Identification of Bladder Cancer at early age in Iraq used by Tumor Marker and Genes Characterization

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Abstract

Cancer is mostly considered as a 30 % genetic disease so much so that this disease, which defines a multi-step neoplasia characterized by tumor formation and cell proliferation becomes uncontrolled, depends on the individual's genetic predisposition. Bladder tumors are clinically characterized by their high recurrence rate once they invade the muscle layer which added clinical value as diagnostic and prognostic biomarkers in bladder cancer. Progress of the high-impact technologies for molecular analysis of tumors allow explore genetic profiles, epigenetic and protein characteristic different tumor subtypes and identify targets and molecular pathways that define a specific clinical behavior. Objective to evaluate the Bladder Tumor Marker (BTM) as a new developed tumor marker in comparison to cytology in the selected area. The present study contributes to a growing body of work on gene expression signatures in bladder cancer and urine samples collection from Bladder cancer early detection center at Nineveh health department, Al-khansa Teaching Hospital in Mosul during the study period patients N: 101 and controls N: 19. The final study is to establish a concept of stratification of patients with bladder cancer. For this, we have developed a multi-parameter test for the quantification of the selected biomarkers.

Keywords: Bladder Cancer, Tumor Marker, Gene Characterization.

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Introduction

The bladder is a hollow organ, the main function of which is to act as a reservoir and allow the evacuation of urine produced in the kidneys. The bladder wall, about half a centimetre thick, is made up of several layers of tissue. Bladder cancer is the transformation of a normal cell in the bladder that becomes cancerous. The anarchic multiplication of the cancer cell leads to the formation of a more or less organized assembly called a malignant tumor. If left untreated, this cancerous tissue can continue to grow and invade surrounding tissue. This spread is called metastatic spread. Bladder cancer appears in 90% of cases in the mucosa, this is called urothelial tumor or urothelial carcinoma. At the start of the disease, cancer cells are few and limited to the mucosa. They can either stay at this local stage, or the tumor can infiltrate the muscle layer or nearby organs (Anthony, 2009). The inside of the bladder is lined with transition cells that are responsible for most bladder cancers. Progression depends largely on
the aggressiveness of the tumor. Advanced age and coexisting diseases are associated with higher mortality rates from any cause. Urinary bladder cancer represents a serious health problem, especially in developed and highly industrialized countries where it reaches a relatively high prevalence. It ranks second in order of frequency and by cause of death within urological tumors. It is characterized because usually (in 75% of cases) it is diagnosed in incipient stages, which would explain its good prognosis and, therefore, its high survival. Like all cancers, the development of bladder cancer involves the acquisition of various mutations in oncogenes and tumor suppressor genes. Genes that can be altered in bladder cancer include FGFR3, HRAS, RB1, and TP53. Thus, a family history of bladder cancer is also a risk factor for the disease. Some people are believed to inherit a decreased ability to break down some chemicals, making them more sensitive to the effects (P. Sanchez, 2004). In order to define the stage of bladder cancer, doctors examine the size of the tumor and its possible infiltration into the internal wall of the bladder, in particular into the bladder muscle. In addition, they examine a possible spread to the lymph nodes, neighboring organs or at a distance in the body, in the form of metastases. It is estimated that 75% of bladder tumors are purely superficial and therefore non-infiltrated. In addition, there are other tumors qualified as infiltrating, because they invaded the wall of the bladder until the muscle (Felix et al., 2008). The development of molecular biology applied to tumors has enabled the identification of somatic genetic (and epigenetic) alterations in bladder tumors and has been an essential step towards understanding the molecular mechanisms of bladder carcinogenesis. Ten years ago, the urothelial bladder carcinogenesis model was based on two distinct pathways of genetic alteration: the pathway of papillary hyperplasia and non-infiltrating and low-grade tumors (with preponderant mutations of the HRAS and FGFR3 genes), and the pathway of dysplasia and muscle-infiltrating tumors (with structural and functional abnormalities of the tumor suppressor genes TP53 and / or RB1). These two molecular pathways must be reconsidered with the recent results of genome-wide studies identifying new genes involved in urothelial bladder carcinogenesis, (Chou et al, 2015). These alterations are also potentially usable as markers in clinical cancerology. Indeed, some can be useful for diagnostic and prognostic evaluations, for assessing the response to treatment and are currently opening the way to new therapeutic approaches. Genetic alterations are responsible for the abnormal activation of oncogenes (gain of function) or the deactivation of tumor suppressor genes (loss of function). These two classes of genes are distinguished by their mechanism of action. The mode of action of oncogenes is considered to be dominant, it is sufficient that only one of the two alleles is activated for a positive effect to be observed on the tumor. Conversely, the mode of action of tumor suppressor genes is considered to be recessive. Their inactivation requires the alteration of their two alleles (Van Tilborg, 2009). The gap between the resolving power of cytogenetic and molecular analysis has narrowed considerably in recent years thanks to the development of new hybridization techniques: comparative genomic hybridization (CGH for comparative genomic hybridization, then CGH-array) and fluorescent in situ hybridization (FISH technique). In the early 2000s, CGH on metaphasic chromosomes increased resolution and avoided cell culture prior to cytogenetic analysis, which is a source of selective bias in tumor cells with a high mitotic index. The CGH (10 Mb resolution) has made it possible to highlight, at the chromosomal level, the amplified or lost regions of the genome with which oncogenes or tumor suppressor genes should be associated respectively. CGH was quickly supplanted by CGH-array techniques which are more and more resolutive (resolution of 1 Mb for BAC-arrays to a few Kb for oligonucleotide-arrays) and
which made it possible to highlight new regions amplified or lost, smaller in size, in tumors. The development of technologies derived from DNA chips (microarrays) has made it possible to change the scale of work by carrying out exhaustive analyzes of all the genes in the human genome (genome-wide analyzes), making it possible to identify a considerable number of new events molecular, genetic and epigenetic, in tumors (Rosser et al, 2014). The exhaustive analysis of tumor DNA and RNA by microarray technologies and especially by sequencing of exomes or whole genomes (resolution of 1 bp) should allow us to quickly clarify to what extent certain alterations play an important role in the development of cancer. Despite the significant advance made by next generation sequencing technologies, these techniques are still too expensive to regularly consider large-scale whole genome (3 Gb) sequencing. An alternative, used for the past five years, is the analysis of the exome sequence, corresponding to all of the coding parts of the genome (1% of the genome, 28 Mb). In bladder cancer, recent studies on the exome have made it possible to identify very recently new genomic alterations concerning genes implicated in urothelial bladder carcinogenesis. Finally, more recently, a genome-wide analysis was carried out within the framework of The Cancer Genome Atlas project including 131 bladder tumors, all of which are infectious and of high grade. The number of genomic alterations was particularly important, bladder tumors coming in third position in terms of mutation rate (around 7.7 per Megabase) after melanomas (10 / Mb) and lung cancers (8 / Mb). This study has identified a number of mutations, deletions and amplifications involving genes never previously reported as damaged in bladder tumors, and which may constitute potential new therapeutic targets (Wu, 2005).

**Aim of current study**

The present research aimed to study new tumor marker, based upon monoclonal antibody, has been described as a simple, rapid and non-invasive test for early detection of a specific antigen of bladder cancer. To assess whether hypermethylation of selected genes is associated with the loss of expression of the selected genes, has been treated bladder cancer cell lines with azacitidine a demethylating agent to retrieve the expression of the genes they are methylated if. It is confirmed to BDNF, the selected gene, the presence of methylation not only by the array of CpG, but PCRs methylation, sequencing bisulphite, and confirming the recovery of the expression of the gene by RT-PCR, western blot (WB) and immunofluorescence. Objective to evaluate the Bladder Tumor Marker (BTM) as a new developed tumor marker in comparison to cytology in the selected area. The present study contributes to a growing body of work on gene expression signatures in bladder cancer.

**Material and Methods**

Patients diagnosed for the first time with a bladder tumor obtained from a hospital registry carried out by coding discharge reports by the Archives Service of the Medical City Teaching Hospital consisting of those coded as bladder tumor according to the ICD-10 classification. This work is a retrospective study of 101 cases of prostate invading bladder tumor over a five months period from March 2020 to July 2020. It consisted of identifying all the tumors of the invading prostate bladder that were collected in the urology Bladder cancer early detection center at Nineveh health department, Al-khansa Teaching Hospital in Mosul during the study period. All markers are recombinant human proteins with the exception of E.N-2 (Lin et al, 2005) C.D.H-1 (Cadherin 1) E.G.F.R (Epidermal Growth Factor Receptor) E.N-2 (Engrailed-2) Er.bB-2 (Receptor 2 for epidermal growth factor) I.L-6 (Interleukin 6) I.L-8 (Interleukin 8) I.L-10 (Interleukin 10) M.M.P-9 (Matrix metalloprotease 9) P.T.G.S-2
Samples

Urine samples from Bladder cancer early detection center at Nineveh health department, Al-khansa Teaching Hospital in Mosul during the study period patients N: 101 and controls N: 19.

Detection Antibodies


ELISA Kits

All ELISA kits AVISCERA BIOSCIENCE, INC are intended for the detection of human proteins. Used kits are AP1M2; BIRC5; CD44; C.D.H-1; DCN; E.G.F.R; E.N-2 and Er.bB-2 (HER2).

Methods

Several types of immunoassays have been performed. Most of the tests are in sandwich format. The first part concerns the classic immunoassays carried out with ELISA kits. The second part describes the methods developed for multi-parameter and automated immunoassays on various supports.

Preparation of samples

Urine samples from patients and healthy donors were analyzed as received. These samples were collected according to the hospital procedure: the first stream of urine is collected and then centrifuged at 1500 rpm for 10 minutes. The supernatant is then aliquot and stored at -80 °C.

General Principle

All the ELISA kits AVISCERA BIOSCIENCE, INC used are based on the same principle of immunoassay type, the microtitration plates supplied have already undergone a step of coating the capture antibodies. Each kit also contains all the reagents necessary for the analysis. The reagents were prepared and diluted according to the supplier's instructions (Fuge, 2015).

The tests were carried out with the Freedom-100 pipetting machineTecan Trading AG Corporate ProfileSeestrasse 103CH-8708, MännedorfSwitzerland, depending on the number of samples to be analyzed. The latter has a manipulator arm to move the microtiter plates and covers, a pipetting arm to transfer the solutions from the reservoirs to the plate by suction and ejection and a heating block for incubating the plates. The instrument is also associated with a washing station for rinsing the wells and a plate reader. Once the plate is placed in the pipetting machine, the protocol is therefore fully automated. It involves the following steps: the urine samples and the diluent are transferred to the microtiter plate (50 μL of each, for a total of 100 μL / well). The incubation parameters vary according to the kits: from 30 minutes to 2.5 hours, at room temperature or at 37°C. The biotinylated detection antibody solution is then incubated (100 μL / well, 30 minutes or 1 hour, at room temperature or at 37 °C.) then there is incubation of the streptavidin solution labeled with horseradish peroxidase (100 μL / well, 30 minutes or 1 hour, at room temperature or at 37 ° C). Finally, the addition of the TMB substrate (50 μL to 100 μL / well, 10 to 15 minutes, at room temperature and protected from light) allows the generation of the colorimetric signal. The
enzymatic reaction is then stopped by the addition of a sulfuric acid solution at a concentration of 0.2 M, pH 1.5 (50 μL to 100 μL / well). Between each step of the protocol and until the addition of TMB, washing of the wells with PBS (3 x 300 μL / well) is carried out to remove the molecules not captured. These ELISA kits are used for the selection of urinary bladder cancer biomarkers (Kelloff, 2012). Once the markers have been selected, a multiparameter immunoassay platform is developed for their detection. A single selected marker is not integrated into this platform. The E.N-2 marker indeed has its own monoparametric immunoassay in direct mode. The first step is the coating of the well bottoms of polystyrene plates with the urine sample (50 μL diluted in 50 μL of PBS / well). The sample is thus incubated for 2 hours at room temperature. Then there is added the solution of detection antibodies labeled by horseradish peroxidase (at a concentration of 1.5 μg / ml in Buffer, 100 μL / well, 30 minutes at room temperature). Finally, the addition of the TMB substrate (100 μL / well, 10 minutes, at room temperature and protected from light) allows the generation of the colorimetric signal. The enzymatic reaction is then stopped by the addition of a sulfuric acid solution (50 μL / well). Between each step of the protocol and until the addition of TMB, washing of the wells with PBS (3 x 300 μL / well) is carried out to remove the molecules not captured.

Results and Discussion
Below is a summary of the strategy and the results that it has given time to carry out in this project. After organizing the collection of urine samples and characterizing their microhematuria, the epigenomics methylation profiles were first analyzed using a methylation array with 6900 CpG islands in 100 of the collected urine samples. The analysis of these profiles has served to identify candidate genes associated with the presence of bladder cancer in the selected urine samples. Then it is optimized methodology of epigenetics to characterize the methylation by MS-PCR and to determine whether selected genes are methylated by techniques alternatives both tumor lines cellular and in future series of urines independent to those already collected. The 10 candidate markers were evaluated in urine samples from bladder cancer patients and healthy donors. The measurability of the markers was first assessed. Indeed, urine is a complex biological matrix which can have a negative impact on the assay method, in particular by its pH, the presence of salts and proteins. This measurability is described in Table 1. For each marker, a calibration range in the diluent of the ELISA kit and a calibration range in human urine were established. Four of the 10 ELISA assays did not detect their target in the urine. These are the dosages for the following markers: CD44, AP1M2, C.D.H-1 and Er.bB-2. A calibration range in the diluent having been established for these assays, it is therefore possible that the non-detection of the targets in the urine is due to denaturation of the target or of the antibody by the biological matrix.
Table (1) First evaluation of markers in urine samples

<table>
<thead>
<tr>
<th>Marker</th>
<th>Calibration range in the absence</th>
<th>Calibration range in urine</th>
<th>Detection limits (pt/mL)</th>
<th>Detection of marker in patient samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP51++</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>BCL2</td>
<td>+</td>
<td>+</td>
<td>20 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>CD44</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>GSTT1</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>DNMT1</td>
<td>+</td>
<td>+</td>
<td>250 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>EGF2</td>
<td>-</td>
<td>+</td>
<td>2 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>E2F2</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>EVIL</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>FBLN5</td>
<td>+</td>
<td>+</td>
<td>&lt; 1.5 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>FIDK3</td>
<td>+</td>
<td>+</td>
<td>&gt; 250 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>IL6</td>
<td>+</td>
<td>+</td>
<td>0.1 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>IMPA4</td>
<td>+</td>
<td>+</td>
<td>5,000 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>NF21</td>
<td>-</td>
<td>+</td>
<td>1 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>pH5</td>
<td>+</td>
<td>+</td>
<td>2.5 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>STX102</td>
<td>+</td>
<td>+</td>
<td>3.0 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>TAC101</td>
<td>+</td>
<td>+</td>
<td>1.25 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>LINUX</td>
<td>-</td>
<td>+</td>
<td>100 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>VSIP1</td>
<td>+</td>
<td>+</td>
<td>0.1 ng/mL</td>
<td>-</td>
</tr>
</tbody>
</table>

+, presence of a calibration curve or marker detection
-, absence of calibration curve or no detection of N/A marker, not applicable
* E.N-2 could not be detected with the ELISA kit but by a direct mode immunoassay
** The detection limits have been determined graphically on the calibration curves

Figure (1) Marker concentrations in samples from bladder cancer patients and healthy donors

Where 88 urine samples have been processed from which 44 patients were included with bladder cancer and 49 individuals without bladder cancer. The bladder cancer patients had low grade in 25 cases, and 19 cases high and stages were pTa (n = 19), pT1 (n = 17) and pT2 + (n = 8). After optimization of the protocol to be used in urinary samples, the 500 ng of DNA from the collected and selected urine were labeled and hybridized. Once extracted DNA from all urines and after assessing their quality in a NanoDrop, we proceeded to the marking of the DNAs of the urines and their hybridization arrays (Fuge, 2015). First, a quality control analysis of the different processes carried out in the treatment, marking and hybridization of the arrays was carried out, as well as some initial initial analyzes that are shown below in the following panels: A) verification of the allele specific extension; B) verification of bisulfite conversion; C) verification of methylation of sex-specific genes (associated with X and Y chromosomes); D) Control of the extension; E) control of the first hybridization; F) control of the second hybridization; G) negative control; H) contamination control I) control of the intensity of the array. All the controls turned out to be favorable indicating a correct process in all the procedures of the use of the CpG arrays used. This
first bioinformatic analysis has been carried out with Illumina's Beadstudio programs. Below is an unsupervised clustering analysis of the methylation profiles of all CpG islands analyzed in the arrays in all samples analyzed. These analyses were performed with programs developed in Standford Eisen, and for the analysis of microarrays. Each of the genes are shown vertically throughout each of the samples shown horizontally. The relationship between what hybridizes to Cy-3 (green) and Cy-5 (red) will establish the methylation status of each CpG island to differentiate cases and controls.

**Conclusions**
The research presented in this study focused on the development of a protein chip for the multiparametric analysis of urinary biomarkers for bladder cancer. They are divided into two parts corresponding to the selection of markers and the development of a multi-parameter immunoassay platform for the detection and quantification of selected markers, the markers to be evaluated were first identified using a molecular model of bladder cancer created by one of the project partners (emergentec biodevelopment). The identified markers were then evaluated using commercial ELISA kits allowing their quantification in urine samples.

**References**