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ORIGINAL RESEARCH

Unravelling bladder cancer in Uganda: insights from the discrepancies in *TP53* assessment between immunohistochemistry and whole exome sequencing

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Background: Bladder cancer (urothelial carcinoma) is highly heterogeneous. Despite several cutting-edge treatment practices, the prognosis of muscle-invasive urothelial carcinoma (MIUC) remains very poor. Immunohistochemistry (IHC) and whole exome sequencing (WES) have been used to understand the heterogeneous nature of this aggressive cancer. Unfortunately, *TP53,* the guardian of the genome, is the most mutated gene in MIUC. Overexpression of its gene products is a predictor of poor prognosis in urothelial carcinoma. This study assessed *TP53* gene mutations and their protein expression using IHC and WES techniques.

Methods: We conducted a cross-sectional study where 50 formalin-fixed paraffin-embedded (FFPE) tissue blocks, processed from biopsy specimens from patients admitted to Mulago National Referral Hospital (MNRH) with MIUC, were consecutively selected and re-examined. *TP53* IHC and WES were done according to standard protocols.

Results: All patients presented with haematuria and a bladder mass. The female-to-male ratio was 1:1.5. The mean age of the participants was 59.3 years (standard deviation [SD] 12.9). Using IHC, 26 (52%) of the 50 samples stained positive for *TP53,* yet only one had a *TP53* missense gene mutation at WES.

Conclusion: In this sample of Ugandan patients, mutations in the *TP53* gene do not drive MIUC as they do in various parts of the world. The overexpressed *TP53* proteins seen at IHC are possibly from the wild-type gene. Therefore, regarding clinical practice, this high discordance between *TP53* immunopositivity and *TP53* gene mutations for MIUC makes IHC (a cheaper and readily available option in our setting) a poor surrogate for *TP53* WES.

Keywords: bladder cancer, *TP53,* immunohistochemistry, whole exome sequencing

Introduction

Bladder cancer is the ninth leading cause of cancer globally and the second most common malignancy of the urinary system, presenting as either muscle-invasive or non-muscle-invasive disease.[1,2] About 81 400 new bladder cancer patients were diagnosed in 2020, of which 17 980 died. These deaths are primarily attributable to muscle-invasive urothelial carcinoma (MIUC).[2] Moreover, even in good clinical settings, the five-year overall survival rate ranges between 10% and 20%.[2,3]

Immunohistochemistry (IHC) determines various cancer-associated biomarkers involved in several biological pathways.[4] Several IHC markers have been investigated and proposed for predicting the prognosis of bladder cancer; however, none are routinely used in current clinical practice.[5-8] Nonetheless, this does not undermine the value of IHC signatures in supporting the diagnosis of MIUC, its management, and overall prognosis.

TP53, a tumour suppressor gene (guardian of the genome) located on chromosome 17p, regulates the cell cycle processes and maintains genomic stability by averting genotoxic stimuli.[9,10] Its overexpression is known to be associated with life-threatening clinical outcomes.[11] Therefore, mutations in the *TP*53 protein-coding gene play significant roles in bladder cancer growth and progression.[12]

Next-generation sequencing is a reliable technique for detecting gene mutations; however, it is costly and not readily available in many bladder cancer treatment centres in sub-Saharan Africa. Hence, IHC analysis of the mutant *TP53* protein expression is commonly used as a surrogate marker for mutations in the *TP53* gene in these settings. However, some studies have reported various reasons that can stabilise the wild-type *TP53* protein, leading to overexpression and rendering it a poor surrogate marker.[13-17]

Because of urothelial carcinoma's known heterogeneity, its immunobiology is not always similar in different human populations.[8,18] Therefore, this study aimed to determine if IHC analysis of *TP53* proteins seen in MIUC could be used as a surrogate marker for predicting mutations in the *TP53* gene.

Methods

A descriptive, cross-sectional study was conducted at the Mulago National Referral Hospital (MNRH) complex. After sample size calculation, we consecutively selected 50 archived formalin-fixed paraffin-embedded (FFPE) tissue blocks of confirmed MIUC from the biorepository of MNRH from 2019 to 2022. Only FFPE tissue

blocks of patients aged 18 years and above were selected. Damaged tissue blocks, those with extensive necrosis, and those with missing vital demographic data were excluded. Two laboratories were used. MNRH ran the histopathology and IHC studies, while Unipath Specialty Laboratory Ltd. (Ahmedabad, India) carried out all whole exome sequencing (WES) studies.

The histopathologic staging was performed per the 2004 World Health Organization (WHO) classification.[19] The retrieved FFPE tissue blocks were trimmed and cut into 4 µm thickness on a microtome machine. The sections were spread on the surface of the water (5–10 °C) below the melting point of wax to remove wrinkles, then mounted onto labelled, silanised glass slides, fixed by dry heat from an oven at 55–65 °C for 30–60 minutes to melt the wax. Later, they were dewaxed in xylene for about five minutes and rehydrated in a series of graded alcohol as follows: absolute 100%, 90% alcohol, and 70% alcohol, for 1–3 minutes each. They were then washed in distilled water for a minimum of 30 seconds. Haematoxylin and Eosin (H&E) staining was done using standard operating procedures.

For IHC, extra 4 µm thick sections were taken on Poly-L-lysine-coated slides and subjected to antigen retrieval by the microwave heat method. IHC staining of *TP53* was performed using an anti-*TP53* protein monoclonal antibody, BP53-11 (GSI: 760-2542J28654-0043), which recognises both mutant and wild-type *TP53* proteins. Sections were incubated with the secondary biotinylated antibody and avidin-biotin-peroxidase complexes for 30 minutes. A Tris-buffered saline solution was used to wash off the primary and secondary antibodies.

Reaction products were revealed with diaminobenzidine as the chromogen, and sections were counterstained with Harris haematoxylin to enhance nuclear detection. Sections of colonic carcinoma tissue were used as positive controls. Nuclear positivity was seen as a dark brown colour on a bluish background. The percentage of immunopositive cells was calculated by counting at least 1 000 tumour cells in areas of maximum positivity. A cut-off value of 15% was considered positive.[19,20] The percentage of nuclear staining was grouped into three categories: > 50%, 25–49%, and 15–24%, while the intensity of staining was grouped as strong, moderate, and mild.

Two consultant pathologists independently examined the tissues without knowledge of the clinical data. In case of a disagreement, a consensus was reached by re-examining the tissues on a multi-head microscope.

Isolation and quantitative DNA analysis

Deoxyribonucleic acid (DNA) was isolated from the samples using the Alexgen FFPE DNA extraction kit (CAT# AGFF50). The quantity of DNA was then measured using a Qubit® 4.0 fluorometer, and quality was analysed using gel electrophoresis. Importantly, sections used for the IHC and WES were from different parts of the same tumour sample.

Library preparation

The exome libraries were prepared using Twist Bioscience 2.0 (CAT# 104207). A TapeStation was used for quality checks.

Cluster generation and sequencing

After obtaining the library's Qubit concentration and mean peak size from the TapeStation profile, the library was loaded onto Illumina NovaSeq 6000 for cluster generation and sequencing. Raw reads were obtained after the NovaSeq 6000 run was demultiplexed.

Bioinformatics analysis

Raw reads were quality-checked and filtered to remove sequencing adapters and those with low-quality bases using Fastp v0.20.0. The high-quality reads obtained were mapped on the hg37 human reference genome. Each sample's Sequence Alignment/Map files were generated and later converted to Binary Alignment/Map files. Variant annotation was carried out using the Franklin (Genoox) tool. The RefSeq database was used to identify and characterise gene-associated variants. The disease association for variants was derived using the COSMIC and ClinVar databases. For this specific study, only the *TP53* gene is discussed.

Statistical analysis

Data was collected using a questionnaire, entered into REDCap (Research Electronic Data Capture), and later exported as a comma-separated values (CSV) file into RStudio for analysis. Patients' ages were categorised into groups (i.e. < 40, 40–49, 50–59, 60–69, 70–79, and > 80) and summarised as mean and SD. Categorical data were summarised as frequencies and proportions and then presented in table format. Crude associations between *TP53* immunoreactivity and the independent variables (age groups, sex, lymphovascular invasion [LVI]) were assessed using Pearson's chi-square test. A *p*-value less than 0.05 was considered statistically significant.

Table I: The immunohistochemical staining patterns of *TP53*

Cell staining (%)	Intensity				Total	
	Strong	Moderate	Weak	Zero		
≥ 50	8	3	0	0	11	
25–49	7	4	1	0	12	Positive 26/50 (52%)
15–24	0	2	1	0	3	
< 15	0	1	3	20	24	Negative 24/50 (48%)
Total	15	10	5	20	50	

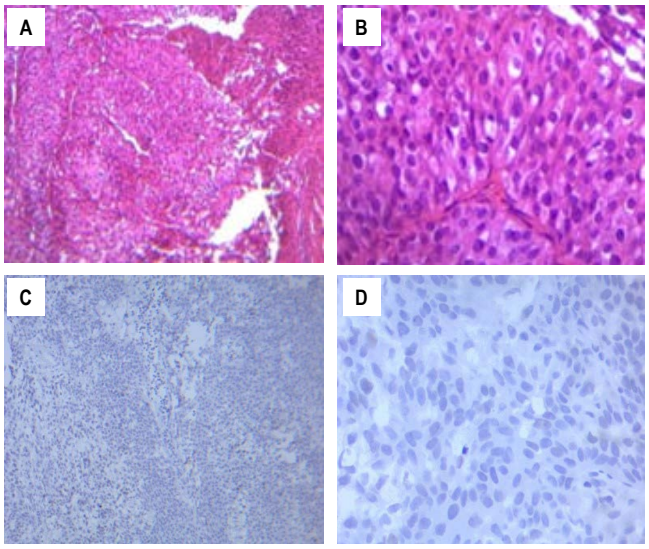

Figure 1: Muscle-invasive urothelial carcinoma; nested variant

Results

Overall, 50 participant samples were included in the study. All patients presented with haematuria and a bladder mass. The participants' mean and SD were 59.3 ± 12.9 years. The majority (60%) were males. Of the 50 samples, 26 (52%) stained positive for *TP53* at IHC analysis, and only one sample with a strong immuno-intensity had a *TP53* missense gene mutation at WES. There was no significant relationship between *TP53* immunoreactivity and the participant's age, sex, or LVI. Most of the samples that stained positive for the *TP53* protein exhibited strong or moderate intensity.

Immunohistopathological characteristics of some of the tissues examined

Images in Figure 1 (A – H&E X60, B – H&E X100) show highly dysplastic urothelium forming fragmented papillae and nests, detrusor muscle invasion, ≥ pT2, and no LVI; hence, the lower likelihood of regional nodal metastasis. IHC images (C – X40, D – X100) show negative *TP53* immunoreactivity.

Images in Figure 2 (A – H&E X60, B – H&E X100) show the tumour extending into the detrusor muscle, ≥ pT2, and prominent LVI; hence, possible regional nodal metastasis. Variable-sized cysts are lined with flattened cuboidal cells and intraluminal necrotic material. IHC images (C – X40, D – X100) show moderate *TP53* immunopositivity.

In Figure 3, image A (H&E X60) shows the tumour extending into the detrusor muscle, ≥ pT2. Image B (H&E X100) shows solid papillary nests of dysplastic urothelium infiltrating and inciting a desmoplastic stromal reaction. IHC images (C – X40, D – X100) show intense *TP53* immunopositivity.

Discussion

Of the 50 analysed samples, only one missense *TP53* mutation was seen at WES. However, when we carried out IHC on the same samples, 52% tested positive for the *TP53* protein (Table I, Figures 1–3). Firstly, the *TP53* mutation seen in just one sample is infrequent. Secondly, the discordance of having a high immunopositivity

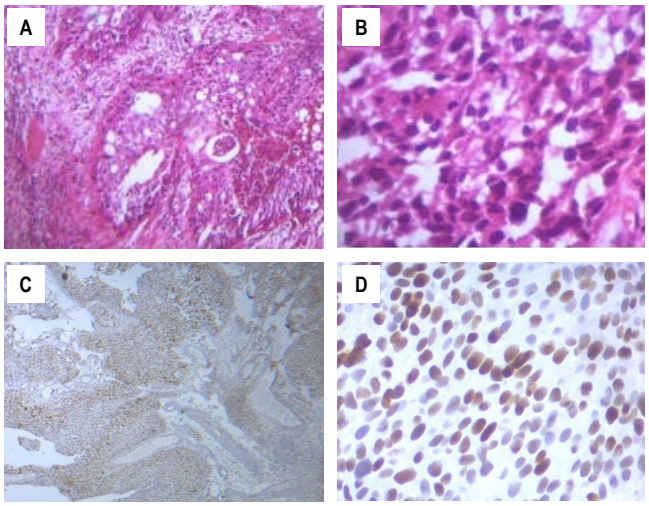

Figure 2: Muscle-invasive urothelial carcinoma, Microcystic variant

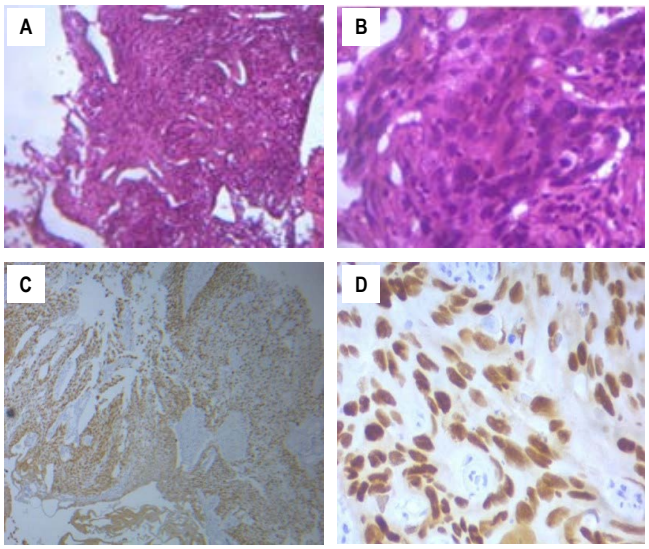

Figure 3: Muscle-invasive urothelial carcinoma with squamous differentiation

of *TP53* with nearly no corresponding mutations is uncommon. According to recent data from The Cancer Genome Atlas (TCGA), *TP53* is the most frequently mutated cancer driver gene across most human cancer types.[16,21] This finding is consistent with the few published papers in this area, notably 49% and 60.7%.[22,23]

The findings from this study are not very common because, traditionally, when there is a strong immuno-expression of the *TP53* protein in a sample, a *TP53* gene mutation is expected.[17] The mutant *TP53* protein has a long half-life and quickly accumulates in the cell, creating a stable target for IHC detection.[10,17] In contrast, the wild-type *TP53* protein (i.e. the normal protein) has a short half-life, making it almost undetectable by IHC.[17]

However, a few studies not necessarily related to bladder cancer explain scenarios that can lead to the stabilisation of the wild-type *TP53* protein. For instance, it is now known that mechanisms that lead to the stabilisation of the mutant *TP53* protein also stabilise the wild-type *TP53* protein.[13,16] This heterogenous stabilisation of the wild-type *TP53* is not related to the functional or structural defects of the *TP53* gene but rather to agents that promote exogenous

stress.[16,24] Foods such as soybeans, eggs, sweet potatoes, and brown rice, among others, contain ceramides that bind to the *TP53* gene and disrupt *MDM2-TP53* interactions, leading to wild-type *TP53* stabilisation.[24] Likewise, when levels of β-catenin increase in the cell nucleus, *TP53* proteolytic degradation reduces, and *TP53* transcriptional activity increases, further leading to the accumulation of wild-type *TP53* proteins.[25]

It should also be noted that several cancers contain large numbers of epigenetic changes affecting DNA or chromatin proteins.[21] In other words, there is a possibility that our samples exhibited epigenetic changes that led to overexpression of the wild-type *TP53* gene products. Similarly, results from the study of comprehensive molecular characterisation of MIUC showed that samples in cluster 4 had no *TP53* or *RB1* mutations. These tumours were associated with the luminal-papillary messenger ribonucleic acid (mRNA) subtype, displayed papillary histology, were predominantly node-negative, and tended to occur in younger patients.[22]

Therefore, although rare, it is likely that the overly expressed proteins (Table I, Figures 1–3) seen in our study were normal (non-mutant) and, hence, products of the *TP53* wild-type gene. Alternatively, it might be the effect of epigenetic dysregulation, including alterations in DNA methylation, histone modification, and chromatin remodelling, which have an essential role in controlling gene expression.

Study limitations

The portion of the tumour sample on which mutational analysis was carried out differed from the immunostained part. Therefore, it is conceivable that tumour heterogeneity could account for the discordant findings. Furthermore, the inability to re-sequence the samples using a newer system, such as AVITI, might have provided additional comparative insights. However, it is essential to note that the Illumina system's accuracy remains highly reliable, with over 85–90% of bases typically exceeding a Q30 quality score (indicating 99.9% accuracy) and mapping specificity consistently above 99%.[26] This robust performance ensures the credibility of our sequencing results despite the lack of cross-platform validation.

This was a single-institution study with a small sample size and possible selection bias (consecutive sampling). However, given that it was conducted at the national referral hospital, which manages most of the cases of bladder cancer in the country, the results provide a good insight into the Ugandan patient population.

Conclusion

Our findings contradict many studies that report *TP53* IHC positivity to correspond with *TP53* mutations in various cancers, underscoring that positive *TP53* proteins at IHC may not necessarily indicate the presence of *TP53* mutations in the same tissue.[16,27,28]

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Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

Ethical approval was obtained from Makerere University School of Medicine research and ethics committee under protocol reference number: Mak-SOMREC-2021-257.

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