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DNA Methylation in Human Genes for *Schistosoma*-Associated and Non-*Schistosoma*-Associated Bladder Cancer

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Abstract

This study is to analyze the usefulness of DNA methylation in eight candidate genes as tumor markers in bladder cancer of Schistosoma-associated and non-Schistosoma-associated bladder cancer. Methy Light assay was utilized to investigate the DNA methylation status of eight cancer related genes using DNA extracted from paraffin-embedded (FFPE) tissues of Saudi patients with bladder cancer of both Schistosoma-associated and non-Schistosoma-associated. These genes include TIMP3, RASSF1A, SLIT2, SOCS1, RUNX3, NEUROG1, IGF2 and CACNA1G. 85% of the investigated samples displayed detectable methylation level in one up to six genes. None of the 45 samples reacted positively to all genes. On other hand, only seven cases did not display any methylation. The correlations between methylation and the investigated genes were illustrated. SLIT2 was the most frequently methylated gene and none of the investigated cases showed methylation to all eight genes. Methylation in Saudi patients with non-Schistosoma-associated bladder cancer was higher than the Schistosoma-associated bladder cancer. There is a need for further work covering panel of genes to correlate them with further factors related to the clinical and pathological aspects.

Keywords: Parasite, schistosoma, cancer, bladder, genes, markers.

Introduction

Schistosomiasis or bilharzia is a widespread endemic parasitic disease caused by blood flukes trematode belonging to the genus *Schistosoma*. According to the world health organization, more than 200 million people are infected, 20 million people with severe complications, about 650 million people at risk of infection with schistosomiasis and 15000 people die annually due to schistosomiasis, mostly due to bladder cancer[1].

The major three human schistosome species are *Schistosoma mansoni*, *S. japonicum* and *S. haematobium*. The last one is the most prevalent and widespread species in Africa and the Middle East[2]. Eggs of these parasites pass out in the urine or faeces of infected people into water. Larval stage from the egg, known as a miracidium penetrates proper snails. These intermediate snail hosts release infective forms cercariae, which penetrate the skin of people in the water.

In the body, the immature schistosomula develop into adult schistosomes, which live in the blood vessels surrounding the intestine and bladder. The females then begin releasing eggs, some of which are passed out of the body in the urine or faeces then into water and life cycle is repeated. Other eggs are trapped in body tissues, causing inflammatory response. In intestinal schistosomiasis, there is progressive enlargement of the liver and spleen, intestinal damage, and hypertension of the abdominal blood vessels. In urinary schistosomiasis, there is progressive damage to the bladder, ureters and kidneys[3].

Routine microscopic diagnosis depends mainly on the morphological features and size of the eggs. Control of schistosomiasis is based on drug treatment, snail control, improvement of sanitation and health education.

Bladder cancer is associated with several causes including exposure to certain chemicals, cigarette smoking, bacterial infections, immunological status, environmental exposure and gender of patient (mainly in males)[1, 4-7]. The mechanisms that by which *S. haematobium* induces bladder cancer are not fully understood, however, evidences to support the relationship between schistosomiasis and bladder cancer was discussed including epidemiological evidence, experimental induced schistosomiasis, histopathological findings, etiological factors, age and gender of patients[2, 7-10].

Bladder cancer is the ninth most common cancer in the world. In Saudi Arabia, bladder cancer is ranked as the fifth most frequent cancer among men representing 5.7% of the cases[11, 12]. This cancer occurs mainly as transitional cell carcinomas or as squamous cell carcinoma. The major histological cell type of bladder cancer associated with schistosomiasis of the urinary tract is squamous[13,14].

In recent years, several studies from research laboratories around the world have been published to compare between

cellular pathology and molecular biology regarding bladder cancer[15-28]. These studies enriched the knowledge for a better understanding of the genetic data of carcinogenesis and therapy.

A study was published in 2004 by Gutierrez and others[25], which used methylation-specific PCR to characterize 12 cancer-related genes using DNA from samples of *Schistosoma*-associated and non-*Schistosoma*-associated bladder cancer. The authors reported that Methylation of at least one gene was detected in all squamous cell tumors except two, and 45% of samples had at least three methylated genes. The average methylation index was 0.24, corresponding to three of the 12 analyzed genes. *Schistosoma*-associated bladder tumors had more genes methylated than non-*Schistosoma* bladder tumors. The overall profile of methylation was similar, with *Schistosoma*-associated cases having a higher methylation index. Then they suggested that schistosomal involvement associates with a greater degree of epigenetic changes in the bladder epithelium.

DNA methylation of CpG islands, a CG rich region located around the 5' promoter region of the gene, where cytosine and guanine are connected by "p" a phosphodiester bond. The CG sequences in inactive genes are usually methylated to inhibit their expression. Analysis of DNA methylation depends on the detection of 5-methyl-cytosine residue in the context of a CpG dinucleotide concentrated in CpG island. MethyLight is a major advance in the form of real-time detection of methylation using qPCR technology. This technique uses the TaqMan approach to amplify and quantify methylation levels in any sample. In MethyLight, two methylation-specific primers are used to amplify a region of interest from bisulfite-converted DNA. The primers span a region targeted with a fluorescence-labeled oligonucleotide probe targeting the methylated CpG within that sequence. This probe is synthesized with a 5'-fluorochrome and a 3' quencher. The 5'–3' exonuclease activity of the Taq polymerase would cleave the probe, releasing the fluorochrome from the proximity of the quencher moiety and freeing to emit light upon excitation[29].

In our study, MethyLight assay was used to analyze the usefulness of DNA methylation in eight candidate genes as tumor markers in bladder cancer in paraffin-embedded tissues specimens of *Schistosoma*-associated bladder cancer and non-*Schistosoma*-associated bladder cancer.

Material and Methods

Specimens: This study included 45 paraffin-embedded tissues specimens of *Schistosoma*-associated bladder cancer (n=19) and non-*Schistosoma*-associated bladder cancer (n=26). These specimens were obtained from Saudi patients from several hospitals in different areas in Saudi Arabia, some of which are endemic with *S. haematobium*. Diagnosis of was confirmed by specialized histopathologists.

DNA Methylation: DNA was extracted from 10 µm-thin formalin-fixed paraffin-embedded slices using the Qiagen QIAMP Formalin-fixed Parafin-embedded Tissue DNA extraction kit, following the manufacturer's guidelines. Up to 0.5 µg of DNA was used for bisulfite conversion using the Qiagen Epitect Bisulfite Conversion kit. DNA methylation analysis was performed using MethyLight as described by Dallol et al., 2011[29].

Tested Genes: The methylation levels of eight genes were investigated in this study using the primer-probe combinations listed in table-1: TIMP3, RASSF1A, SLIT2, SOCS1, RUNX3, NEUROG1, IGF2 and CACNA1G.

A probe targeting bisulfitemodified Alu repeat sequences was used to normalise for input DNA. The specificity of the reaction was ascertained using sssl-treated and bisulfite-modified positive control DNA (Qiagen) and the negative control DNA (Qiagen). The percentage of fully methylated reference (PMR) was calculated by dividing the gene:Alu ratio of a sample by the gene:Alu ratio of the positive control DNA and multiplying by 100. Samples with PMR>10 were considered positive for methylation, whereas samples with PMR<10 were considered negative (i.e. unmethylated).

Statistical Analysis: All statistical tests were performed using PASW_Statistics 18.03. (SPSS, Inc., Chicago, IL USA).

Ethical Approval: This study was approved by the Ethics and Research Committee in the Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia.

Results and Discussion

In this study, 45 bladder cancer specimens were analyzed, 19 (42.2%) were *Schistosoma* associated and 26 (57.8%) were non-*Schistosoma* associated. Methylight technology was used for analysis of DNA methylation in 8 different genes; TIMP3, RASSF1A, SLIT2, SOCS1, RUNX3, NEUROG1, IGF2 and CACNA1G. It was very clear that six genes undergo frequent methylation. SLIT2 (68.89%), RASSF1A (60%), RUNX3 (60%), NEUROG1 (46.67%), SOCS1 (31%), and IGF2 (26.67%). For the remaining two genes; TIMP3 was methylated in 4 specimens (8.89%) and CACNA1G in 2 specimens (4.44%). None of the samples showed methylation to all genes and only seven cases (15.56%) did not demonstrate any methylation, while 84.45% of the cases demonstrated one to six genes methylated (figure-1,2,3).

We identified strong correlation between methylation of RASSF1A with RUNX3 (P= 0.003), NEUROG1 (P= 0.007) and SLIT2 (P= 0.025). Highly significant association was observed between the methylation of RUNX3 with SLIT2 (P< 0.0001) and NEUROG1 (P< 0.0001), while with SOCS1 (P= 0.002) and IGF2 (P= 0.05). Methylation of SOCS1 was also associated with SLIT2 (P= 0.02) and NEUROG1 (P= 0.004).

Epigenetic abnormalities are associated with cancer development. DNA Methylation pattern of promoter region of

tumor suppressor genes is used as a target for recognition of many types of human cancer. According to that, not all same genes are hypermethylated in all cancer cases.

Methylation of five genes (TIMP3, RASSF1A, SOCS1, RUNX3 and IGF2) were analyzed in previous bladder cancer studies[2, 9,10,30-45], while to the best of our knowledge, three genes in our study (SLIT2, NEUROG1 and CACNA1G) are investigated for the first time in bladder cancer; however they were investigated in other cancer types[46,47]. SLIT2 was the most frequently methylated gene in about 70% of the samples in the present study, however none of the previous workers investigated this gene in bladder cancer.

In the present study, none of the genes showed methylation in all samples, while seven samples did not reveal any methylation at all. There was no methylation of TIMP3 in *Schistosoma*-associated bladder cancer specimens, while CACNA1G was methylated in one specimen from each group of samples.

Non-*Schistosoma* associated bladder cancers revealed higher extent of methylation (median methylation index = 0.5, average methylation index = 0.4) than *Schistosoma*-associated bladder cancers (median methylation index = 0.25, average methylation index = 0.29). Methylation of SLIT2 and IGF2 were equally distributed between *Schistosoma* associated and non-*Schistosoma* associated bladder cancers, while methylation of RASSF1A, RUNX3, NEUROG1 and SOCS1 tends to be more common in non-*Schistosoma*-associated bladder cancers. CACNA1G methylated only in one sample of each group of cancer, while TIMP3 methylated only with four samples of non-*Schistosoma* associated bladder cancers. These observations contradict with previous report[25]. Other factors such as age, geographical variation, cancer stage may play role in the variability of reported results.

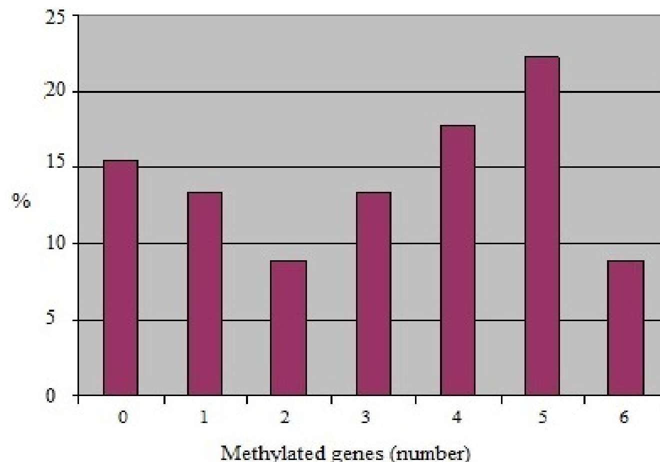

Figure-1
Sample Frequency (%) for the number of methylated genes

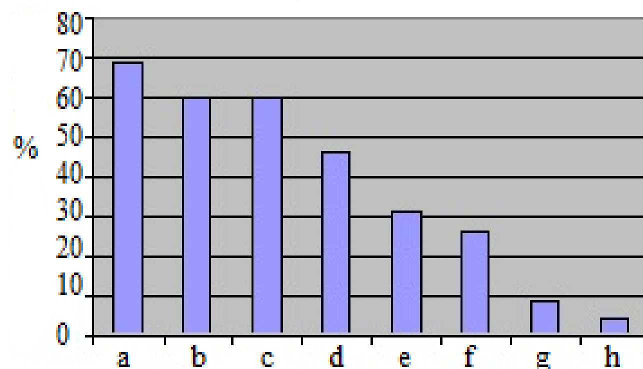

Figure-2
Methylation frequency (%) of each gene. a=SLIT2, b=RASSF1A, c=RUNX3, d=NEUROG1, e=SOCS1, f=IGF2, g=TIMP3 and h=CACNA1G

Table-1
Primers and probe sequences used for methylation analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe oligo sequence (5'-3')
IGF2	GAGCGGTTTCGGTGTCGTTA'	CCAACTCGATTTAAACCGACG	VIC-CCCTCTACCGTCGCGAACCCGA-TAMARA
SOCS1	GCGTCGAGTTCGTGGGTATTT	CCGAAACCATCTTCACGCTAA	6FAM-ACAATTCCGCTAACGACTATCGCGCA-TAMARA
NEUROG1	CGTGTAGCGTTCGGGTATTTGTA	CGATAATTACGAACACACTCCGAAT	6FAM-CGATAACGACCTCCCGCGAACATAAA-TAMARA
CACNA1G	TTTTTTCGTTTCGCGTTTAGGT	CTCGAAACGACTTCGCCG	6FAM-AAATAACGCCGAATCCGACAACCGA-TAMARA
RUNX3	CGTTCGATGGTGGACGTGT	GACGAACAACGTCTTATTACAACGC	VIC-CGCACGAACTCGCCTACGTAATCCG-TAMARA
RASSF1A	ATTGAGTTGCGGGAGTTGGT	ACACGCTCCAACCGAATA CG	6FAM-CCCTTCCCAACGCGCCCA-BHQ1
SLIT2	CAATTCTAAAAACGCACGACTCTAAA	CGGGAGATCGCGAGGAT	6FAM-CGACCTCTCCCTCGCCCTCGACT-BHQ1
TIMP3	GCGTCGGAGGTTAAGGTTGTT	CTCTCCAAAA TTACCGTACGCG	6FAM-TTCGGCGGGCGAGCGAGTT-BHQ1
ALU	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA	ATTAACTAAACTAATCTTAAACTCCTAACCTCA	VIC-CCTACCTTAACCTCCC-MGBNFQ

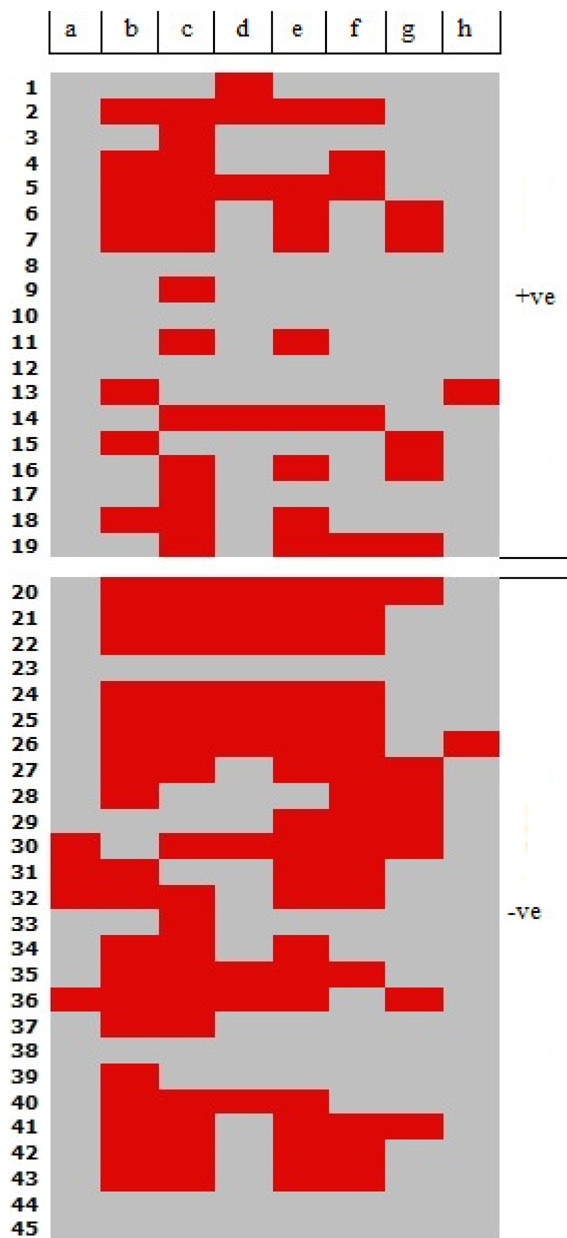

Figure-3
Methylation of genes in *Schistosoma* associated (1-19) and non-*Schistosoma* associated (20-45) bladder cancer. Red areas demonstrate methylation and gray areas represent unmethylation. a=TIMP3, b=RASSF1A, c=SLIT2, d=SOCS1, e=RUNX3, f=NEUROG1, g=IGF2 and h=CACNA1G.

Conclusion

The pattern in DNA methylation between *Schistosoma*-associated and non-*Schistosoma* associated bladder cancer confirm that the parasite has an etiological factor. However, there is a need for further studies covering panel of genes to correlate them with further factors related to the age, sex, histological stages and clinical aspects of the patients. This will widen our understanding of the diagnosis and prognosis of bladder cancer caused in presence and absence of the parasite.

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