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Mutational analysis of *p53* gene in cervical cancer and useful polymorphic variants in exons 3 and 4

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Abstract

Background: Factors contributing to the pathogenesis and progression of cervical cancer include poor attitude to screening and health intervention, late presentation, among others. Mutations in *p53* gene have been attributed to several cancer cases. The present study was designed to find relationships between the mutation patterns in *p53* gene and cervical carcinoma staging. Such knowledge could contribute to early diagnosis of cervical cancer.

Results: From the sequence analysis of *p53* gene fragment isolated by polymerase chain reactions (PCR), nineteen (19) polymorphic variants were identified. Missense mutations occurred in 47% of the samples, 32% were silent mutations, 16% were frameshift mutations and 5% nonsense mutations. Socio-biological characteristics of the study participants revealed that 60% have husbands with multiple sexual partners and that only 23.3% of the participants have ever had the Papanicolaou (Pap) smear test prior to diagnosis, whilst 20% were unaware of the screening test.

Conclusions: Increased severity of cervical carcinoma staging as revealed from the histopathological analysis was found to be associated with accumulation of higher levels of mutations in the *p53* gene. Molecular analysis of p53 gene mutations may prove useful as a screening biomarker for cervical cancer.

Keywords: Genomic DNA, Cervical carcinoma, Gene mutation, Polymorphic variation

Background

Cancer is a multi-factorial disease that remains a serious global concern. Cervical carcinoma is the fourth most frequent cancer in women with a global incidence rate of 570,000 reported in 2018, and representing 6.6% of all cancers in females [1]. Approximately 90% of cancer-related deaths occur in low- and middle-income countries [2], with about 85% of the 250,000 deaths recorded annually from cervical cancer are reported in developing countries where most cases are presented in the late stages of the disease. The incidence of cervical cancer in Nigeria is about 250/100,000 with about 8000 deaths annually [3].

Three major factors have been identified in cervical cancer pathogenesis out of which two are related to human papillomavirus (HPV) infection. These include the consequences of HPV DNA integration in the host genome, the effects of viral oncoproteins and the accumulation of cellular genetic damage not associated with HPV infection [4]. High risk of HPV infection, late diagnosis due to poor screening uptake and prognosis and low uptake of HPV vaccination have been attributed as the major factors culpable in the cervical cancer burden in women [4].

HPV is the primary causative biological agent involved in cervical cancer pathogenesis [4, 5]. The high-risk HPV's DNA encodes the oncoproteins E6 and E7 which bind to p53 and retinoblastoma proteins, respectively. The binding of the E6 protein, through its interaction with the E6-associated protein (E6AP), to p53 induces

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the degradation of p53, the tumour suppressor protein [6] (as schematically shown in Fig. 1). This proteasomal degradation pathway could play a crucial role in cervical carcinogenesis through the promotion of cell cycle entry that leads to unregulated proliferation of invasive cells [7, 8].

On the other hand, the protooncogenes and tumour suppressor genes play vital roles in carcinogenesis [9]. The p53 protein (commonly referred to as the guardian of the genome), which contains 11 exons, is a nuclear phosphoprotein whose gene is located on the short arm of chromosome 17 at the 17p13.1 locus [10–12]. It plays an important role in the regulation of cell proliferation, the cell cycle, as well as apoptosis and senescence in response to DNA damage [13, 14], and is frequently mutated in most human cancers [15]. Most mutations observed in the *p53* gene are single base-pair substitutions and occur predominantly between codons 130 and 290 [5]. The *p53* gene has been rarely observed to be mutated in early cervical tumours [6]. In addition, some p53 mutant proteins are unsusceptible to E6-mediated degradation, thereby suggesting that p53 polymorphisms might be culpable in the variation of cell responses to the HPV infection [6].

Mutations in *p53* are also associated with genomic instability [16] and delay in cell cycle progression [17, 18]. The proline-rich region of the p53 protein is essential for induction of apoptotic response to cellular stressors [19] and inhibition of tumourigenesis [20]. However, the E6 oncoprotein causes more efficient degradation of the arginine polymorph at codon 72 than the proline polymorph, thereby, reducing cellular levels of p53 protein and increasing the risk of HPV-associated cervical cancers in individuals with the arginine polymorph [21]. The first *p53* gene mutation in human cancer was described by Bakers and his team [22]. Codon 72 is located within the proline-rich region of the gene and may have a significant effect on the putative (sarcoma homology) SH3-binding domain [14]. Studies have linked genetic

polymorphism of the codon 72 of *p53* to cervical cancer progression [8], and the variation in this codon can either be arginine (GCG) or proline (GGG).

The divergence in the polymorphic variations that exist in *p53* with respect to the pathogenesis of cervical cancer has been attributed to varying geographic locations, and the different environmental exposures [23]. It has also been reported that greater than 85% of known cancer-related *p53* mutations are missense mutations [2, 4]. Some of the missense mutations resulting from single nucleotide substitutions can affect p53 conformation and result in inactive protein.
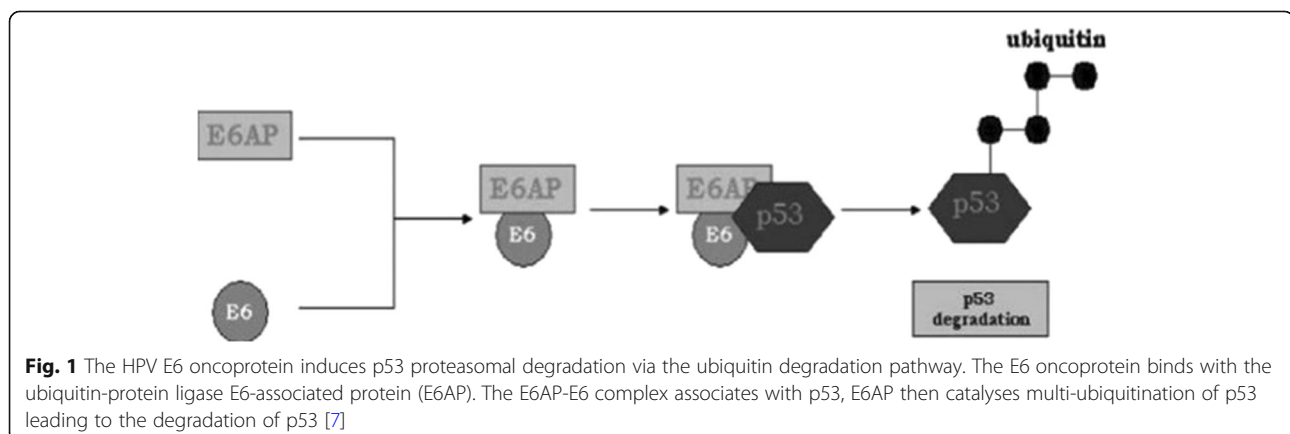
The exon 4 in the *p53* gene is one of the largest, spanning 311 nucleotide base pairs (bp). It contains the proline-rich region and is essential for DNA specific binding, apoptosis, and transcription [10, 16].

To understand further the role of this tumour-suppressor gene mutation-pattern in cervical cancer, we performed a mutational analysis for the polymorphicvariations in the exons 3 and 4 of p53 gene in patients diagnosed with cervical cancer in Ibadan, Nigeria.

Methods

Study site, ethical review and samples collection

The University College Hospital (UCH)-University of Ibadan Institution Ethics Committee approved the study. Written informed consents wereobtained from all study participants before samples were taken. Cervical carcinoma tissues were obtained from such patients who presented at the day-casetheatre of the Obstetrics and Gynaecology Department for examination under anaesthesia, staging and cervical tissue biopsy as required for definitiveapproach to management. The tissue biopsies for the genomic DNA isolation were preserved in a DNA/RNA shieldTM, while about 5 g of the sampleswere preserved for histological studies at the Pathology Department of UCH. Molecular biology procedures were carried out at the Professor AbideenOluwasola Special Diagnostics Centre, UCH, Ibadan.

Fig. 1 The HPV E6 oncoprotein induces p53 proteasomal degradation via the ubiquitin degradation pathway. The E6 oncoprotein binds with the ubiquitin-protein ligase E6-associated protein (E6AP). The E6AP-E6 complex associates with p53, E6AP then catalyses multi-ubiquitination of p53 leading to the degradation of p53 [7]

Histological analysis

Cervical tissue biopsy samples (5 g per sample) obtained from the study subjects were sent to the Pathology Department, College of Medicine for analysis for tumours. This involves histological staining, sectioning and examination under a microscope.

DNA isolation

High molecular weight genomic DNA samples were purified from the cervical tissues using Quick-DNA™ Miniprep Plus Kit as specified in the kit's manual. The purified DNA was quantified on a NanoDrop™ 2000 spectrophotometer.

PCR amplification of *p53* gene fragment

The *p53* gene fragment was amplified from the genomic DNA using forward (5′ TGAAGTCTCATGGAAGCC AGC 3′) and reverse (5′ GCTCTTTTTCACCCATCT ACAG 3′) primers at the optimum PCR conditions in a total reaction volume of 25 μl. Purified genomic DNA from an established cancer-free individual was used as the control. The amplification reactions were carried out in a programmable thermal cycler using the following sequence: Initial denaturation at 95° C for 5 min, followed by 35 cycles of denaturation at 94° C for 45 s, annealing at 55° C for 60 s, and extension at 72° C for 60 s, and a final extension step at 72° C for 7 min, making a total run of about 1 h, 56 min at 9600 ramp to ensure a complete extension of all amplicons.

Analysis of the PCR products

Four (4)-microliter aliquots of the PCR products were applied to 1.5% agarose gel to check that the right size product is obtained. Electrophoresis was performed in TAE (tris-acetate-ethyl diamine tetra acetate) buffer at a voltage of 100 volts, a current of 400 Amps for 45 min. The ethidium-bromide stained gel was viewed on a VIVI LBER transilluminator. The two strands of DNA were then sequenced from the representative samples.

DNA sequencing and sequence analysis

Based on the findings from the histological analyses, amplified DNA from representative samples for normal cervical epithelium, well-differentiated cervical cell, moderately differentiated malignant cells and poorly differentiated malignant epithelial neoplasm were sequenced. The chromatograms were viewed and edited using the combination of FinchTv version 1.4.0 and SnapGene Viewer version 4.2.9 for specificity and accuracy. The nucleotide sequences were then exported from the chromatogram and identified by submitting to the NCBI BLAST-N (National Centre for Biotechnology Information Basic Local Alignment Search Tool for nucleotide) page. Reference nucleotide sequences were

also mined from the same web site. Furthermore, T-COFFEE was used for the multiple sequence alignment of the sequences. EXPASY was used to translate the DNA nucleotide sequence to the protein amino acids sequence. Catalogue of Somatic Mutations in Cancer (COSMIC), International Agency for Research in Cancer (IARC) and NCBI databases were used for the analysis of the gene mutations. Nucleotide sequences from the present study have been submitted to the NCBI public database under the accession numbers MN982161 to MN982165.
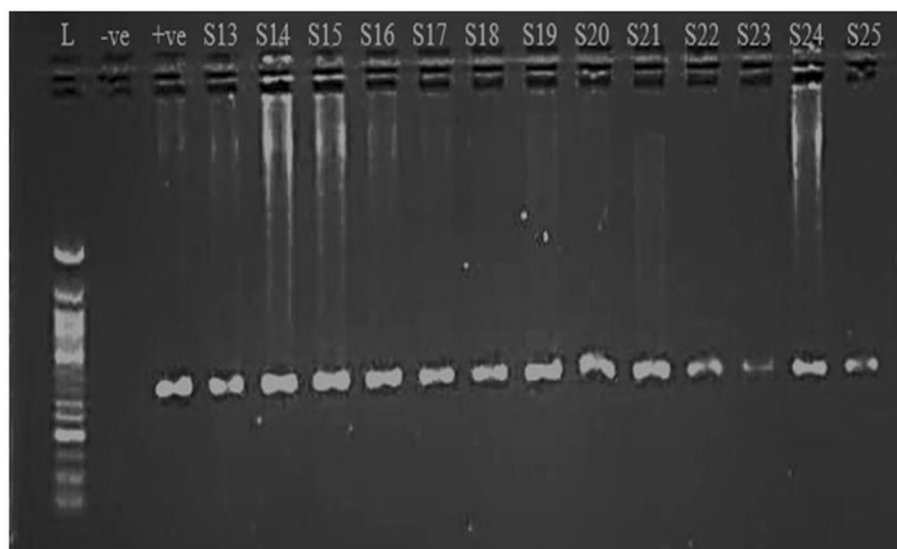
Results

The socio-biological characteristics of the participants are as presented in Table 1.

Using genomic DNA isolated from the cervical tissues of the study participants, highly specific amplification of exons 3 and 4, which spans the coding region of the gene that encompasses the proline-rich region, activation domain, and the sequence specific DNA binding domain were successful. The PCR products analysis on agarose gel revealed that the correct sized (~350 bps) gene fragments were amplified in most positive reactions (Fig. 2).

Thirty selected cases (representing the differentiation stages) of human cervical cancer DNA sequences were analysed for polymorphic variations in the proline-rich

Table 1 The socio-biological characteristics of the participants (*N*=30)

	Frequency	Percentage (%)
Age range (in years)		
36-45	7	23.3
46-55	3	10.0
56-65 years	8	26.7
66-75 years	3	10.0
76-85 years	6	20.0
86-95 years	3	10.0
Marital status		
Married	15	50.0
Divorced	1	3.3
Widowed	14	46.7
Pap smear test awareness/uptake		
Never aware	6	20.0
Ever done	7	23.3
Aware but never done	17	56.7
Husband's known number of sexual partners		
1	12	40.0
2	13	43.3
Greater than 2	5	16.7

Fig. 2 Representative ethidium bromide-stained agarose gel of the PCR products (350 bps) amplified from genomic DNA isolated from the cervical tissues using primers as specified in the "Methods" section. The first lane (L) represents the marker (50 base pair DNA ladder), lane (–ve) is the negative control (no DNA template), lane (+ve) is another control (template is genomic DNA from individual pre-tested to be cervical cancer free) and lanes S13-S25 are PCR products using genomic DNA templates from respective biopsy samples

region, activation domain and the sequence specific DNA binding domain of the *p53* gene. Result of the multiple sequence alignment of the sample sequences and the reference sequence (accession number: NG_017013) [24] is shown in Fig. 3.

The photomicrographs from the histology studies of different staging of the cervical cancer are presented in Fig. 3. The photomicrograph in Fig. 3a shows normal cervical epithelium, with the epithelial wall of the cervix still intact and normal proliferation of cells within the cervix, whilst the one in Fig. 3b shows well-differentiated cervical cells. These cells show extensive areas of necrosis and haemorrhage alongside areas revealing keratin pearls formation by the tumour cells and underlying infiltration of the stroma. Figure 4c shows moderately differentiated malignant cells with focal areas of necrosis as well as stroma infiltration by eosinophils, lymphocytes and plasma cells, and invasion of the fibro-collagenous stroma. Poorly differentiated, malignant epithelial neoplasm is shown in Fig. 3d with mass encroachment of proliferating cells beyond the epithelial wall of the cervix. It was observed that the samples accumulated more mutations with increased severity of differentiation.

Nucleotide variations and polymorphisms from the DNA mutation analysis

The observed distribution of nucleotide substitutions and polymorphic variations in the samples were in the following orders: Missense mutations occurred in 47% of the samples, 32% were silent mutations, 16% were frameshift mutation and 5% nonsense mutation was observed in the cervical tissue samples. The specific codons involved are listed below, using the standard one-letter code for amino acids (W—Tryptophan; L—Leucine; K—Lysine; N—Asparagine; I—Isoleucine; M—Methionine; R—Arginine; P—Proline; A—Alanine; V—Valine; Q—Glutamine; E—Glutamate). In addition, fs* stands for frameshift.

Missense mutations

- At codon 30 (N30K), there was a substitution of Lysine (K) for Asparagine (N)
- At codon 35 (L35W), there was a substitution of Tryptophan (W) for Leucine (L)
- At codon 66 (M66I), Isoleucine (I) substituted for Methionine (M)
- At codon 72 (P72R) and 82 (P82R), Arginine (R) substituted for Proline (P)
- At codon 73 (V73A), Alanine (A) substituted for Valine (V)
- At codon 77 (P77A/Q), Alanine (A) and Glutamine (Q) substituted for Proline (P)
- At codon 86 (A86E), Glutamate (E) substituted for Alanine (A)

Silent mutations

- codon 74 (A74A)
- codon 79 (A79A)
- codon 81 (T81T)
- codon 83 (A83A) and 84 (A84A)
- codon 85 (P85P)

Fig. 3 Multiple sequence alignment (using T-COFFEE MSA tool) of the nucleotide sequences (P1-P10) showing the nucleotide sequence with respect to the reference sequence (accession number: NG_017013). The con shows the conservation pattern of the sequences; an asterisk illustrates a conserved nucleotide across the sequences whilst an empty con shows a variation (SNV or SNP) in the sequences

Frameshift (insertion and deletion)

• At codon 26 (L26fs*), 66 (M66fs*) and 119 (A119fs*)

Nonsense mutation

• A stop codon was introduced at codon 103 (Y103X) instead of Tyrosine (Y)

Discussion

Cervical cancer is not spontaneous but occurs through a multistep process [22] and develops with age [25, 26]. The relationship between age and cervical cancer development is evident in the present study. The mean age of study participants is 59 ± 16.9 years; the age category

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Fig. 4 Photomicrograph of biopsies from Pathology Department; P10 (**a**), P7 (**b**), P3 (**c**) and P2 (**d**) are representives of normal cervical epithelium and cervicitis (26.7%), well-differentiated/polyp (30%), moderately differentiated (30%) and poorly differentiated (13.3%) respectively (magnification ×400). P10, P7, P3 and P2 represent the derived sequences from the PCR products

56-65 years has the highest incidence (26.7%) of cervical cancer cases reported in this study. This is in agreement with the previously documented peak age of incidence [26, 27].

There is a 92% chance of survival if cervical cancer is detected early but as documented by early researchers, most women still present late [3]. Although the Papanicolaou (Pap) smear test is an efficient screening tool that helps in early detection of cervical cancer or its predisposition, there was a poor cervical cancer screening (CCS) uptake by participants in this study. This is similar to the report by Ifemelumma et al. [3].

Most of the mutations detected in the samples in the present study are located within the central DNA binding domain (residues 102–292) of *p53* and are particularly common in the four highly conserved domains in this region. This is consistent with previous reports [28, 29]. Silent mutations in the *p53* gene have been reported to be associated with poor prognosis of breast cancer [2] but a definitive link for cervical cancer is yet to be established. There is a possible synergism between silent mutations and missense mutations in cancer development [2, 14]. The effect of missense mutations in the *p53* gene brings about single amino acid substitutions, resulting in defective p53 conformation, leading to an inactive protein. Missense mutations are the most common mutations observed in our study, which is in line with report by Skaug et al. [30] of about 90% missense mutations in their study.

The codons Phe19, Trp23, and Leu26 are present on the transactivation domain of *p53*, which makes them critical for MDM2 homologue binding, the principal *p53* cellular antagonist [31]. The present study revealed a frameshift mutation in codon 26, which may lead to

deregulation of MDM2, and its overexpression [32, 33] targeting *p53* for rapid degradation [28]. An earlier report has suggested the primary involvement of point mutations in the *p53* gene, resulting in the substitution of amino acids in the protein's central region [34]. It was observed that the E6 oncoprotein inactivates the codon 72 arginine polymorph more easily compared to the proline polymorph, which has been associated with the outcome of HPV infections. Hence, women that have the arginine polymorph might be more susceptible to the effects of the oncogenic HPV types [8]. Ten (10) sequences, representing the four cancer differentiation stages were selected for analysis. Six sequences carry arginine polymorphism. This is corroborated by findings in a study on the Greek population [23]. There was a high presence of p53 arginine polymorphs in the tumour suppressor, which influences the susceptibility of an individual to cervical cancer [23]. Another finding [35] reported that the E6 oncoprotein from high-risk human papillomaviruses (HPVs) resulted in more efficient degradation of the arginine polymorph (R72) than the proline polymorph (P72). This drastically reduced cellular levels of p53 protein and increasing the risk of HPV-associated cancers in arginine homozygotes.

The T350G sequence variation leads to the amino acid substitution L83V in the E6 oncoprotein. Previous studies have suggested that the missense substitution of valine for leucine at codon 83 observed in our study (L83V) is attributed to viral persistence and thus it is regarded as a considerable risk factor for the development of severe dysplasia and cervical cancer [23]. In addition, tumour suppressor associated single nucleotide polymorphisms might directly or indirectly affect tumour progression as well as further

interruption of cell cycles. Allelic polymorphisms that occur in the regulatory regions of these genes are closely associated with malignant cellular alterations [17]. Whereas tumour suppressors are commonly inactivated by frameshift (insertions or deletions) or nonsense mutations, most *p53* mutations are missense and cause single amino-acid changes at many different positions [14].

Although several studies have reported the association of p53 protein accumulation and mutations with cancer prognosis [14, 17, 23], few have explored the *p53* gene mutational landscape with respect to the histopathological analyses in cervical cancer, especially in African populations. Most of the observed mutations in the present study were missense and silent mutations which suggest that the tumour suppressor gene, *p53*, does not accumulate mutations so easily or spontaneously in cervical carcinogenesis (consistent with the findings of Tommasino et al. [28]). Therefore, mutation in this gene can be a very important biomarker to evaluate the development and extent of progression of cervical cancer [4]. An earlier report has suggested that advanced stage and cancer subtypes with aggressive behaviour have more frequent mutations leading to poor prognosis [14].

Conclusions
In this study, 19 mutational polymorphic variations in exons 3 and 4 of the *p53* gene were identified. Increased accumulation of mutational polymorphic variability in *p53* gene was found to be associated with increased severity of cervical cancer; especially in the sequence-specific DNA-binding domain. This tends to have a considerable impact on cervical cancer progression. The present study is limited by the sample size. Further study, using larger cohorts, will help to pinpoint specific polymorphic variability in *p53* gene with specific staging of cervical cancer. It may also be possible to investigate the clinical significance of the observed mutations on drug response amongst cervical cancer patients in the study population.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s43042-021-00144-1.

Additional file 1: Figure S1. Other ethidium bromide-stained agarose gel of the PCR products (350 bps) amplified from genomic DNA isolated from the cervical tissues using primers as specified in the methods section. The first lane (L) represents the marker (50 base pair DNA ladder), lane (-ve) is the negative control (no DNA template), lane (+ve) is another control (template is genomic DNA from individual pre-tested to be cervical cancer free) and lanes S1-S12 are PCR products using genomic DNA templates from respective biopsy samples.

Additional file 2: Table S1. The nucleotide base sequence and parameters of the forward and reverse primers used for PCR amplification of the region of interest of p53 gene.

Additional file 3: Table S2. The composition of the polymerase chain reaction for gene amplification.

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