Homozygous Deletion of the Fhit Gene, p21WAF1 Protein Expression and Apoptosis in Bilharzial Bladder Cancer

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Abstract: The FHit gene alterations may occur as early consequences for the urinary bladder cancer. The present study aimed to identify and analyze the role of homozygous deletion (HZD) and transcriptional alterations of fragile histidine triad (FHit) gene in the development and progression of bilharzial bladder cancer in Egyptian patients. The possible associations between FHit abnormalities and some clinical variables that have prognostic impact in bilharzial bladder cancer patients were determined. In addition, the relations between homozygous deletion (HZD) of FHit gene, FHit protein, p21WAF1 expression and apoptosis were demonstrated. We investigated 42 human bladder cancer and 10 normal adjacent tissues as a control group. HZD of the FHit gene, FHit protein, p21 protein and apoptosis were assessed by PCR, immunohistochemistry and DNA ladder method, respectively. FHit HZD deletions have expressed a significant correlation with FHit protein (p<0.04), p21WAF1 protein expression (p<0.005) and apoptosis (p<0.03). On the other side, no correlations were detected between FHit homozygous deletions and tumor type, tumor grade and gender. Therefore, the FHit gene deletions could be important in the development and/or progression of urinary bladder cancers and may be used as an independent prognostic indicator for the clinical outcome in patients with these tumors.

Key words: Cell death, immune suppressive, protein, malignancy, shistosomiasis

INTRODUCTION

Urinary bladder cancer is the fifth most common cancer; it represents 3% of cancer deaths. Its incidence increases rapidly all over the world, about 53,000 cases were diagnosed in 2002 and 60, 240 cases were diagnosed in 2004 in the United States (Greenlee et al., 2000; Jemal et al., 2004). Although 60, 240 bladder cancer cases will be diagnosed in the United States in 2004, very little is known about its molecular etiology. Most bladder cancers arise in the bladder epithelium and regardless of treatment with surgery, chemotherapy or immunotherapy, may recur and/or metastasize. The search for improved therapies remains a high priority in this disease. For most experimental therapies, animal tumor models represent an essential link between vitrotesting and clinical trials (Jemal et al., 2004). Intravesical gene therapy, primarily with adenoviral-mediated gene transfer or other potential viral vectors (Gomella et al., 2001) is a very promising approach for the treatment of refractory superficial bladder cancer (Kuball et al., 2002). Indeed, intravesical administration enables an efficient delivery of therapeutic genes to cancer cells with minimal systemic exposure. Furthermore, the response to treatment is easily done by analysis of urine samples, cystoscopic examination and biopsy. Cytogenetic studies of bladder cancer have helped to define two clinically distinct subtypes: benign tumors with few genetic mutations and a stable karyotype and aggressive cancers with chromosomal instability and many non-random cytogenetic aberrations. While the cytogenetic data does not provide complete information, these studies have been important for suggesting pathways for bladder carcinoma initiation and its progression. In addition, molecular cytogenetic studies have proven useful for diagnosing bladder cancer and for monitoring patients for cancer recurrence (Wolff, 2007).

S. haematobium infection affects the bladder by deposition of ova causing chronic granulomatous injury and permanent loss of mature superficial cells producing hyperplastic urothelium consisting of immature cells with high tendency to undergo malignant transformation (Fadl-Elmnula et al., 2002).

The molecular genetic investigations have revealed that the incidence and prevalence of different types of urinary bladder cancer were emerged by many different genetic chromosomal alterations predisposing different

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tumor genes and its effect on the tumor suppressor genes. The suppressor activity of FHIT could be related to apoptotic cascades and to the alteration of cell cycle regulatory factors and its down regulation has been correlated with a variety of clinicopathologic factors such as disease stage, tumor progression and recurrence in several cancers (Capuzzo et al., 2000) but the value of FHIT as a potential predictor marker of survival is not clear. The fragile histidine triad (FHIT) gene located on chromosome 3p14.2 is frequently deleted in human tumors (Andrea et al., 2004).

Various morphological changes of apoptosis in bladder cancer subjects receiving intravesical chemoinmunotherapy or systemic chemoradiation, include compaction of nuclear hromatin (pyknosis), loss of cellular volume and chromatin condensation on the nuclear envelope (nuclear crescents) After chromatin condensation, convolution of the nuclear cellular outlines, referred to as Meriosis, proceeds the collapse of the nucleus and its disintegration into sphere like fragments. These fragments become sealed by plasma membrane to form several apoptotic bodies in which closely packed organelles appear intact. Apoptosis can be expressed as an apoptotic index i.e. the number of apoptotic cells divided by the population of cancer cells Apoptotic index is related to tumour prognosis. Various cellular and biochemical changes in apoptosis can be measured by DNA electrophoresis and flow cytometry (Bashir, 2005).

The present study was conducted to assess the correlations between FHIT homozygous deletions (H2D), tumor grade, programmed cell death (apoptosis), p21\textsuperscript{WAF1} and FHIT protein expression in bilharzial bladder cancer.

**MATERIALS AND METHODS**

**Patients and tissue collection:** Samples were obtained from 42 patients suffering from bladder cancer and 10 adjacent normal tissues as control. All subjected samples were collected from patients undergoing bladder cancer surgery at the National Cancer Institute (NCI), Cairo University, Egypt during the period year 2003 to 2005. All cases were classified and graded according to the World Health Organization criteria and all patients presented with an approval for the research aims. Patients enrolled in the current study were classified as follows according to sex 73.8% (31/42) males and 26.2% (11/42) females, age (Males from 59-69 years and females from 59-74 years), type of the pathological tumor (squamous cell carcinoma SCC and transitional cell carcinoma TCC) and the grade of tumor (Grade II and III). Fresh tumor and normal tissues were obtained at the operation and divided into two pieces. One fragment was immediately snap frozen and stored at -80°C for subsequent DNA extraction. The other was fixed in neutral buffered formalin and processed for histological examination to determine the routine histopathological examination and immunohistochemistry. Determination of Schistosomiasis in patients studied was according different aspects as follows: previous clinical history of Bilharziasis, history of previous hematuria not related to the current malignancy and histological verification of the presence of schistosomal cystitis in the bladder mucosa close to the tumor. All collected samples were subjected to immunohistochemical analysis of p21\textsuperscript{WAF1} and FHIT protein, DNA fragmentation for apoptosis and PCR for detecting FHIT homozygous deletions.

**Immunohistochemical detection of FHIT and p21\textsuperscript{WAF1} proteins**

**II.B.1. FHIT protein:** Tumor tissues were fixed in a 10% formaldehyde solution and embedded in paraffin. Four-micrometer sections were cut and mounted on slides. Staining was performed using the avidin-biotin method. Anti-FHIT polyclonal antibody (F-130, 1:80 dilutions; IBL, Gunma, Japan) was used for FHIT detection. Sections were incubated 12 h at 4°C with antibody in PBS containing 5% goat serum and were counterstained with hematoxylin. The absorption test was done using FHIT antigen peptide according to manufacturer’s instruction to evaluate the specificity of the antibody. For the negative control, the primary antibody was replaced with normal rabbit serum. As positive control we used tumor tissue with the wild-type of FHIT gene.

**p21\textsuperscript{WAF1} protein:** Immunohistochemical detection of p21\textsuperscript{WAF1} protein activity was done as described by Pavelic et al. (1990). The tissue sections were fixed and the endogenous peroxidase activity was quenched by 15 min incubation in methanol containing 3% hydrogen peroxide (Sigma, Taufkirchen, Germany). Non-specific binding was blocked by applying normal rabbit serum in a humidity chamber at a dilution of 1:10 for 30 min. Primary mouse monoclonal antibody to p21\textsuperscript{WAF1} (Oncogene Science, Cambridge, MA) at a concentration of 5µg mL\textsuperscript{-1} were applied overnight at 4°C. The secondary antibody (rabbit to mouse immunoglobulins; DAKO, Glostrup, Denmark) was applied for 1 hr at room temperature. Peroxidase-antiperoxidase (PAP, DAKO, Glostrup, Denmark) conjugate diluted 1:100 in Phosphate-Buffered Saline (PBS) was applied for 45 min at room temperature. The slides were stained with diaminobenzidine tetrahydrochloride (DAB, Sigma) and then counterstained with hematoxylin. Finally, the slides were mounted in Canada balsam. Specificity controls comprised
pre-absorption of monoclonal antibodies with their appropriate antigen/peptides as recommended by the manufacturer. Antibodies were titrated to the lowest dilution having acceptable background staining. The localization and level of specific immunostaining for each slide was evaluated in the whole tumor area.

The relative level of specific immunostaining and its localization were judged. The relative intensity of cell immunostaining was evaluated, semi-quantitatively, so that no staining was denoted (0), weak staining was denoted (+1), moderate (+2) and strong (+3).

DNA extraction: Tumor and normal tissues were rapidly frozen after excision in liquid nitrogen. Tumor tissues (transitional cell tumors, TCC) and (squamous cell carcinoma, Sqcc) between 200 mg to 1 g were grinded with a prechilled mortar and pestle to fine powder. The powdered tissues were suspended in 1.2 mL digestion buffer (100 mM NaCl, Sigma, USA, 100 mM Tris·Cl pH 8 Sigma, USA, 25 mM EDTA, pH 8 Sigma, USA 0.5% sodium dodecyl sulphate, sigma, USA, 0.1 mg mL⁻¹ proteinase K. The proteinase K is labile and must be added fresh with each use) per 100 mg tissue and incubated in shaking water bath at 50°C for 12 to 18 h in tightly capped tubes and prepared for the DNA extraction. DNA extraction from tissues was carried out according to the standard method of Pavelic et al. (2001) using the phenol, chloroform. Extracted DNA samples were stored at -80°C until use.

FHIT gene homozygous deletions analysis: FHIT gene was examined by multiplex-PCR for the seven coding exons from 3-9 for detecting the homozygous deletions of fragile histidine triad genes using the specific set of primers (STRATA-GENE, Alameda, California, USA) for each exon as mentioned in Table 1. PCR reactions were done in 25 μL reaction in Perkin Elmer thermocycler. Gene Amp 2400 (Norwalk, CT, USA). The PCR reactions were carried out with the following conditions, denaturation 94°C for 4 min, 35 cycles (denaturation 94°C for 1 min, annealing for 1 min and extension 72°C for 2 min), extension 72°C for 10 min. The products of amplification were then analyzed in 1.5% agarose gel electrophoresis in a submrame (Biometra gel system, USA), using 100 bp DNA Ladder to detect the specific bands according to primers used in test.

Apoptosis analysis: DNA Ladder method: DNA fragments were isolated according to the method described by Herman et al. (1994). Tumor and normal adjacent tissues were disaggregated, pelleted by centrifugation and washed two times in PBS. The cells were resuspended for 10 sec in 100 μL of lysis buffer and centrifuged for 5 min at 3000 g. The supernatant was transferred to a new eppendorf tube and incubated with 100 μL of lysis buffer and centrifuged again. The supernatants were pooled together and incubated for 2 h in 1% SDS and RNase (5 μg mL⁻¹) at 56°C followed by proteinase K addition in final concentration of 2.5 mg mL⁻¹ and incubated again overnight. DNA fragments were pelleted by addition of 1/2 volume of 10 M ammonium acetate and 2.5 volume of prechilled absolute ethanol and centrifugation was carried out 40 min at 12000 g. The pellet was washed with 70% ethanol more than one, centrifuged for 10 min at 12000 g, dried and dissolved in 20 μL of TE buffer. The fragmented DNAs were seen as a ladder DNA in 1.5% agarose gel.

Statistical analysis: Statistical analysis and calculations of data was assessed using Microsoft software program (SPSS 12.0, Chicago, IL, USA). For ordinary data, descriptive statistics are presented with percentages from a contingency table. Fisher's exact test was used to compare the determined FHIT gene alterations (homozygous deletions) with tumor type, tumor grade,
sex, FHIT protein, p21WAFT protein expression and apoptosis. Differences with p<0.05 were accepted as statistically significant.

RESULTS

The homozygous deletions in the FHIT gene distributed among different exons from 3-9 in chromosome 3 were twenty five samples out of forty two (59.5%). Among the thirty three tumor tissues characterized pathologically as squamous cell carcinoma, we detected twenty samples (60.6%) with FHIT homozygous deletions and thirteen samples (39.3%) expressed with normal FHIT gene pattern (Fig. 3). For the nine tumor tissues identified as transitional cell carcinoma, it was found five samples (55.5%) with deletions and four samples (44.4%) with normal pattern (Table 2).

On the level of histopathological tumor grade, there were twenty two samples (62.85%) out of thirty five with grade II tumor expressing homozygous deletions, while thirteen samples (37.1%) were detected as normal FHIT gene without any deletions. Among seven samples with grade III, we detected two samples (28.57%) and five samples (71.4%) were FHIT homozygous deletion and FHIT normal, respectively.

Thirty one male samples with urinary bladder cancer were characterized as follows, seventeen (54.8%) and fourteen (45.1%) with positive FHIT deletion and negative FHIT gene deletion, respectively. Among the eleven females tumor tissues, FHIT gene with deletions were seven samples (63.6%) and four samples (36.3%) expressed normal FHIT gene pattern (Table 2).

Therefore, previous results revealed no significant statistical correlations between the FHIT gene homozygous deletions and type of tumor (SCC and TCC), histopathological tumor grade (II and III) and the gender differences (male and female) (Table 2).

For further characterization of FHIT gene involvement in the apoptotic process, we looked for anti-apoptotic proteins such as p21WAFT protein and characterization of the DNA fragmentation. The detection of the p21WAFT protein expression was carried out with Immuno-histochemistry staining. All normal adjacent tissues showed positive strong staining for p21WAFT protein, while the tumor tissues with grade II and III and squamous cell carcinoma SCC and transitional cell carcinoma TCC revealed different scales of staining (Fig. 1a, b) as follows: five samples (11.9%) with score +1, nine samples (21.4%) with score +2 and three samples (7.1%) with score +3. Moreover, there was no statistical significant correlations between the different scores of p21WAFT protein expression density and the type and the grade of tumor. Among the twenty five urinary bladder cancer samples with negative p21WAFT protein expression, twenty two (88%) samples were detected as tumor tissues with homozygous deletions for FHIT gene distributed among different coding exons and three samples (12%) with normal FHIT without any homozygous deletions. While, among the seventeen samples with positive p21WAFT protein stain, two samples (11.7%) and fifteen samples (88.2%) were detected with FHIT deletion and normal FHIT gene, respectively.

On the other hand, FHIT protein expression was detected (40.4%) positive in all cases, three samples out of sixteen (18.75%), (81.25%) were detected positive and negative HZD respectively. Twenty six samples (61.5%) were discovered loss of expression of FHIT protein, twenty-one samples (80.76%) were presented positive for HZD and five samples(19.2%) were expressed FHIT gene detection of DNA fragmentation. In present study, we get eighteen samples (42.8%) with positive apoptosis markers and twenty four (57.1%) expressed negative apoptotic markers without DNA fragmentation (Fig. 2). Among the

Table 2: Correlations between homozygous deletions, clinicopathological features of the patients, p21WAFT1, apoptosis and FHIT protein

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No 42</th>
<th>FHIT(HZD)</th>
<th>-17N (%)</th>
<th>+17N (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology type</td>
<td>SCC</td>
<td>33</td>
<td>13 (39.3)</td>
<td>20 (60.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>TCC</td>
<td>9</td>
<td>4 (44.4)</td>
<td>5 (55.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td>GII</td>
<td>35</td>
<td>13 (37.1)</td>
<td>22 (62.9)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>GIII</td>
<td>7</td>
<td>5 (71.4)</td>
<td>2 (28.6)</td>
<td>NS</td>
</tr>
<tr>
<td>p21WAFT</td>
<td>Male</td>
<td>31</td>
<td>14 (45.1)</td>
<td>17 (54.8)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11</td>
<td>4 (36.3)</td>
<td>7 (63.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>+</td>
<td>17</td>
<td>15 (88.2)</td>
<td>2 (11.8)</td>
<td>0.005*</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>25</td>
<td>3 (12)</td>
<td>22 (88)</td>
<td>NS</td>
</tr>
<tr>
<td>FHIT protein</td>
<td>+</td>
<td>16</td>
<td>13(81.25)</td>
<td>3 (18.75)</td>
<td>0.04*</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>26</td>
<td>5 (19.23)</td>
<td>21 (80.76)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*p value<0.05 significant, NS: Not significant

Fig. 1: The immunohistochemistry of the p21WAFT protein among urinary bladder cancer. (a) The positive Immuno-histochemistry staining of p21WAFT (b) The negative immuno-histochemistry staining of p21WAFT.
Therefore, 25 different types of aberrant FHIT involving various exons were observed.

DISCUSSIONS

Cancer cell is malicious and characterized by its different complex interactions of environmental and infectious causes that trigger chromosomal genetic disorders. Furthermore, the chromosomal fragile sites mutations and deletions have been implicated to have a pivotal role in cancer proliferations and progression (Webster et al., 2002). In the present study we have identified 60.6% of the squamous cell carcinoma and 55.5% of transitional cell carcinoma expressing the homozygous deletion of FHIT gene. This revealed that there is no significant statistical correlation for homozygous deletion of FHIT gene with the histological tumor type. Also, Tanimoto et al. (2000) did not showed any significant correlation between the status of FHIT deletions and any epidemiological factor, including age at cancer onset, sex and sub site among oral cancer samples. As they dedicated the reasons for that there is more than one carcinogenic reason or pathway for the emergence of the oral tumorgenesis.

Whereas, squamous cell carcinomas of the head and neck cell lines revealed high degree of abnormal FHIT with loss of homozygous pattern (Franceschi et al., 1990; Ah-See et al., 1994). The presence of normal FHIT gene fragile sites in squamous cell carcinoma lines was also detected by Virgilio et al. (1996). The occurrence of normal and aberrant transcripts in the same cell line might be explained by the presence of several distinct cell populations, which would obscure the detection of homozygous deletions in a subpopulation (Virgilio et al., 1996). Several documents have repeatedly supported our TCC demonstrations. On the other hand, Buffa et al. (2000) have recorded 87% abnormal transcripts of FHIT gene with significant correlation with progression of primary TCC bladder cancer.

The inactivation of FHIT gene and reduction in FHIT protein expression in bladder tumors is probably a result from deletions within both alleles, as described for other carcinomas, particularly tumors resulting from exposure to environmental carcinogens. Further studies will be required to define the exact mechanisms leading to loss of FHIT homozygosity in urinary bladder cancer.

In present study, the different histological tumor degrees (II and III) and the gender differences (male and female) did not show a significant correlation with the HZD of FHIT gene, early stages of tumor and viability of more different types of tumor with different stages and activity. Conversely, Campiglio et al. (1999) stated high
significant correlation (p<0.0013) between the absence of FHIT expression and advanced stage of the primary tumor among transitional cell carcinoma of the bladder. In addition, reduced FHIT expression is correlated with a more aggressive disease in both bladder and breast cancers (Capuzzo et al., 2000).

For the p21 WAF1 expression protein the significant correlation between the FHIT homozygous deletion and activity of p21 WAF1 expression was apparent with (p<0.005). We detected about 88% of cases with homozygous deletions in fragile sites of FHIT gene were negative for the expression of p21 WAF1 to 12% were with normal FHIT pattern. Further, we could not report any correlation between expression of p21 WAF1 and clinical or pathological parameters of histological tumor type and grade. This may be attributed to the excision time of tumor or may be early squamous or transitional tumors.

p21 WAF1 is one of the important factors regulating malignant cells progression in oral tongue squamous cell carcinoma. Eissa et al. (1999) have shown that the lower expression of p21 WAF1 was evident and prevalent in Squamous Cell Carcinoma (SCC) and schistosomal subtype than in Transitional Cell Carcinoma (TCC) and nonschistosomal tumors. These results have concluded that patients with p21 WAF1 positive tumors had a decreased probability of tumor recurrence and progression, so loss of p21 WAF1 expression is a statistically significant and independent predictor of bladder cancer progression.

Present study provided a significant correlation of the FHIT deletion and the apoptotic pathway through the DNA fragmentation (p<0.03). 83% of the samples did not express DNA fragmentation and expressed high level of FHIT deletion. From our current results we reported the role of the homozygous deletion in FHIT gene and its resulting protein expression impairments will lead to influence tumorgenesis and will affect on the consequences chain of the apoptotic cascade and p21 WAF1 apoptotic protein that inhibit the cell cycle and proliferation.

On the level of distribution of exons deletion in FHIT gene and its effect on the expression of the FHIT protein, p21 WAF1 and apoptosis, we found 44 and 52% of the cases were with two exons and more than two exons deletion, respectively. Moreover, there was not a relation between the increased rate of exons deletions and the different levels of p21 WAF1 protein expression or apoptosis. Further, we found that the most prevalent deletions in exons were expressed in exon 9, 5 and 4 with 68, 60 and 52%, respectively among the all cases demonstrated with homozygous deletion for FHIT gene. This may suppose the cooperative and segregated effect of deletions on the tumor genesis. Present observations were in accordance not only to those described by Ohba et al. (1996) with the involvement of exons 5 and 9 in low expression of FHIT protein but also to Lux et al. (1997) for his five novel expressed sequence tags within 3p14.2 related to exon 9. Further, Zekri et al. (2005) have reported the highest incidence of deletion in FHIT gene with 78.2% were distributed in exon 9 and exon 7 with 52 and 4.3%, respectively among cases with hepatitis C virus-associated hepato-cellular carcinoma.

The HZD of highest incidence among males of grade II was in exons 5 and 9. The lowest value was in exons 3, 4, 7, 8. In contrary, female samples of grade II expressed high prevalent deletions among exon 3 and 4 more than the deletions rate among 5 and 9 exons.

Present findings are similar to those described by Sarad et al. (1999) who have indicated that the observed growth inhibitory effect in FHIT-reexpressing cells could be related to apoptosis and cell cycle arrest. Pavlic et al. (2001) have revealed also that tumors with aberrant FHIT gene showed much lower intensity of apoptosis in comparison to tumors with normal FHIT gene.

CONCLUSION

The Present study prove and formulate a very attractive hypothesis that the homozygous deletion of FHIT gene allele, chromosome rearrangement at FRA3B and translocations are associated with development of urinary bladder cancer through the disturbance of related cell cycle inhibitors and low index apoptotic proteins leading to more aggressive tumor behavior. Therefore, it could be considered as one of early prognostic biomarkers and events contributing to/or reflecting the malignant transformation of Bilharzial bladder cancer. Moreover, it is argues to suggest establishing some of these parameters that would probably be effective in public demand and related risk factors of Schistosomiasis to emerge urinary bladder cancer. Present results clearly indicate a correlation between aberrant FHIT gene, increased cell proliferation (low p21 WAF1, FHIT protein) expression and low rate of apoptosis.

The ultimate goal is to develop reliable prognostic markers which will accurately predict not only the expected clinical course of an individual bladder tumor but also the response of that tumor to currently available therapies. More importantly, this information may be employed in the future to dictate altogether new treatments for the prevention and/or stabilization of the early molecular events that lead to the development of bladder cancer.
REFERENCES


