Microsatellite (D9S905) Instability in Two Urogenital Schistosomiasis Patients with Abnormal Squamous Cells in the Urine

Olaoluwa P. Akinwale¹*, Pam V. Gyang¹ and Morakinyo B. Ajayi²

¹Public Health and Epidemiology Department, Nigerian Institute of Medical Research, P.M.B. 2013, Yaba, Lagos, Nigeria.
²Microbiology Department, Nigerian Institute of Medical Research, P.M.B. 2013, Yaba, Lagos, Nigeria.

*Correspondence should be addressed to Olaoluwa P. Akinwale: pheabian@yahoo.co.uk
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Abstract

Background: Schistosomiasis ranks second to malaria among parasitic diseases of socio-economic and public health importance. Schistosoma haematobium infection-causing urinary schistosomiasis is widespread in Nigeria. Literature has shown that bladder cancer of squamous cell carcinoma type could be associated with long-term S. haematobium infection. Many studies have also reported genomic alterations in cancers and have detected identical microsatellite instability in bladder cancer and corresponding urine sediment from the same patients. Therefore, this study aimed to detect microsatellite instability in genomic DNA obtained from exfoliated urine cells of S. haematobium infected participants using microsatellite marker D9S905, which is one of the genetic markers located around regions of frequent chromosomal loss in bladder cancer.

Methods: Genomic DNA was extracted from urine and blood of 24 S. haematobium infected study participants. Microsatellite marker D9S905 located on locus 9q34.2 with allele size 294 base pair (294bp) was analyzed to identify genetic variation between exfoliated urine cells and corresponding blood samples from the infected participants.

Result: Microsatellite alterations due to allelic loss were seen in the DNA of exfoliated urine cells of 2 of the participants. Also, previous cytopathological examinations of exfoliated urine cells of these 2 participants revealed squamous cell abnormalities.

Conclusion: Bladder cancer is one of the severe complications of chronic S. haematobium infection. Since not all cases of the infection are chronic, this might have accounted for the low number of cases of microsatellite instability seen among participants in this study.

Keywords: S. haematobium infection, urogenital schistosomiasis, microsatellite instability, bladder cancer.
1.0 INTRODUCTION

*Schistosoma haematobium*, a species of digenetic trematode belonging to the genus *Schistosoma* caused urinary schistosomiasis. The disease ranks second to malaria among parasitic diseases of socio-economic and public health importance. It is a water-borne disease transmitted by freshwater snails. It is endemic in 76 countries of the tropics and sub-tropics, and it is estimated that there are more than 200 million people infected. Of these, about 120 million suffer severe consequences of the infection, and a further 600 million are at risk, with an estimated annual mortality rate of about 20,000 worldwide [1]. In most endemic areas, the highest intensities of infection are found in children between 5 and 15 years of age [2]. Urinary schistosomiasis is endemic in Nigeria, is more prevalent in the South-Western part of the country, while an estimated 30 million Nigerians need to be treated annually for the disease [3, 4]. There are very few studies on the epidemiology of urinary schistosomiasis-related bladder cancer in Nigeria. Two studies reported cases of bladder cancer, predominantly squamous cell carcinoma (SCC), among urinary schistosomiasis patients in North-Western and North-Eastern parts of the country [5, 6]. Bladder cancer, mostly Squamous Cell Carcinoma (SCC), has been associated epidemiologically and clinically with long-term infection with *S. haematobium* infection [7]. It is difficult to diagnose without invasive measures such as cystoscopy, ultrasonography, and cytology. As a result of which there is little information on its epidemiology. However, most human cancers are characterized by specific genomic alterations, accumulation of multiple genetic alterations, and allelic imbalance throughout the genome. Characterization of these alterations associated with cancer initiation and progression using molecular techniques such as polymerase chain reaction (PCR) will help develop molecular assays for cancer detection. PCR is a highly sensitive and specific molecular technique for detecting genetic changes in a limited amount of samples. It also provides a minimally invasive approach that may be useful for cancer screening, diagnosis, subclinical disease surveillance, and monitoring response to therapy.

Loss of heterozygosity (LOH) is a common form of genetic alteration, and the standard method for detecting LOH is PCR amplification of a specific locus, followed by size separation of the allele products on a denaturing polyacrylamide gel. Genomic rearrangements have also been reported to occur frequently in humans, and their accumulation is a sign of cancer progression. In contrast, affected bladders have been observed to slough cells more readily into urine than normal bladder mucosa due to their increased cell turnover [8]. In bladder Transitional Cell Carcinoma (TCC), recurrent LOH at chromosomes 3,4,8,9,11,13,17 and 18 involving tumour suppressor genes such as p53 and p16, and chromosome 9 alterations have been observed to occur early in bladder cancer [9]. The suppressor p53 gene has been reported to play a vital role in regulating cell cycle progression and apoptosis under genotoxic conditions, and its mutations are known to be the most common genetic defect in human cancers [10]. Recent studies have used molecular markers such as cytokeratin, CD44 antigen, mucin genes, and microsatellites to detect urothelial and bladder TCC in exfoliated urine cells of patients [11-13]. However, SCC is the most common malignancy in many tropical and subtropical countries due to endemic infections by *S. haematobium*, and Schistosoma-related bladder cancer defines a characteristic pathology, which differs from non-Schistosoma-related bladder cancer [14].

Microsatellites are highly polymorphic tandem repeat DNA sequences, representing a widespread class of genetic elements within the human genome. Their alterations are an important part of cancer progression and are valuable as clonal markers for detecting human cancers [15]. Microsatellite instability has been reported as a hallmark of defective DNA mismatch repair of genes, and mutations in their sequences confirm neoplastic transformation, which have been reported in most human cancers [16-18]. Multiple genetic alterations occur in bladder cancer due to clonal expansion of cancer cells. Alterations in microsatellite regions are also common and offer the possibility of early tumor detection by examining the DNA of urinary sediment [19]. Microsatellite markers have been used to study genome instability in exfoliated urine cells, and their potential use to study genetic variations in bladder tumours and their corresponding urine sediments has been demonstrated [13, 20]. Therefore, this pilot study aims to detect microsatellite alterations in exfoliated urine cells from *S. haematobium* infected participants using polymorphic microsatellite marker D9S905, one of the genetic markers located around regions of frequent chromosomal loss in bladder cancer [21].

2.0 METHODOLOGY

2.1 Study Location
The study was conducted in Imala Odo, a community with a population of about 1250 people, located in Abeokuta North Local Government Area (LGA) of Ogun State, South-Western Nigeria. Imala Odo is one of the communities situated around Oyan Dam. The dam is located at latitude 7º14’N and longitude 3º13’E. It is a multipurpose dam used mostly for water, flood control, fishing, and irrigation. The main occupations in this community are fishing and farming. Like many rural areas in Nigeria, Imala Odo lacks basic infrastructures such as pipe-borne water and safe waste disposal.

2.2 Ethical Approval, Study participants and Selection Criteria

Approval was received from the Institutional Review Board of the Nigerian Institute of Medical Research, and permission was also obtained from Ogun State Ministry of Health and Abeokuta North LGA authorities. Informed consent was obtained from each participant under a protocol approved by the Ethical Review Committee of the World Health Organization. The study participants were made up of adult males and females aged between 40 and 70 years. This age group was chosen for the study based on the observation that bladder cancer caused by *S. haematobium* infection occurs especially in the fifth decade of life [22]. Other selection criteria were haematuria and the presence of *S. haematobium* eggs in all three rounds of urine collected from each participant. Twenty-four out of 73 infected patients, made up of 17 (70.8%) males and 7 (29.2%) females, met the selection criteria, and none of the participants was diagnosed with bladder cancer before the study.

2.3 Sample Collection and Preparation

About 50 ml of voided urine and 2 ml of whole blood were collected from each participant. Blood samples were collected in EDTA bottles and stored at 4°C. To obtain urine cell pellets, the voided urine samples were centrifuged at 5,000 x g for 10 minutes, the urine was decanted, the pellets were washed three times with 25 ml PBS (0.8% NaCl, 2.7 mM KCl, 1.8 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.4) and stored immediately at -80°C until used.

2.4 DNA Extraction

Urine cell pellets and whole blood were digested with 1% SDS and 50µg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) at 48°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of the DNA. The extracted DNA pellet was resuspended in 25 µl double distilled water and stored at 4°C until used.

2.5 Microsatellite Analysis

Microsatellite marker D9S905 located on chromosome 9 according to the Genome database (http://www.gdb.org) was selected and the primers used for the PCR were commercially synthesized by Invitrogen (Brazil). The characteristics of the microsatellite marker are shown in Table 1. PCR amplifications were performed with genomic DNA (10 ng) in a 10 µl total reaction volume containing 2 pmol each of forward and reverse primers, Taq polymerase (0.1 U), 1X Tris-HCl PCR buffer, MgCl₂ (1.5 mM), dNTP (200 µM) and ddH₂O (3.5µl) and two independent PCR amplicons were generated from each of the blood and urine samples. The PCR conditions were: initial denaturation 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, and final extension at 72°C for 7 minutes. Amplification was confirmed by running 3 µl of each PCR amplicon in an 8% polyacrylamide gel (PAGE) and silver stained. The PCR products were then run on an automated sequencer (ALF, Pharmacia) on an 8% PAGE with 7m urea to detect microsatellite alterations in the matched urine and blood DNA. The amplified products were detected by laser using the Allelic Links software, and allelic size was determined by running a 50bp ladder in every 10 lanes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Localisation</th>
<th>Primer sequence (5' - 3')</th>
<th>Annealing temperature</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D9S905</td>
<td>9q34.2</td>
<td>GTGGGAAATTTGCCTAA GT</td>
<td>55</td>
<td>294</td>
</tr>
</tbody>
</table>

3.0 RESULTS

A total of 24 *S. haematobium* infected adult male and female inhabitants of Imala Odo, a rural predominantly fishing community in South-Western Nigeria, and who were positive for haematuria and *S. haematobium* infection took part in the study. The participants were 17 (70.8%) males and 7 (29.2%) females aged between 40 and 70 years, with a mean age of 47.5 years. Genomic DNA obtained from exfoliated urine cells and blood samples of each participant were subjected to PCR using mi-
crosatellite marker D9S905 on locus 9q34.2 with allele size 294bp. The result showed genomic alterations in the urine DNA of 2 participants, and their blood DNA showed homozygosity for the allele (294bp) on the same locus (Figure 1). The 2 participants’ urine DNA showed LOH with allele sizes 290bp and 280bp, respectively. This follows our earlier study [23], where we performed cytopathological examinations on exfoliated urine cells from the 24 participants, and the results confirmed squamous cell abnormalities in these 2 participants.

Figure 1. Results of the LOH Analysis of 2 Urogenital Schistosomiasis Patients Using Marker D9S905 on Locus 9q34.2, Allele Size 294bp

On the left of the gel is the DNA marker, urine DNA (1, 2) and blood DNA (3, 4) from one patient; urine DNA (5, 6) and blood DNA (7, 8) from another patient. The blood DNA (3, 4; 7, 8) from the two patients showed homozygosity, having alleles 294bp on the same locus while their urine DNA (1, 2, 5, 6) showed LOH with allele sizes 290bp (1, 2) and 280bp (5, 6) respectively. Lane 9 is the negative control.

4.0 DISCUSSION

The This study was carried out to identify genetic alterations in exfoliated urine cells of 24 S. haematobium infected patients in relation to bladder cancer. The study, which involved analysis of paired DNA from blood and voided participants' urine, used microsatellite marker D9S905 as the genetic marker. D9S905 is mapped to chromosome 9 and is one of the genetic markers located around regions of frequent chromosomal loss in bladder cancer [21]. The LOH or microsatellite instability in DNA from exfoliated urine cells of two participants observed in this study agrees with other studies [24-27], which reported extensive and more frequent deletions on chromosome 9 of bladder cancer patients of all stages and grades, and suggested a link between chromosome 9 abnormalities and recurrence of superficial bladder cancer.

In addition, earlier examinations of cytological preparations from the urine samples of the 24 participants also revealed squamous cell abnormalities in the urine cells of only these two participants [23]. This agrees with other studies [7, 22] that reported schistosome-related bladder cancer to differ from non-schistosome-related cancer in its squamous cell differentiation. Although urine cytology is a standard non-invasive procedure for detecting bladder cancer, its sensitivity is poor, particularly for low-grade tumours [28]. On the other hand, cystoscopy is the gold standard for the initial diagnosis of bladder cancer because it allows visualization and direct biopsy of suspicious bladder lesions [29]; however, it is expensive and invasive. Bladder cancer is one of the severe complications of chronic S. haematobium infection. Many of the participants might have acute infection at the time of the study, which might account for the low rate of microsatellite instability observed this study.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

OPA conceived and designed the study, contributed to data collection, analysis of data and manuscript writing. PVG contributed to data collection and analysis of data. MBA performed sample collection. All authors approved the final copy of the manuscript.

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