Medical Research Updates Vol. 4, Issue 2. 2026

Original Article https://doi.org/10.70084/mru/pmrcc/041.P21

Detection of Microsatellite Markers (LOH) in Urine Samples from Sudanese Patients with Cervical Cancer

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Cite: Ben Sumaidea, et al. Detection of Microsatellite Markers (LOH) in Urine Samples from Sudanese Patients with Cervical Cancer.Medical Research Updates Journal 2026;4(2):10-20. https://doi.org/10.70084/mru/pmrcc/041.P21

ABSTRACT

Background: Cervical cancer is one of the leading causes of cancer-related death in women globally. Early discovery of cervical cancer is largely curable; delayed detection lowers survival rates. The current study examined whether microsatellite markers could detect cervical cancer-associated genetic changes in urine samples. **Methodology:** A total of 23 matched blood and urine samples were taken from Sudanese women with cervical cancer. Four microsatellite markers on separate chromosomal regions—D3S1300 (3p14.2), D3S1260 (3p22.2), D11S528 (11q23.3), and D11S35 (11q22.1)—were evaluated for loss of heterozygosity (LOH), which is known to be common in cervical cancer. To determine the existence of predicted allelic bands, a polymerase chain reaction (PCR) was used, followed by 8% polyacrylamide gel electrophoresis separation. **Results:** There was no LOH identified in any of the blood samples. In contrast, LOH was detected in urine samples from 18 of 23 individuals (78.3%) at one or more of the four loci studied. The LOH frequencies for these markers were as follows: D3S1300 (3p14.2) in 12 samples (52.2%), D11S35 (11q22.1) in 7 samples (30.4%), D3S1260 (3p22.2) in 8 samples (34.8%), and D11S528 (11q23.3) in 9 samples (39.1%). Two samples showed LOH at two loci, three samples at three loci, and three samples at all four loci, with a predictive value of around 90%. **Conclusion:** This study shows that LOH at one or more of the four microsatellite markers is related to all kinds of cervical cancer and can be diagnosed from urine samples with 78.3% sensitivity. LOH detection at these loci is more accurate, culturally acceptable, and less invasive for cervical cancer risk assessment.

Keywords: Cervical cancer, microsatellite markers, loss of heterozygosity, Sudan

Introduction

Cervical cancer is the fourth most frequent malignancy in women worldwide, with 660,000 new cases in 2022. In the same year, 94% of 350,000 cervical cancer fatalities occurred in low- and middle-income countries. Southeast Asia, Central America, and sub-Saharan Africa have the highest incidence and mortality. These regional variances reflect Human Papillomavirus (HPV) vaccines, screening, and treatment disparities [1, 2]. Without the expansion of preventative interventions, including the HPV vaccine and cervical cancer

screening, alongside coordinated efforts by government, civil society, and corporate sectors, the global incidence of cervical cancer is projected to rise in the future [3].

Cervical cancer is the second most common cancer among women in Sudan, with more than two-thirds of all women with invasive cervical cancer being diagnosed at an advanced stage (stages III and IV). The lack of a screening program for cervical cancer in Sudan may contribute to the late presentation of this cancer, but other factors potentially associated with advanced stages of cervical cancer at diagnosis are unknown. The purpose of this research was to investigate the relationship between age, marital status, ethnicity, health insurance coverage, residence in an urban vs. a rural setting, and stage (at diagnosis) of cervical cancer in Sudan [4]. Annually, it is predicted that 833 Sudanese women receive a diagnosis of cervical cancer, with 534 dying from the disease [5].

Detection of Loss of Heterozygosity (LOH) through microsatellite markers is a molecular method used to identify the absence of a normal allele in a heterozygous pair, often indicating the inactivation of tumor suppressor genes. This method uses PCR-based assays to detect high-frequency deletions on specific chromosomes, such as 3p, 9p, and 17p, which are linked to cancer progression and prognosis [6, 7]. LOH is a common and early genetic change in cervical cancer development, signifying the deletion of tumor suppressor genes on particular chromosomes, especially 3p, 11q, and 6p. LOH at these locations is associated with aggressive tumor features, high-grade lesions, and poor prognosis [8]. Therefore, this study aimed to screen for LOH in a series of Sudanese women with cervical cancer.

Materials and Methods

This was a prospective descriptive pilot study conducted in the Radiation and Isotopes Centre, Khartoum (RICK). This study enrolled twenty-three patients who were diagnosed with cervical cancer during a specific period. All selected participants were not receiving chemotherapy or radiotherapy during sample collection and had never undergone a hysterectomy. Sample collection began after receiving ethical approval from the IEND Ethical Committee. Formal communication was initiated with the administration of the Radiation and Isotopes Centre Khartoum (RICK), resulting in the acquisition of both samples and clinical data. The director of RICK granted institutional approval.

All participants were apprised of the study's significance and objectives. Informed consent was obtained from each participant before enrollment. Participants were interviewed utilizing a structured questionnaire. Biological samples of two types were collected from each participant. The initial sample comprised urine, utilized for identifying microsatellite instability markers. Urine samples were collected in sterile containers, with the incorporation of 0.5 mg of EDTA per 30 ml of urine. Samples were maintained in ice-filled containers during transport to ensure the stability of DNA. The second sample consisted of a peripheral blood specimen, utilized as a control to distinguish between genuine homozygosity and loss of heterozygosity.

Extraction of DNA

DNA extraction from urine samples utilized the guanidine chloride method after centrifuging 15–35 ml of urine at 3000 rpm for 20 minutes. The supernatant was removed, and the resultant pellet underwent two washes with phosphate-buffered saline (PBS). Blood samples underwent three washes with red blood cell (RBC) lysis buffer.

To the pellet, 2 ml of lysis buffer, 0.5 ml of proteinase K, 1 ml of guanidine chloride, and

300 µl of ammonium acetate were added, and the mixture was incubated at 37°C overnight. Following this, 2 ml of pre-chilled chloroform was added, and the mixture underwent vortexing and centrifugation for 10 minutes. The upper aqueous layer was transferred into new tubes, followed by the addition of 10 ml of cold absolute ethanol. The mixture underwent incubation at −20°C for 24 hours, subsequently followed by centrifugation for a duration of 20 minutes. The supernatant was removed, and the tubes were inverted onto tissue paper. The DNA pellet underwent a wash with 70% ethanol, was air-dried, and subsequently resuspended in 50 µl of distilled water. The resuspended DNA was incubated at 4°C overnight and then stored at −20°C for subsequent analysis.

Detection of Loss of Heterozygosity (LOH) by PCR

Detection of Loss of Heterozygosity (LOH) via PCR involved the analysis of DNA extracted from exfoliated cervical epithelial cells in urine samples and from blood samples. This analysis utilized four microsatellite markers situated on various chromosomal regions commonly associated with loss of heterozygosity in cervical cancer. The markers comprised D3S1300 (3p14.2), D3S1260 (3p22.2), D11S528 (11q23.3), and D11S35 (11q22.1) (see Table 1).
Primers were developed to closely flank the tandem repeat regions, resulting in small amplicons appropriate for amplification from DNA extracted from urine and blood samples. Each PCR reaction included 1 µl of DNA (30–40 ng), Ready Mix with Taq™ DNA Polymerase (5 U/µl; final concentration 2.5 U), deoxynucleotide triphosphates (dNTPs) at 2.5 mM each, 10× reaction buffer at 1× concentration, gel loading buffer at 1× concentration, 17 µl of distilled water, and 2 µl of primer mix.
PCR amplification was conducted for 37 cycles with the following parameters: initial denaturation at 94°C for 30 seconds, annealing at 57°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes using a thermocycler

Table 1: Primers used for LOH analysis of microsatellite loci

Target	Chromosomal location	Forward primers	Reverse primers
D3S1300	3p14.2	5'GCTCACATTCTAGTCAGCCTG3'	5'TGTCACAGAATAGTCTTTCCCA3'
D3S1260	3p22.2	5'GCTACCAGGGAAGCACTGTA3'	5'GCTAAACTGAAGACCCTGCA3'
D11S35	11q23.3	5'GAGGAAAGTCATGAACGCAG3'	5'ATCGATTAACCAACTTCACACA3'
D11S528	11q22.1	5'GCCTAACTAATGGTGTCCCC3'	5'GACCCCAGTGTGAGATGAAT3'

PCR products were verified using 1.5% agarose gel electrophoresis, which was prepared with 8 ml of 10× TBE buffer, 67 ml of distilled water, 1.5 g of agarose powder, and 1.5 µl of ethidium bromide. PCR products were separated on an 8% polyacrylamide gel to detect the expected allelic bands. The gel was prepared using 3.2 ml of 30% acrylamide, 6.4 ml of distilled water, 2.4 ml of 5× TBE buffer, 200 µl of 10% ammonium persulfate (APS), and 10 µl of TEMED. Electrophoresis was conducted at 120 V for a duration of 30 minutes, followed by staining of the gels with ethidium bromide. Negative controls were systematically incorporated during PCR setup in each

experimental run to mitigate the risk of cross-contamination.

Results

The analysis of 23 participants revealed an age range of 45–80 years, with a mean age of 64.74 years. In terms of current residence, 16 patients were located in Khartoum State, two in Darfur State, two in Kordofan State, one in Sennar State, one in Gezira State, and one in Blue Nile State.

The participants exhibited diverse ethnic backgrounds. Nine participants were from Darfur States, seven from Kordofan States, three from Khartoum State, one from Blue Nile State, one from White Nile State, one from Juba, and one from Sennar State.

The majority of participants indicated a history of recurrent gynecological issues, encompassing pain, bleeding, inflammation, and vaginal discharge. During the study, participants primarily reported complaints of pain, back pain, inflammation related to vaginal secretions, and, in certain instances, vaginal bleeding.

Histopathological analysis revealed that the majority of participants were diagnosed with squamous cell carcinoma (SCC), displaying a range from well-differentiated to poorly differentiated variants. Both keratinizing and non-keratinizing subtypes of squamous cell carcinoma (SCC) were identified, including large cell non-keratinizing SCC. Two cases were identified as adenosquamous carcinoma.

DNA extracted from urine samples exhibited variability in both concentration and purity, as assessed by NanoDrop spectrophotometry. The DNA concentration varied from 17.6 to 4792.0 ng/ul, while purity values were between 1.21 and 1.89. DNA extracted from blood samples exhibited variable concentrations and purity levels, with concentrations ranging from 49.0 to 2785.3 and purity values between 1.74 and 1.89.

Four microsatellite markers were analyzed to identify loci where loss of heterozygosity (LOH) is anticipated to correlate with the disease and may have prognostic significance. All twenty-three samples underwent successful polymerase chain reaction (PCR) amplification. No LOH was observed in any of the blood samples analyzed. LOH was identified in urine samples from 18 of 23 patients (78.3%) at one or more of the four markers investigated, as shown in Figure 2. Of the four microsatellite markers examined, D3S1300 (3p14.2) exhibited loss of heterozygosity (LOH) in twelve samples (12/23; 52.2%), D11S35 (11q23.3) in seven samples (7/23; 30.4%), D3S1260 (3p22.2) in eight samples (8/23; 34.8%), and D11S528 (11q22.1) in nine samples (9/23; 39.1%), as shown in Table 2 and Fig. 1. The analysis of LOH distribution across various loci indicated that two samples displayed LOH at two loci, three samples exhibited LOH at three loci, and three samples revealed LOH at all four loci. Additionally, four urine samples exhibited one prominent band, with the corresponding allele manifesting as a faint band.

A study conducted in 2010 at the Institute of Endemic Diseases, University of Khartoum, evaluated a potential screening method for cervical cancer using approximately nineteen urine samples from patients without the disease. The positive predictive value, defined as the proportion of individuals with a positive test result who truly have the disease, was estimated to be approximately 90% based on this comparison.

Images 1, 2, 3, and 4 illustrate the patterns of positive and negative results observed in the electrophoretic bands.

Table 2 presents the eighteen urine samples and their corresponding LOH results, analyzed using four markers. Samples exhibiting loss of heterozygosity are indicated with (+), while those demonstrating a normal pattern with the presence of heterozygosity are marked with (-).

LOH in D11S35	LOH in D11S528	LOH in D3S1260	LOH in D3S1300	Number of samples
+	+	+	+	3
+	+	+	-	1
+	+	-	+	1
+	-	-	+	1
_	+	+	+	1
_	+	_	+	1
+	+	_	_	1
+	_	_	_	1
_	+	_	_	1
_	_	+	_	2
_	_	_	+	5

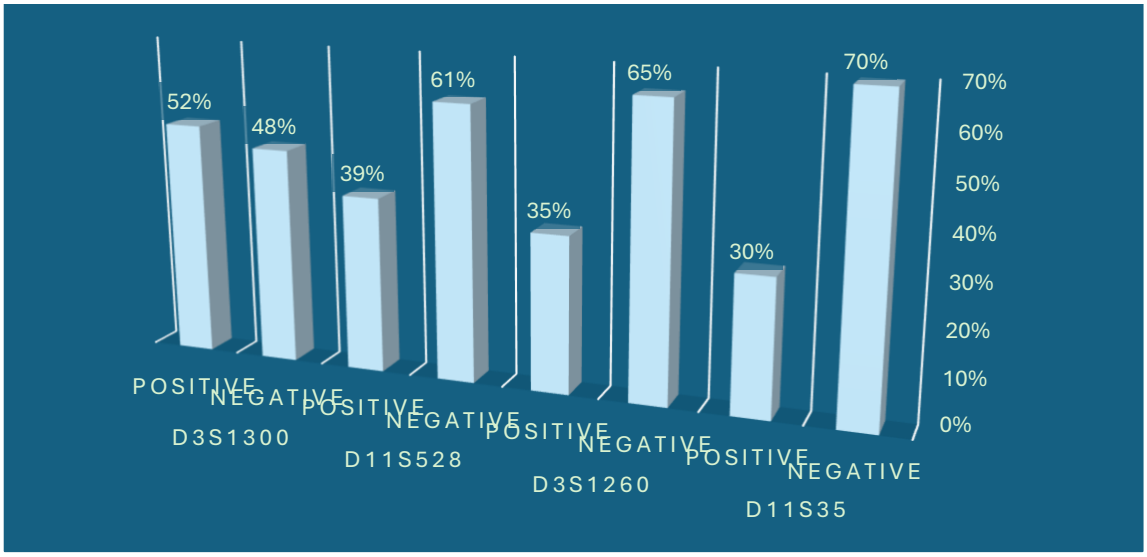

Figure 1 shows the LOH of each marker in all samples.

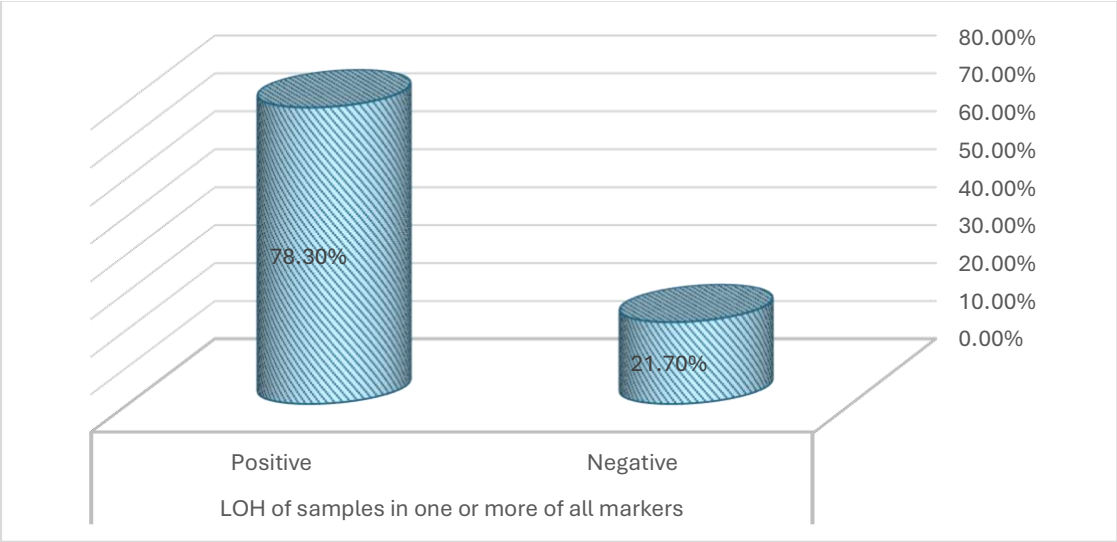

Figure 2 shows the LOH of all samples in any one or more of the markers.

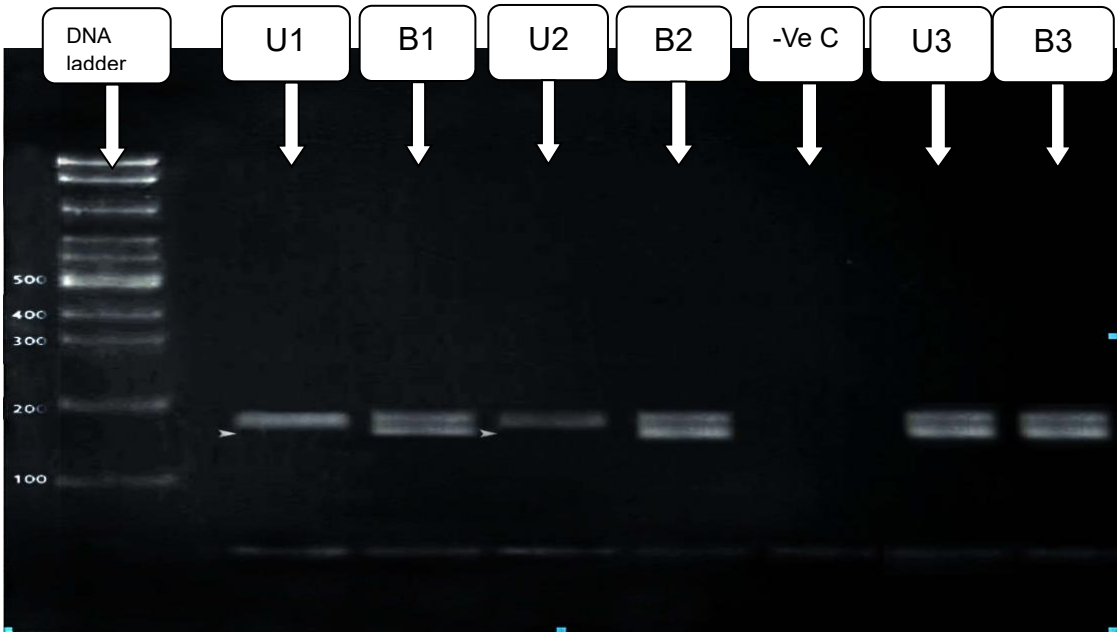

Image 1: Representative gel of microsatellite analysis demonstrating loss of heterozygosity (LOH) on urine DNA using primer D3S1300 (3p14.2) (165 bp). The arrow indicates the deletion of one allele from urine samples U1 and U2, which demonstrates the loss of heterozygosity in these samples. In contrast, B1, B2, U3, and B3 exhibit no loss of heterozygosity, and the negative control (-Ve C) shows no band.

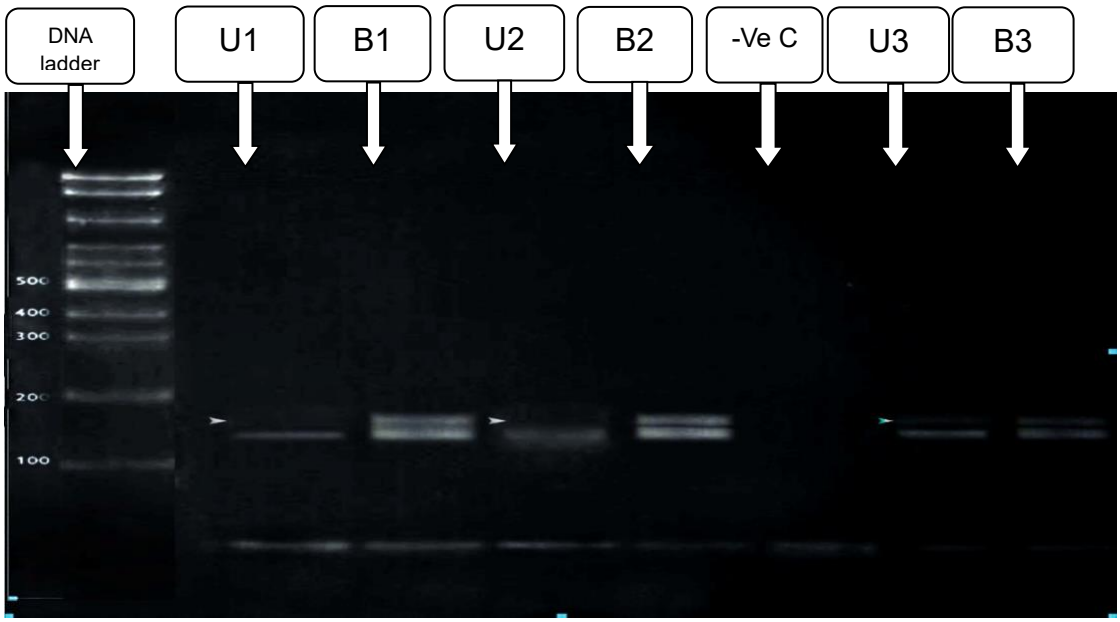

Image 2: Representative gel of microsatellite analysis (loss of heterozygosity) on urine DNA using primer D11S528 (11q22.1) (base pairs). The arrow indicates the deletion of one allele from urine samples U1 and U2, which exhibited loss of heterozygosity. In contrast, samples B1, B2, U3, and B3 did not show LOH, although a faint band was observed in the urine sample U3, and no band was present in the negative control (-Ve C).

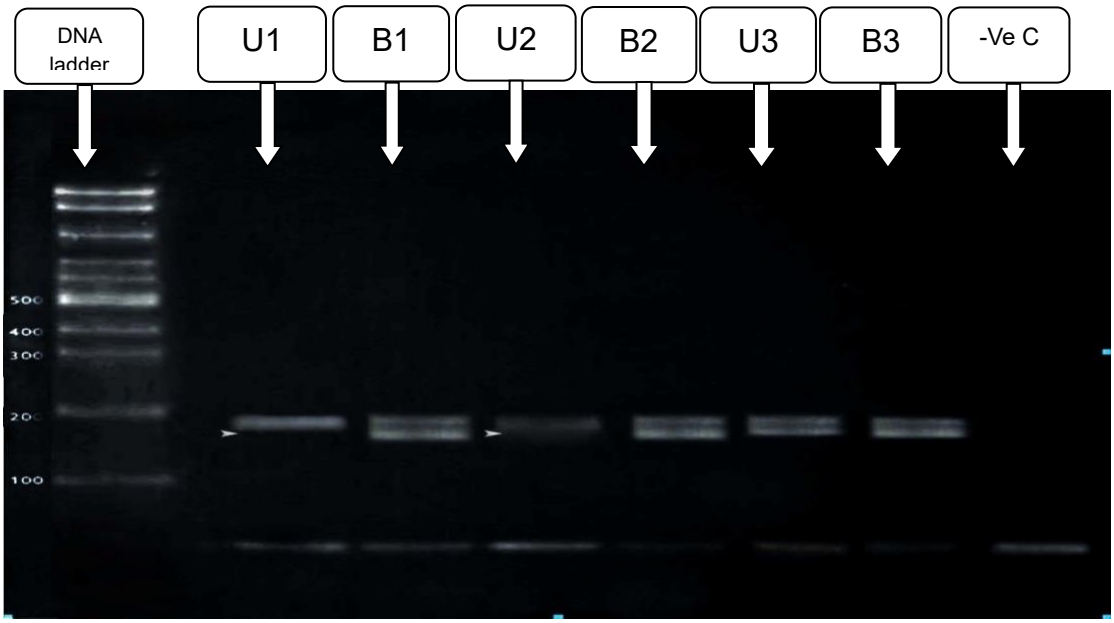

Image 3: Representative gel of microsatellite analysis demonstrating loss of heterozygosity (LOH) on urine DNA using primer D3S1260 (3p22.2). The arrow indicates the deletion of one allele from urine samples U1 and U2, which demonstrates the loss of heterozygosity in these samples. In contrast, samples B1, B2, U3, and B3 exhibit no loss of heterozygosity, and the negative control (-Ve C) shows no band.

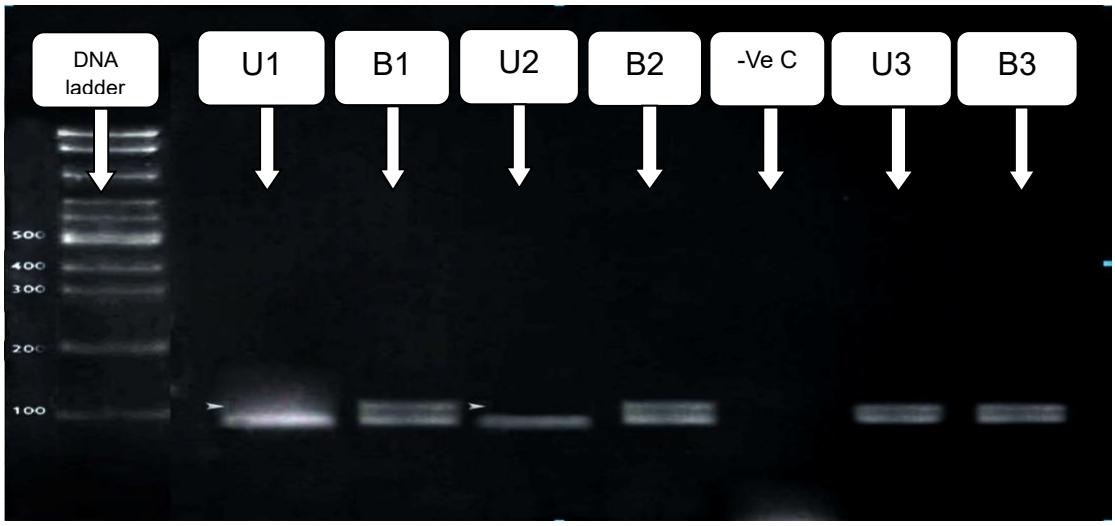

Image 4: Representative gel of microsatellite analysis demonstrating loss of heterozygosity (LOH) in urine DNA using primer D11S35 (11q23.3) (100 bp). The arrow indicates the deletion of one allele from urine samples U1 and U2, which exhibited loss of heterozygosity. In contrast, samples B1, B2, U3, and B3 showed no loss of heterozygosity, and the negative control (-Ve C) displayed no band.

Discussion

Urine has historically been regarded as a significant biological specimen for laboratory analysis, as it reveals information about various physiological and pathological conditions of the body. This text provides insightful information about the well-being of the urinary tract and reproductive system. Urine comprises hormones, metabolic substances, shed epithelial cells, crystals, casts, and bacteria, each of which can provide valuable information about both systemic and localized pathological conditions.

The identification of microsatellite markers in urine samples from Sudanese women signifies a promising approach for cervical cancer screening. A prior pilot study carried out in 2010 at the Institute of Endemic Diseases, Khartoum University, revealed a significant association Numerous studies have documented LOH in primary cervical carcinomas, consistent with between loss of heterozygosity in four microsatellite markers and cervical intraepithelial neoplasia (CIN), with reliable detection achievable in urine samples.

This pilot study delves deeper into the sensitivity of loss of heterozygosity detection at four microsatellite markers situated on chromosomal regions 3p14.2, 3p22.2, 11q22.1, and 11q23.3 throughout the process of cervical tumorigenesis. In the analysis of 23 samples, it was observed that eighteen samples (18/23; 78.3%) demonstrated loss of heterozygosity (LOH) at one or more loci, whereas five samples (5/23; 21.7%) did not exhibit any LOH. The estimated predictive value of the test stands at around 90%, with sensitivity reflecting 78.3% of LOH-positive samples.

observations in other tumor types where frequent LOH occurs at multiple loci.

Combined LOH analysis at D3S1300, D3S1260, D11S35, and D11S528 has been shown to identify cervical changes. A study has documented a notable occurrence of loss of heterozygosity (LOH) at chromosomal regions 3p, 3q, 4q, 5p, and 5q in primary cervical cancer, with frequencies at these loci varying between 31.0% and 56.3%. The analysis examines the frequent-deletion sites in the context of HPV infection [9-11].

The current results indicate that LOH at one or a few microsatellite loci may still serve as an early indicator of tumorigenesis across different types of cervical cancer, including squamous cell carcinoma (both keratinizing and non-keratinizing) and adenocarcinoma. Research suggests that genomic instability occurs as a later phenomenon in the carcinogenic process of cervical cancer and correlates with the transition from cervical intraepithelial neoplasia to an invasive form. On the contrary, loss of heterozygosity on chromosome 3p plays a preliminary role in the progression of cervical intraepithelial neoplasia [12].

Samples that showed no LOH or only faint bands may reflect the presence of non-epithelial cells, such as white blood cells or pus cells resulting from bleeding, which can potentially produce false-negative results. Most solid tumors have genome-wide heterozygosity loss. Nonrandom LOH may indicate the loss of genes that promote neoplastic growth and be prognostic. LOH has been difficult to characterize in large clinical and public health studies. Clinical biopsies and thin needle aspirates create little tissue, decreasing LOH-assessing loci. Genotypically diverse premalignant and malignant neoplastic cell populations are found in human biopsies. Traditional autoradiographic LOH analysis is laborious, inhibiting high-throughput locus, sample, and patient analysis. Thus, clinical or public health science LOH analysis requires reliable, high-throughput technologies that can swiftly evaluate many loci in microscopic tissue samples and purify homogeneous cell populations [13].

Nevertheless, the high predictive value observed suggests that urine-based LOH detection may have practical utility as a non-invasive screening method or for early detection of cervical changes in women at risk of developing cervical cancer. Urine samples present a promising alternative to both physician-administered and self-collected cervical samples for the purpose of cervical screening. Primary hrHPV testing necessitates a supplementary evaluation of risk, commonly referred to as triage, for women who test positive for hrHPV [14].

The findings of the current study indicate that urine sampling serves as a compelling alternative method for cervical cancer screening. Nevertheless, additional research is essential to enhance the protocol, reduce the incidence of false-negative results, and substantiate the technique in broader and more varied populations. To increase DNA concentration for analysis, collect morning urine samples. More research with larger sample sizes is needed to prove that LOH (loss of heterozygosity) detection in urine samples can diagnose cervical cancer early. Further research should examine potential relationships between LOH at various indicators, tumor grade, and patient prognosis. For Sudanese LOH detection, tissue samples from confirmed positive cases at different disease stages are recommended. PCR output should be examined with a DNA sequencer and GeneScan software to better identify allelic variants.

In conclusion, this study shows that LOH at one or more of the four microsatellite markers is related to all kinds of cervical cancer and can be diagnosed from urine samples with 78.3% sensitivity. LOH detection at these loci is more accurate, culturally acceptable, and less invasive for cervical cancer risk assessment.

Acknowledgement

The author extends gratitude to all personnel in IEND, with appreciation for those in the molecular biology laboratory. I extend my gratitude to the staff of the clinics at the Radiation and Isotopes Centre, Khartoum, for their invaluable support and assistance in the collection and processing of samples.

Funding

The Prof. Medical Research Consultancy Center (PMRCC) funded this project. Grant Number: PMRCC/2025A5.

Conflict of Interest

The author declares that they have no conflict of interest to disclose.

Ethical Considerations

Ethical approval was obtained from the local government authorities, and administrative authorization was received before data collection. The study adhered to the principles of confidentiality and responsible use of routinely collected health information.

Ethical Approval

The protocol of this study had been approved by the Human Ethics Committee at Prof MRCC. Approval number: HREC 00012/MRCC.4/26).

Disclosure

This research was conducted without the use of artificial intelligence or assisted technologies, including the generation of figures.

Data Availability

The data supporting the conclusions of this article are included within the article, and further inquiries can be sent to the corresponding author.

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