Anti and pro-inflammatory cytokine gene expression in patients suffering from cancer bladder associated with chronic schistosomiasis haematobium.

1Abd El Hamid A, Sabry, 1Marwa El - Dardiry, 1Yossra Nabil, 3Mohamed S. Badr and 3Marwa Nabil

1Department Medical Parasitology, Faculty of Medicine, Al-Fayoum University, Al-Fayoum, Egypt
2Department of Molecular Biology2, Medical Research Center, Faculty of Medicine, Ain Shams University, Cairo, Egypt
3Department of Oncology, National Cancer Institute, Faculty of Medicine, Cairo University.

Address For Correspondence:
Abd El Hamid A, Sabry, Department Medical Parasitology, Faculty of Medicine, Al-Fayoum University, Al-Fayoum, Egypt.
E-mail: ahamdatawab@yahoo.com

ABSTRACT
This work was a case-control study applied on 24 patients with chronic complicated Schistosomiasis haematobium with bladder carcinoma, versus 10 subjects with history of S. haematobium without complications as a control group. Gene expression of 2 anti-inflammatory cytokines (IL-10 and TGF-β) and 2 pro-inflammatory cytokines (IFN-γ and TNF-α) was done using quantitative real time PCR. The results revealed marked increase in the level of (IL10 and TGF β), in contrast to marked decrease in the level of (IFN γ and TNF α). Therefore, cases in the current work were reported to be poorly controlled by unbalanced Th1/Th2 in which Th2 was dominated. However, possibly failed to eliminate the damaging impact of Schistosoma infection in our cases, instead counteract Th1 cytokines (significant negative correlation between Th2 anti-inflammatory cytokine IL-10 & the chief Th1 pro-inflammatory cytokine IFN γ was reported). This possibly led to loss of Th1 control in defending the host against both parasite and carcinogenic changes. This study suggested a vital regulatory role for IL-10 in such serious infection which certainly needs further elucidation as regard its prognostic and therapeutic potential.

INTRODUCTION

In reality, schistosomiasis is a prevalent infectious disease in developing countries in the tropics and subtropics. It affects more than 270 million people worldwide, with over 700 million living under conditions supporting transmission (Yosry, 2006). Although the manifestations are varied, the majority of the morbidity and mortality can be attributed to the host immune response against Schistosoma eggs deposited within the walls of the urinary tract which may lead to urinary tract inflammation and increased susceptibility to urothelial carcinoma. In fact, the annual deaths are about 150,000 due to urogenital schistosomiasis-induced obstructive renal failure makes S. haematobium one of the most lethal worms worldwide (Fu et al., 2012).

Numerous immunological works supported the concept that individuals living in endemic areas have altered immune responses, making them either challenging or vulnerable to various complications. Evidence accumulated has underlined the existence of functionally distinct T helper cells that play a meaningful role in immune responses to infection with schistosomes via the different members of the cytokine classes they produce (Mduluza et al., 2001). However, there is no evidence concerning the exact role of these immune cells and their mediators and despite the global burden of schistosomiasis, there remains little known about the basic mechanisms underlying the pathophysiology in complicated infection. This is primarily due to the lack of an experimentally tractable animal model and limited research on human cases (Anwar et al., 2008 and Airfax et al., 2012). Carcinogenesis is a slow multistep and multifactorial process, usually as a consequence of a long-
term inflammation, which involves the changes in the cellular genome. The accretion of irreversible structural alterations in genes results in the development of carcinomas (Thorgeirsson and Grisham, 2002). Infected human cases with S. haematobium is still reported in Egypt with a different degree of morbidity. This indicates an urgent need of research studies to elucidate the factors behind the occurrence of different levels of morbidity. Studying the immunological factors is critical to explain the outcomes of S. haematobium infection in Egyptian cases. Therefore, the aim of the current work was to investigate gene expression profiling of 2 pro-inflammatory cytokines (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-10 and TGF) in patients suffering from complicated schistosomiasis haematobium by bladder cancer, using reverse transcription quantitative real time PCR (RT-qPCR). In addition, to compare different expression patterns in complicated & uncomplicated schistosomiasis cases.

MATERIAL AND METHODS

This work was a case control prospective study conducted in the period of time from the first of March 2013 to end of April 2016. This study was carried out on patients attending the outpatient clinic of Medical oncology in Fayoum health insurance hospital. Cases were subjected to the following; Full clinical examination, detailed history taking, complete urine analysis, serological testing for anti-bilharzial antibodies, histopathological examination for bladder biopsies for chronic cases with bladder carcinomas. RNA extraction from blood samples and reverse transcription using quantitative real time PCR (RT-qPCR). Finally gene expression profiling of 2 pro-inflammatory cytokines (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-10 and TGF) was done, applying qPCR.

Study groups:

Group 1 (cases): Patients suffering from chronic schistosomiasis haematobium complicated by bladder carcinomas (24 cases).

Group 2 (control): Patients with history of schistosomiasis and didn’t suffer from any complications (10 cases).

Inclusion criteria:

a-Male or female patients with chronic Schistosoma haematobium infection and complicated by neoplasm in the bladder. b-The bilharzial association must be confirmed by histopathological examination. c-The cases should be serology positive for anti-schistosomal antibody.

Exclusion criteria: a-Immunocompromized cases infected with S. haematobium. B-Cases with malignant growth in other organs.

Collection of samples:

Approximately 5 ml of blood sample was collected from each individual and was divided into 2 parts. The first part (3ml) was collected on EDTA tube and subjected to separation of mononuclear layer as fresh as possible and then kept at -20°C until used for nucleic acid extraction and gene expression by qPCR. The second part (2 ml) was collected in plain vacutainer tubes, then were centrifuged at 4,000 rpm and subsequently stored at -20°C until further processing and serological testing. Mononuclear layer was separated from each blood sample, by Lymphoprep (Axis-Shield PoC, Norway) density gradient centrifugation, which is a simple and rapid method of purifying Peripheral Blood Mononuclear Cells (PBMC). Serum samples were subjected to ELISA for anti Schistosoma serum IgG antibody level using commercially available kits was performed. On the other hand, urine samples were collected and subjected to microscopic examination using a light compound microscope at a magnification power 40x and 10x to examine after centrifugation at 5,000 rpm for 10 min for the presence of Schistosoma haematobium eggs or other parasites.

RNA extraction and reverse transcription PCR (RT-PCR):

According to manufacturer’s instructions, RNA extraction was done using Commercial kits (The SV Total RNA Isolation System) Promega CorporaƟ on on 2800 Woods Hollow Road, Madison, WI 53711-5399 USA. Reverse transcription PCR was performed using HYBAID OmniGene 3 Block Thermal Cycler (Mid-Atlantic Lab.equip., UK). The recommended cDNA synthesis reaction mixture consisted of; M-MLV Reverse Transcriptase RNase H-200 U/µl (Solis BioDyne) (1µl, 4U/µl), 20mMdNTP mix (1.25µl, 500µM), 100mM DTT (2.5µl, 5mM), random hexamer µg of RNA (2.5µl, 500ng). RNase inhibitor (Solis BioDyne) (1µl, 25U), For 5x RT Reaction buffer 1 (10µl , 1X), with MgCl2 and DTT) 0.25 M Tris-HCl, 0.5 M KCl, 30 mM MgCl2, 25 mM DTT. RNA(5 µl, 2.5µg) and DEPC treated H2O to a 50 µl final reaction volume (Schwabe et al., 2000).
Microfluidic LabChip RT- real-time PCR:

The procedure was applied on UltraFast LabChip Real-time PCR G2-3 System (NanoBioSys Inc., Korea). Real-time PCR reaction was prepared using SYBR Green I Real-Time PCR Master Mix Kit (NanoBioSys Inc., Korea) For every patient, two PCR reaction mixtures were prepared, for assay of cytokines mRNA and β-actin mRNA expression levels in RNA isolated from the samples collected from all cases. A 15 μL reaction mixture containing 10% (v/v) of synthesized cDNA, and 1 μM of each primer were loaded into each channel of LabChip. LabChip filled with reaction mixture was placed onto the LabChip case and further injected into the ultra-fast LabChip real-time PCR G2–3 system. Real-time PCR was performed according to software protocol (pre denaturation at 95°C for 8 s then 30 cycles at 95°C for 8 s and 72°C for 14 s). As real-time PCR proceeded, real-time data were plotted on the screen and then sigmoidal curves for amplified DNAs appeared. After curve fitting, Ct values and reaction time of real-time PCR (15 min for 30 cycles) appeared on the screen. Each reaction included the no template control (NTC) to determine whether it was positive or negative. A sample with a Ct value less than 31 was considered positive, in cases where the Ct value of the NTC was not assigned; β-actin was treated as the internal control and results were expressed as cytokines/β-actin ratio. Target concentration was expressed in relation to the concentration of the housekeeping gene. For relative quantization, the values obtained were compared to those from the standard RNA dilutions which were amplified by the RT-PCR in parallel. Relative cytokines expression = copy no of cytokines/copy no of β-actin = concentration of cytokines/ concentration of β-actin (Suzuki et al., 2000).

Ethical considerations: Each patient individually signed an informed written consent, before being included in the present study. The current study was conducted according to the institutional ethical and professional guidelines in management and follow up of cases by the committee of ethics, Faculty of Medicine, Al-Fayoum University.

Statistical analysis:

Data collected in this study were evaluated using the PRISM® 5.01(GraphPad software Inc., San Diego, CA, USA.) Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparisons between two groups were made using the Student’s t test. For the analysis of numerical data when samples failed to show a normal distribution, the Mann-Whitney U test was employed for comparison between two groups. The Pearson chi-square test was used for categorical data. The relation between variables was examined with correlation analysis using Spearman rank correlation equation. P values of < 0.05 were considered as statistically significant.

Results:

In the current work, a list of inclusion criteria was allocated, as a trial to minimize biased results and to properly assign the included groups as much as possible. Therefore, the subjects described as cases were those complaining of chronic schistosomiasis haematobium complicated by bladder carcinoma (24 cases). Confirmation was done by imaging, serological (higher ELISA O.D readings reported in our 24 cases than the control subjects) and histopathological, in addition to the clinical diagnosis. Past history of all these cases was proved to be Schistosoma infection as well. Histopathological examination confirmed the presence of neoplastic lesions associated with old bilharzial lesions in all the 24 samples related to the chronically infected patients. Regarding bladder cancer, 18 patients suffered from squamous cell carcinoma (SCC) associated with bilharziasis (75%), while 6 patients suffered from transitional cell carcinoma (TCC) associated with bilharziasis (25%). Reverse Transcription quantitative –PCR (RTq-PCR) products for IFN-γ, TNF-α, IL-10, TGF-β, in addition to β-Actin gene were analyzed in the 24 cases and the 10 controls. There was variability in the expression of pro-inflammatory cytokines (IFN-γ, TNF-α) and anti-Inflammatory cytokines (IL-10, TGF-β) in the samples obtained from the study groups. The mean value of gene expression related to the TGF-β was 1.134±0.25 and for IL-10 was1.254±0.31. While the pro- inflammatory cytokines; IFN-γ revealed a mean of 0.057±0.028 expression value and 0.031±0.021 for TNF-α (Table 1 & Figure 1).

Table 1: Mean values for gene expression of different cytokines TGF-β IL-10 TNF-α IFN-γ.

<table>
<thead>
<tr>
<th></th>
<th>TGF-β (mean ±SD)</th>
<th>IL-10 (mean ±SD)</th>
<th>IFN-γ (mean ±SD)</th>
<th>TNF-α (mean ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>1.134±0.25</td>
<td>1.254±0.31</td>
<td>0.057±0.028</td>
<td>0.031±0.021</td>
</tr>
<tr>
<td>Control</td>
<td>0.047±0.038</td>
<td>0.054±0.030</td>
<td>0.084±0.018</td>
<td>0.065±0.033</td>
</tr>
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</table>
The anti-inflammatory cytokines (TGF-β & IL-10) were significantly of higher expression value than the pro-inflammatory cytokines (IFN-γ, TNF-α); 1.134±0.25, 1.254±0.31 versus 0.057± 0.028 and 0.031± 0.021 respectively (p≤0.05). Therefore, there was 19.89 and 20 folds increase in the anti-inflammatory cytokine (TGF-β & IL-10) than the pro-inflammatory cytokine IFN-γ respectively. On the other hand, there was 36.58 and 40.45 folds increase in the anti-inflammatory cytokine (TGF-β & IL-10) than the other pro-inflammatory cytokine TNF-α respectively. There was significant positive correlation between the 2 anti-inflammatory cytokine (TGF-β & IL-10) (r=0.361) (figure 1, left) and the 2 pro-inflammatory cytokines (IFN-γ, TNF-α) (r=0.687) (p≤0.05) (figure 2, right). This positive correlation is possibly signifying the harmony in their action. Significant stronger correlation was noticed between the 2 pro-inflammatory cytokines (IFN-γ, TNF-α) than the 2 anti-inflammatory cytokine (TGF-β & IL-10). This may denotes their cooperative struggle to overcome their extremely significant lower level.

On the other hand, there was a negative correlation between the anti-inflammatory cytokine IL-10 and both pro-inflammatory cytokines (IFN-γ, TNF-α) (r = -0.728 and =-0.0925) respectively (figure 3 A&B). However, the strong significant value was only seen between IL-10 and IFN-γ. This possibly indicates the powerful and controlling authority of the anti-inflammatory cytokine IL-10 over the chief pro-inflammatory cytokine IFN-γ. Moreover there was a negative correlation between the anti-inflammatory cytokine TGF-β and both pro-inflammatory cytokines (IFN-γ, TNF-α) (r = 0.289and r=-0.35) respectively (figure 3 C&D). However, these correlations were insignificant.

Fig. 1: Bar chart represents the mean values for gene expression of different cytokines TGF-β IL-10 TNF-α IFN-γ.

Fig. 2: (Left) Positive correlation between the pro-inflammatory cytokines (IFN-γ and TNF-α). (Right) Positive correlation between the 2 anti-inflammatory cytokines TGF-β & IL-10.
Fig. 3: Negative correlation between: the anti-inflammatory cytokine IL-10 and the anti-inflammatory cytokine IFN-γ (A), the anti-inflammatory cytokine IL-10 and the anti-inflammatory cytokine TNF-α (B), anti-inflammatory cytokine TGF-β and the anti-inflammatory cytokine IFN-γ (C) and the anti-inflammatory cytokine TGF-β and the anti-inflammatory cytokine TNF-α (D).

Concerning the control group, representing chronic uncomplicated group of Schistosomiasis haematobium, there was some cases in which undetectable levels were reported. For the anti-inflammatory cytokines TGF-β & IL-10, 3 and 2 out of the 10 cases did not obtain any detectable value concerning gene expression RT-qPCR. As regards the pro-inflammatory cytokines IFN-γ, TNF-α, 5 and 6 out of the 10 cases did show any measurable level. On the other hand there was no statistical significant correlation between the detectable values (P≥0.05). Very weak negative correlation was demonstrated between the 2 anti-inflammatory cytokines (r=−0.0016). There was positive correlation between the 2 pro-inflammatory cytokines (r= 0.014). However, these correlations were insignificant (P≥0.05).

Discussion:

Schistosoma infection is usually progress to a chronic disease because of the ability of schistosomes to evade the immune response and survive in the host for years by a highly polarized Th2 response (Zheng and Blobel, 2011 and Najeeb et al., 2013). Later, the T regulatory cell is responsible for a status of immune modulation during chronic schistosomiasis (Riet et al., 2007). The cellular and/or molecular basis for this process has yet to be distinctly defined. A state of balance between Th1 and Th2 immunological response is usually reached as the infection pass through the chronic phase to avoid abnormal destructive immunological reaction (Rujeni et al., 2012 and Wu et al., 2014). However, this state of balance is clearly not occurred in our cases. Instead, Th2 anti-inflammatory cytokines reported significant higher levels than (Th1). Hence, the contrary was happened, a state of imbalance was recorded between Th2 and Th1; high Th2/low Th1. Therefore, the results of the current study demonstrated that, the Th1/Th2 relation was in favor of Th2 cells domination in this late progressive complicated phase of complicated schistosomiasis infection in the 24 cases included in this work. This Th2 cell mediated the activation and maintenance of both humoral and cellular functions (Zhu and Paul, 2011). This may explain the significant higher ELISA O.D readings reported in our 24 cases than the
control subjects. The dominance of Th2 response instead of usual balance in uncomplicated cases may lead to such increase in humoral response and subsequent antibody production. This activation and maintenance of the Th2 humoral functions and production of related cytokines (IL10 and TGF-β) in addition to the strong antibody production may cause inhibition of several macrophage functions as reported by Nakayamada et al. (2012).

The previous authors also suggested strong role of such Th2 authority to counteract the Th1 responses that explain the significant lower expression level of Th1 cytokines in the current work (IFN γ and TNF α). Despite that, the humoral immunity promoted by Th2, indeed failed to eliminate the Schistosoma egg antigen in the 24 cases included in the current study which has been satisfied with the stage of granuloma formation, which palpably did not prevent the toxicity of the trapped eggs.

In the current study, IL-10 recorded a strong significant negative correlation with the pro-inflammatory IFN-γ. This correlation may indicate the mastering role of the anti-inflammatory cytokine IL-10 in the long standing complicated bilharzial infection (with high expression level) on the pro-inflammatory cytokine IFN-γ (with low expression level). This may confirm the previously mentioned data about the domination of Th2. IL-10 was initially reported to suppress cytokine secretion, however, further investigation has shown that IL-10 predominantly inhibits induction of the pro-inflammatory cytokines TNFα and IFNγ (Varma et al., 2001). The present study suggested a major role for this cytokine in the 24 complicated cases. IL-10 is a cytokine with multiple, pleiotropic, effects in immune regulation and inflammation. Beside its role in down-regulation of the expression of Th1 cytokines, it enhances B cell survival, proliferation, and antibody production. Again this explained the significant higher antibodies in our 24 chronic complicated schistosomiasis cases. Also, its high expression level may corrupt the nature relation between Th1 and T cytotoxic that naturally protects the host against malignant transformation (Zhang and Mosser, 2008). On the other hand, the deficiency in the Th1 cytokines reported in the present study may be behind the arisen complications. This because Th1 is recorded to be beneficial in the differentiation of cytotoxic T lymphocytes (CTL). Moreover, Th1 increase the expression of major histo compatibility complex (MHC-I) on tumor cells, to be more sensitive to lysis by CTLs (Ceri et al., 2014). Furthermore, Th1 activates NK cells and maximizes the killing efficacy of the macrophages and the proliferation of cytotoxic CD8 T cells, thus, Th1 prevents rather than promoting cancer. While this protective function of Th1 was deficient (low expression level) in the 24 cases in the present study. This may clarify the inhibitory role of IL-10 upon the cell mediated immunity against tumor cells. Therefore, tumor cells by the time have a good chance to evade the immune system. The previous findings support, once more the proposed hypothesis in the present work concerning the powerful role of IL-10 during long standing complicated schistosomiasis infection by its controlling effect on Th1 cytokines. These results supported by the accumulating evidence which indicates that patients with chronic inflammation display increased numbers of regulatory cytokines such as IL-10 which counteract anti-tumor immune responses during tumor progression (Shirasaki et al., 2011).

Our findings possibly support the immuno-editing theory reported by Pancione et al., in 2014 who suggested that the immune system is able to recognize and eradicate subclinical tumors, but some point of equilibrium has to be reached to affect tumor cells and remains in situ, in a state of balance with a partially efficacious response. Unfortunately, many tumors escape from this equilibrium state, and cancer becomes clinically apparent, as recorded with cases included in the current research, in which imbalanced immunological response was reported. The findings of the present study suggested that IL-10 may be the key factor in this process due to its only significant strong negative correlation with the chief Th1 cytokine IFN-γ. Therefore the present study recommended the use of this cytokine (IL-10) in the future utilization as prognostic or therapeutic marker in chronic schistosomiasis. Taken, together, these new insights into cytokine gene expression and function in long standing schistosomiasis infection, may offer multiple novel possibilities for intervention in terms of generating a valuable anti-parasitic and anti-neoplasm response. Finally we recommended further studies to explore the roles of other inflammatory cytokines which certainly need more satisfactory budget to perform such expensive technique.

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REFERENCES


Abd El Hamid A, Sabry et al., 2017


