with high cost, substantial patient discomfort, and variable sensitivity. Although, urine cytology has poor sensitivity in detecting both low-grade and low-stage tumors, it remains the method of choice for detection of bladder cancer (Cajulis et al., 1995; Matsumoto et al., 2014).

Recently, it has become possible to obtain a complete picture for cancer biology by array-based molecular profiling. Microarray-based gene expression profiling can help in gene pathway discovery and can determine the molecular signatures with respect to chemo-sensitivity or resistance to anticancer drugs (Bubendorf, 2001; Quackenbush, 2006). The gene expression patterns in tissues, exfoliated cells in urine, or molecules in serum and in circulating cells for bladder cancer has been reported in several studies (Kim and Quan, 2005; Kim and Bae, 2008; Ramshankar and Krishnamurthy, 2013). Also, microarray gene expression analysis could facilitate the identification of molecular prognostic markers that correlate with bladder cancer outcomes. In the current study, we investigated the gene expression profile in Egyptian bladder cancer patients.

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Materials and Methods

The study was conducted in compliance with Helsinki Declaration and was approved by senior staff committee and by a board regulating non-intervention study comparable to an institutional review board. Written informed consent was obtained from all patients included in this study.

The study included 29 patients who attended the National Cancer Institute (NCI), Cairo University, that were consecutively diagnosed as bladder cancer. The clinical characterizations were collected from pathology and medical reports. Fifteen patients were females and fourteen were males. Twelve patients had history of bilharzias, 20 were smokers. According to the pathological type, 8 were SCC, 18 were TCC and 3 were undifferentiated. According to pathological grade, 2 patients had grade 1, 17 had grade II and 10 had grade III.

Tumors and their adjacent non-neoplastic tissues samples were obtained. Tissues were cut into three pieces; one piece was processed for routine pathological examination to confirm diagnosis, determine the pathological features of the tumor and assess tumor: normal ratio. The second and third portions were immediately snap-frozen and stored in liquid nitrogen for RNA extraction.

RNA extraction and cDNA Microarray

Total RNA was isolated by using Trizol (Invitrogen, Germany) followed by RNeasy Mini Kit (Qiagen, Germany). RNA quality and quantity were assessed by electrophoresis and by NanoDrop (Thermo, USA). cDNA, labelled with the Cy3 dye and Cy5 dye (Amersham Biosciences, UK), were prepared from mRNA of cancer and adjacent non-neoplastic (ANT) sample. Each Cy3-labelled cDNA probe was combined with the Cy5-labelled and the mixture was hybridized to the microarray (Zekri et al., 2012). Each sample was tested in triplicate on array 15K (Array-III) supplied from Fox Chase Cancer Center http://www.fcc.edu/rsearch/facilities/biotechnology/DNAMicroarray.htm.

Data Collection: The primary data from image files were obtained using Scan Array Express II (Perkin Elmer, USA), a confocal laser scanner capable of interrogating both the Cy3- and Cy5-labeled probes and producing separate images for each and then normalized using intensity and spatially dependent method, as previously described (Yang et al., 2002).

Statistical analysis: The analysis was performed using "Cluster" and "Tree View" software and confirmed by Genesis software a gift by Dr. Alexander Sturn, Graz University of Technology, Graz, Austria. Hierarchical clustering method was applied to both genes and samples by using the Pearson r test as the measure of similarity and average linkage clustering as described previously (Eisen et al., 1998). For gene expression analysis, Mann Witney Test was used for numeric variables, and Chi square or Fisher's exact Test was used to analyze categorical variables. The p value was considered significant when $p \leq 0.05$. Scan Array Express II (Perkin Elmer, USA) software for image processing was used. Measured intensities were analyzed using the Genesis software and R program that detect the up- and down-regulated genes according to the ratio in their software's.

Results

Different analyses were performed for each of the three replicates experiments. The differentially expressed genes were 899 genes out of the 15,000 genes; 516 of known function and 383 were EST.

Of the 516 genes, 128 showed up-regulation and were involved in different pathways as p53 signaling pathway, Cell cycle pathway, Notch signaling pathway, Adipocytokine signaling pathway, Insulin signaling pathway, Fc epsilon RI signaling pathway, ErbB signaling pathway, GnRH signaling pathway, T cell receptor signaling pathway, Jak-STAT signaling pathway, MAPK signaling pathway and mTOR signaling pathway. Out of the 128 up-regulated genes, 44 were involved in biological processes, 48 in molecular function, 53 in cellular components (Table 1). Ten genes (*SOS1, HDAC2, PLXNC1, GTSE1, ULK2, IRS2, ABCA12, TOP3A, HES1, SRP-68*) were involved in 33 different pathways. The most frequently detected genes were: *SOS1* in 20 different pathways; *HDAC2* in 5 different pathways; *IRS2* in 3 different pathways.

Table 1. Up-regulated Genes Involved in Biological Processes

Pathway Name	Gene Name
ABC transporters	ABCA12
p53 signaling pathway	GTSE1
Cell cycle	HDAC2
Huntington"s disease	HDAC2
Maturity onset diabetes of the young	HES1
Notch signaling pathway	HES1, HDAC2
Type II diabetes mellitus	IRS2
Adipocytokine signaling pathway	IRS2
Insulin signaling pathway	IRS2, SOS1
Axon guidance	PLXNC1
Endometrial cancer	SOS1
Non-small cell lung cancer	SOS1
Acute myeloid leukemia	SOS1
Glioma	SOS1
Renal cell carcinoma	SOS1
Fc epsilon RI signaling pathway	SOS1
Colorectal cancer	SOS1
ErbB signaling pathway	SOS1
Prostate cancer	SOS1
Gap junction	SOS1
GnRH signaling pathway	SOS1
T cell receptor signaling pathway	SOS1
Natural killer cell mediated cytotoxicity	SOS1
Jak-STAT signaling pathway	SOS1
Focal adhesion	SOS1
Regulation of actin cytoskeleton	SOS1
MAPK signaling pathway	SOS1
Chronic myeloid leukemia	SOS1, HDAC2
Pathways in cancer	SOS1, HDAC2
Protein export	SRP68
Homologous recombination	TOP3A
Regulation of autophagy	ULK2
mTOR signaling pathway	ULK2

Table 2. Down-regulated Genes Involved in Biological Processes

Pathway Name	Gene name
PPAR signaling pathway	APOPA1
Focal adhesion	CAV2
Small cell lung cancer	CDKN2B
TGF-beta signaling pathway	CDKN2B(P15)
Cell cycle	CDKN2B, MCM4,MDM2
Pathways in cancer	CDKN2B, MDM2, FGF12,FZD4
Ubiquitin mediated proteolysis	DET1,UBE2K,MDM2
Regulation of actin cytoskeleton	FGF12
MAPK signaling pathway	FGF12
Melanoma	FGF12, MDM2
Basal cell carcinoma	FZD4
Colorectal cancer	FZD4
Long-term potentiation	GNAQ, PLCB2
Long-term depression	GNAQ, PLCB2
Gap junction	GNAQ, PLCB2
GnRH signaling pathway	GNAQ, PLCB2
Melanogenesis	GNAQ, PLCB2,FDD4
Alzheimer"s disease	GNAQ,PLCB2
Calcium signaling pathway	GNAQ,PLCB2
Taste transduction	GNAT3, PLCB2
Tight junction	INADL, CGN
DNA replication	MCM4, POLE4
Bladder cancer	MDM2
Glioma	MDM2
p53 signaling pathway	MDM2
Chronic myeloid leukemia	MDM2
Prostate cancer	MDM2
Non-homologous end-joining	NHEJ1
Phosphatidylinositol signaling	PLCB2
Wnt signaling pathway	PLCB2, FZD4
Base excision repair	POLE2
Nucleotide excision repair	POLE2
RNA polymerase	POLR2E
Huntington"s disease	POLR2E,GNAQ,PLCB2
Protein export	SRP19
Cytokine receptor interaction	TNFRSF6B
Homologous recombination	TOP3B, RAD54L

There were 388 genes down-regulated of them different genes were involved in biological processes, as molecular function, cellular components, PPAR signaling pathway, TGF-beta signaling pathway, Cell cycle, Pathways in cancer, MAPK signaling pathway, GnRH signaling pathway, Calcium signaling pathway, p53 signaling pathway, Phosphatidylinositol signaling system and Wnt signaling pathway (Table 2). *PLCB2* gene in 11 different pathways, *MDM2* in 9 pathways, *FZD4* in 5 pathways, *p15* and FGF12 in 4 pathways, *POLE2*, epsilon 2 in 3 pathways, and *MCM4* and *POLR2E* in 2 pathways.

Out of the 60 pathways detected only 18 pathways were specially up regulated encounter for seven genes; *ABCA12*, *SOS1*, *IRS2*, PLXNCI, HES1, *ULK2* and *HDAC2* and 21 pathways are specially down encounter for 19 genes; *GNAAQ, PLCB2, FZD4, POLE2, TNFRSF6B, MCM4, POLE4, FDD4, FGF12, MDM2, HNEJ1, APOPA1, POLR2E, P15, GNAT3, INADL, CGN, DET1, UBE2K*.

Out of the 516 genes, which were differentially expressed, only 30 showed significant differences between transitional cell carcinoma (TCC) and squamous cell carcinoma (SCC) (Figure 1); 23 of unknown function; one of them is *SOS1*0, which is involved in the FGF-β signaling pathway. In addition 26 showed significant differences between low and high stages (Figure 2); 20 of unknown function; of them is fragile histidine triad (FHIT) gene, Ras and sialyl transferase 8 (alpha) and 16 showed significant differences between low and high grades (Figure 3); Ten of unknown function.

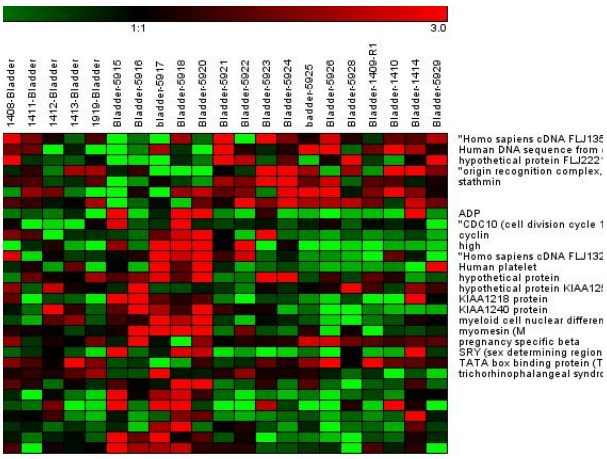

Figure 1. Differentially Expressed between Transitional Cell Carcinoma (TCC) and Squamous Cell Carcinoma (SCC)

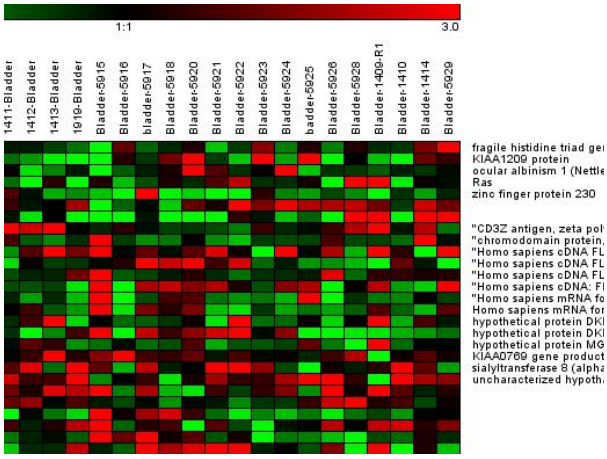

Figure 2. Differentially Expressed between Low and High Bladder Cancer Stages

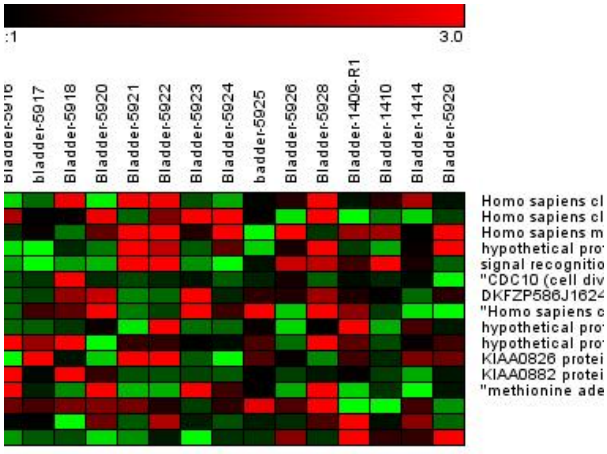

Figure 3. Differentially Expressed between Low and High Bladder Cancer Grade

Discussion

The molecular study of bladder tumors have identified several genetic alterations in different pathways include growth signals, apoptosis, angiogenesis, replication and metastasis. However, only few of these have proven to be potentially positive clinical targets in the prognosis and therapy of bladder cancer (van Rhijn et al., 2002).

Several studies on the associations between genetic variants and bladder cancer investigated few selected variants (Andrew et al., 2006; Garcia-Closas et al., 2006; Figueroa et al., 2007a; Figueroa et al., 2007b). Many genetic markers that are associated with bladder cancer have been subjected to extensive studies examining their biological roles in bladder cancer development and progression. In the current study, the resulted genes of the gene expression profile of bladder cancer and their pathways might help in diagnosis and prediction.

Our results showed that 899 genes were differentially expressed; 516 of known function and 383 were EST. Of the 516 genes, 128 showed up-regulation. In the present study, we analyzed the expression profile of the up-regulated genes involved in p53 signaling pathway, Cell cycle pathway, Notch signaling pathway, Adipocytokine signaling pathway, Insulin signaling pathway, Fc epsilon RI signaling pathway, ErbB signaling pathway, GnRH signaling pathway, T cell receptor signaling pathway, Jak-STAT signaling pathway, MAPK signaling pathway and mTOR signaling pathway. Different studies have indicated that alteration in cell-cycle regulation is a key event in determining the biological behavior of bladder cancer (Cordon-Cardo 1995; Mitra et al., 2006; Zaravinos et al., 2011).

Of the 516 genes 388 genes were down-regulated (109 are involved in biological processes, 124 in molecular function, 131 in cellular components and 21 are involved in 36 different pathways). In concordance with other reports, the current study supported the potential usefulness of microarray study in these fields (Dyrskjot et al., 2003; Sanchez-Carbayo et al., 2006; Dyrskjot et al., 2007).

Similarly, some studies used the cDNA microarrays technology to facilitate the hierarchical clustering of non-muscle-invasive and invasive bladder cancers (Sanchez-Carbayo et al., 2003; Ding et al., 2012). Another study have characterized the global gene-expression patterns of 80 bladder cancers, nine bladder cancer cell lines, and three normal bladder samples using cDNA microarrays containing 10368 human genes (Blaveri et al., 2005).

Using genome expression profiling, we demonstrated that 10 genes related to processes associated with biological processes, molecular function, cellular components, including *SOS1*, *HDAC2*, PLXNC1, GTSE1, *ULK2*, *IRS2*, *ABCA12*, TOP3A, HES1, *SRP68*, were up-regulated in bladder cancer tissues. The most frequently detected genes are *SOS1*, *HDAC2* and *IRS2*. Some of these genes were detected previously in different studies related to cancer; however, the function of other genes related to bladder cancer is unknown.

Among the upregulated genes, *HDAC2* is a member of the histone deacetylase family that mediates the transcriptional repression. According to Yang et al. (2007) study, HDAC 2 regulates the activity of NF-κB (Ashburner et al., 2001). Especially, *HDAC2* is related with regulation of cell cycle and apoptosis in cancer (Huang et al., 2013). As previously reported, inhibition of HADC2 increases apoptosis through p21cip1/WAF1 and p53 in colon cancer (Huang et al., 2005). Our study showed that *HDAC2* gene was upregulated and involved in Notch signaling, cancer and Cell cycle pathways. Similar to our results, Yang et al. (2007) reported that *HDAC2* expression was increased and was involved in apoptosis regulation by tissue specific manner. Another possibility is these cells were increased *HDAC2* expression for cell survival.

ULK2 is identified as the mouse homolog of the UNC51 serine/threonine kinase (Yan et al., 1999). *ULK2* is involved in the neuron elongation and differentiation (Tomoda et al., 1999). In the current study, *ULK2* gene was upregulated and involved in the regulation of autophagy pathway. *ULK2* may be involved in apoptosis through the p53 pathway (Yang et al., 2007).

SOS1 gene, activator of *Ras/MAPK*, is essential for intrauterine development (Timofeeva et al., 2009). In the current, *SOS1* was upregulated in bladder cancer tissue and involved in 20 different pathways. However, the function of *SOS1* gene related to bladder cancer is unknown. *SOS1* gene is increased in prostate cancer with increase in proliferation and migration through activation of ERK signaling that is a factor for cancer aggressiveness and is consistent with higher stages (Timofeeva et al., 2009; Zekri et al., 2012).

Among the up-regulated genes, IRS gene was involved in 3 different pathways. IRS family contains IRS-2 that is expressed in almost all cells and tissues (Withers 2001; Hennige et al., 2003). IRS-2 regulates body weight control and glucose homeostasis (Hennige et al., 2003). *IRS2* is thought to be involved in insulin signaling and glucose intolerance (Withers et al., 1999; Rojas et al., 2003). Within tumors, *IRS2* may be an important risk factor for colon cancer, given its previously reported association with obesity and diabetes (Hennige et al., 2003; Chen et al., 2014).

GTSE1 is a microtubule-localized protein. Its expression is cell cycle-regulated and can induce G2/M-phase accumulation when over-expressed (Monte et al., 2000). In the current study, GTSE1 gene is up-regulated and involved in p53 signaling pathway. Similarly, as reported GTSE1 down-regulates the levels and activity of p53 tumor-suppressor protein and represses its ability to induce apoptosis after DNA damage (Monte et al., 2004).

Several ABC transporters including *ABCA12* is an important mediators of chemo-resistance. The current study showed an up-regulation in *ABCA12* gene and its involvement in ABC transporters pathway. Our results suggest that ABC transporters in human bladder cancer may affect the clinical response to neo-adjuvant chemotherapy. Also among the up-regulated genes is the transcription factor Hes1 gene, which is known to repress endocrine cell formation. Introduction of *DeltaHes1* and pancreatic transcription factor (*Pdx1*) can therefore initiate a partial re-specification of phenotype from biliary epithelial cell towards the pancreatic beta cell.

The current study showed down-regulation in 388 genes and involved in biological processes, molecular function, cellular components and other different pathways. *PLCB2* gene was involved in 11 different pathways, *MDM2* in 9 pathways, *FZD4* in 5 pathways; *p15* and FGF12 in 4 pathways; *POLE2* in 3 pathways; and *MCM4* and *POLR2E* in 2 pathways.

MDM2 gene is a cellular p53-binding protein and is over-expressed in a subset of soft-tissue sarcomas (Oliner et al., 1992). *MDM2* gene is functioned as oncogene when over-expressed *in vitro*. In this study, *MDM2* gene was down-regulated and was involved in nine pathways include the cell cycle and p53 signaling pathways. According to previous study expression of *MDM2* is associated with cell cycle progression and apoptosis (Lohrum et al., 2003; Momand et al., 1992), and affects carcinogenesis. In disagreement to our study, other studies found that its over-expression may alter cell growth and promote carcinogenesis is by inactivating p53 (Haupt et al., 1997; Momand et al., 1992). Over-expression of *MDM2*, seen in a variety of tumors of diverse tissue origins, can result from gene amplification, increased transcription, or enhanced translation (Zhang and Wang 2000).

Phosphoinositide-specific phospholipase C (PLC) is one of the key enzymes in the metabolism of inositol lipids. It plays a crucial role in multiple trans-membrane signal transduction pathways that regulate numerous cell processes, including proliferation and motility (Rhee 2001). PLC-ß2 is one isoform of PLC and is frequently associated with hematopoietic malignancies and neuro-endocrine tumors (Bertagnolo et al., 2002; Brugnoli et al., 2006; Lo Vasco et al., 2004; Stalberg et al., 2003). In this study, in the bladder cancer tissue PLC-ß2 gene was down-regulated and was involved in 11 different pathways. Simillarly, in breast tumors, PLC-ß2 is over-expressed and correlates with a poor clinical outcome, constituting a molecular marker of breast cancer severity (Bertagnolo et al., 2006). Previous study, has reported that, although PLC-ß2 fails to induce tumorigenesis in non-transformed breast-derived cells, it has a major role in promoting migration (Bertagnolo et al., 2007). PLC-ß2 induces transition from G0/G1 to S/G2/M phases of the cell cycle, which appears to be a critical event in cancer progression and is responsible for inositol lipid-related modifications that occur during division, and invasion of tumor cells (Bertagnolo et al., 2007).

POLB gene codes the polymerase β inside the DNA replication. In this study the POLB is down regulated, similarly in another study, the POLB locus is often lost in bladder cancers and numerous splice variants have been reported in tumor tissues (Khanra et al., 2012; Michiels et al., 2009).

In the current study , the down-regulated gene included FGF12, FZD-4, and *MCM4*. In contrast, FGF12 gene was up-regulated in prostate cancer patients (Hansel et al., 2009). The mammalian FZD family has ten members. The pattern of fzd-4 expression in the rat ovary indicates that the granulosa cells of all growing follicles express fzd-4 (Orsulic and Peifer 1996; Wodarz and Nusse 1998). The *MCM4* gene plays essential roles in replication *MCM4* belongs to minichromosome maintenance (MCM) protein family.

In the current study, thirty differentially expressed genes were showed significant differences between Transitional cell carcinoma (TCC) and squamous cell carcinoma (SCC). Of them, the *SOS1*0 gene is involved in the FGF-β signaling pathway. In addition 26 showed significant differences between low and high stages; the fragile histidine triad gene, Ras and sialyltransferase 8 (alpha) and 16 showed significant differences between low and high grades, of them is methionine adenosyltransferase II, beta. As reported, that the loss of FHIT gene is common in TCC of bladder cancer (Baffa et al., 2000). Due to Schistosoma infection and the accompanied inflammation, the SCC seems to follow a distinct development, and is the predominant bladder cancer seen in Africa and the Middle East

In conclusion, the microarray analysis provides approaching of molecules and processes contribute to bladder cancer. To our knowledge, this study is the first looking for the gene expression profile involvement in different cell cycle pathway in Egyptian bladder cancer. This study may help in the development of specific molecular marker for bladder cancer and thereby significantly lowering the morbidity associated with bladder cancer. The present work adds to the current knowledge on molecular signature identification of Egyptian bladder cancer.

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