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ABSTRACT:
Cervical cancer is regarded as a sexually transmitted disease caused by the human papilloma virus (HPV) detected in up to 80 per cent of the cancer biopsies. Genetic susceptibility of a p53 allelic variant has been postulated to play a vital role in carcinogenesis. This study was aimed at determining the allelic frequencies of p53 codon 72 polymorphism in Papua New Guinean women and also assessing the presence of HPV in cervical cancer biopsies. Peripheral blood (3-5 mL) was collected from 53 healthy females of reproductive age (19-37 years) with no known past and current history of HPV infections. Sixty-two cervical biopsies along with cervical swaps were obtained from patients (19-54 years) with clinical symptoms and histopathological confirmation of cervical cancer. DNA was extracted from the peripheral blood samples and cervical samples. Exon 4 was amplified with PCR and further genotypic analyses performed by Restriction fragment length polymorphism (RFLP) and single-stranded conformational polymorphism (SSCP). Of the 53 normal samples analyzed, 3.8 % (2/53) were Arginine homozygous, 58.5 % were Proline homozygous and 37.7 % were heterozygous. For the cancer samples, 14.5 % (9/62) were Arginine homozygous, 54.8 % were Proline homozygous and 30.7% were heterozygous. HPV genome was detected in 83.9 % (52/62) of the cervical cancer samples. The genotypic trend and allelic frequencies were consistent with literature.

Keywords: p53 Codon 72 Polymorphism, Cervical Cancer, Human Papillomavirus
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INTRODUCTION:

The wild-type *TP53* gene is polymorphic on Exon 4 codon 72, having a single nucleotide variation of either CGC or CCC coding for Arginine or Proline amino acid residue, respectively [1]. Allelic frequency distribution globally of exon 4 codon 72 has depicted the preference of Proline homozygotes predominantly around the equatorial regions and with the increase in latitude a transition to the Arginine variant [2]. The significance of the polymorphism stems from the increased

susceptibility to tumourigenesis associated with the Arginine homozygous genotype noted in various cancers including, cancers of the breast [3] bladder [4] , lung [5] , oropharynx [6] and the uterine cervix [7, 8]. It has been demonstrated that the polymorphic variants are functionally distinct, particularly in their ability to induce apoptosis, where the Arginine homozygous has an enhanced apoptotic potential, a property that can translate to cancer risk [9]. Furthermore, it is well documented that the p53 gene is mutated in over 50 percent of all cancers, demonstrating the vital role it plays in the maintenance of cellular integrity via its involvement in cell cycle regulation [10], DNA repair [11] and apoptosis [12].

The *Arg/Arg* genotype *versus Arg/Pro* or *Pro/Pro* genotypes at codon 72 of the p53 gene has been implicated as a risk marker in cervical neoplasia [13]. Cervical cancer is now regarded as a sexually transmitted disease based on the elucidation of human papillomavirus (HPV) oncoprotein E6 associated with premature ubiquitin-dependent proteolytic degradation of the p53 gene products [14] as the initial event in cellular transformation. The fact that only a fraction of females infected with HPV progress to cancer indicates that HPV infection is not a sufficient cause and points to co-factors. Genetic susceptibility is most likely to be one of the important co-factors. The high rate of occurrence of cervical cancer in certain ethnic groups also increases this suspicion. Genetic susceptibility is thought to play a crucial role in the initiation of cervical cancer. The implication of p53 gene and its allelic susceptibility to HPV mediated degradation serves as an appropriate starting point for the screening and detection of preceding events in cervical carcinogenesis. Studies have shown that the Arginine homozygous genotype of the p53 codon 72 polymorphism has being associated with a seven-fold increased susceptibility to HPV-mediated cervical cancer development [7].

In Papua New Guinea (PNG) there is no documentation of the allelic/genomic frequencies of the p53 polymorphism within the population. Since there is high incidence of cervical cancer cases in PNG the need to understand the genomic susceptibility is paramount. This study aimed to determine the allelic frequencies of p53 codon 72 polymorphisms in the Melanesian female population of PNG and assess its association with cervical cancer.

SUBJECTS AND METHODS:

Sample collection and DNA extraction

Peripheral blood (3-5 mL) was collected from 53 healthy females of reproductive age (19-37 years) with no known past and current history

of HPV infections. Sixty-two cervical biopsies along with cervical swaps were obtained from patients (19-54 years) with clinical symptoms of cervical cancer, which included post coital vaginal bleeding, offensive discharge and dyspareunia with further histopathological confirmation. The samples were stored at -20°C until DNA extraction.

DNA extraction was performed depending on the type of sample. DNA was isolated from peripheral blood lymphocytes by the standard phenol/chloroform/isoamyl alcohol method [15]. The biopsies were finely dissected with sterile blades prior to DNA extraction. The swaps were microcentrifuged and pellets collected and lysed with Lysis Buffer containing freshly thawed Proteinase K that was added and incubated at 55°C for 1 hour. The mixture was then heated to 100°C and cooled on ice, ready for polymerase chain reaction (PCR) and subsequent analysis.

Detection of p53 codon 72 polymorphisms by PCR-RFLP and PCR-SSCP

The p53 polymorphic region of exon 4 was PCR amplified from genomic DNA samples utilizing specific sense and antisense primers: p53F 5'-GCTCTTTTCACCCATCTACAG-3' and p53R 5'-AGGCATTGAAGTCTCATGGAAGC-3' (Operon). Each reaction mixture (50 µL) consisted of 1 µL genomic DNA template, 200 µM of each dNTP, 50 mM KCl, 10 µM Tris-HCl pH 8.5, 1.5 mM $MgCl_2$, 26 µM of each primer and 1.25 units *Taq* DNA polymerase (Promega). The mixture was heated to 95°C for 5 minutes then the amplification was carried out by 35 cycles of 30 seconds denaturation at 95°C, 60 seconds primer annealing at 62°C and synthesis at 72°C for 90 seconds. An additional 8 minutes of elongation was allowed at 72°C [15]. The PCR products were analyzed with appropriate DNA markers on a 1.5% agarose gel electrophoresis at 80 volts over 1 hour, stained with 10 mg/mL ethidium bromide and imaged on a UV light transilluminator to ensure correct length of DNA amplified for further analysis (Figure 1A).

PCR-RFLP:

Restriction fragment length polymorphism (RFLP) analysis of the PCR amplified DNA was performed in a reaction mixture (5 µL) containing 0.5 µL *Bst*U1, 0.5 µL 10X NE buffer (BioLabs) and 4 µL of amplified DNA. BstU1 cleaves a 5'-CGCG-3' sequence in the Arginine variant of codon 72. The mixture was incubated at 60°C for 16 hours, electrophoresed on a 1.5 % agarose gel at 80 volts, then stained with ethidium bromide solution and image using a UV transilluminator (Figure 1B) [15].

Figures 1 A, B and C: Determination of the allelic and genotypic frequencies of p53 codon 72 in normal and cancer patients

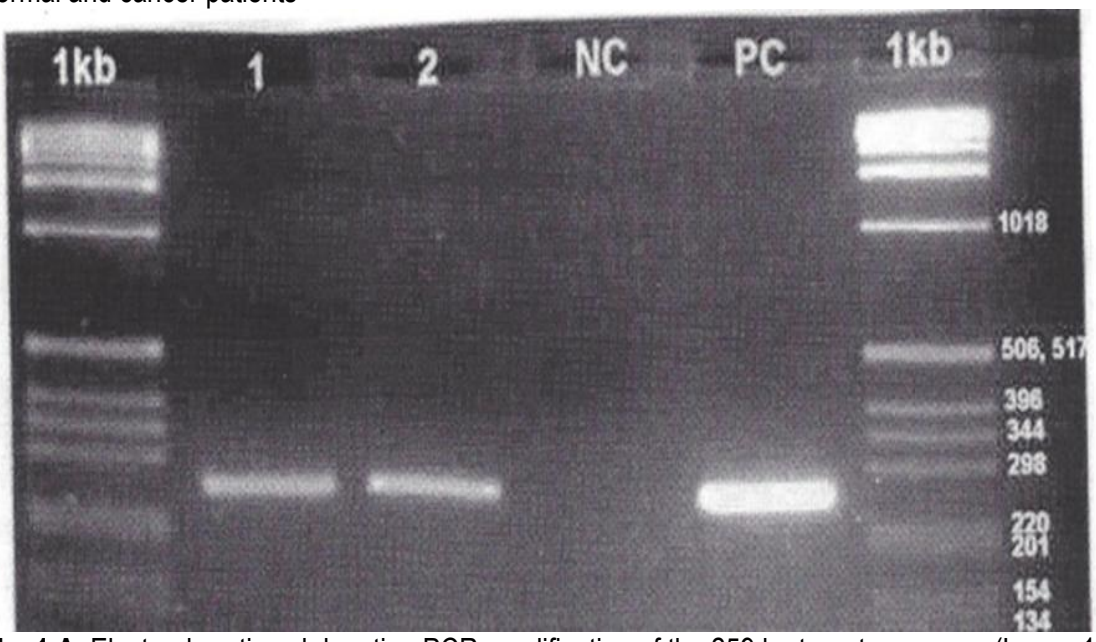

Fig. 1 A: Electrophoretic gel denoting PCR amplification of the 259 bp target sequence (Lanes 1 and 2), negative control (NC) and positive control (PC).

Fig. 1 B: RFLP analysis utilizing *Bst*U1 digestion of PCR amplified p53 product to demonstrate codon 72 polymorphism of homozygous Arginine or Proline or their heterozygous genotype.

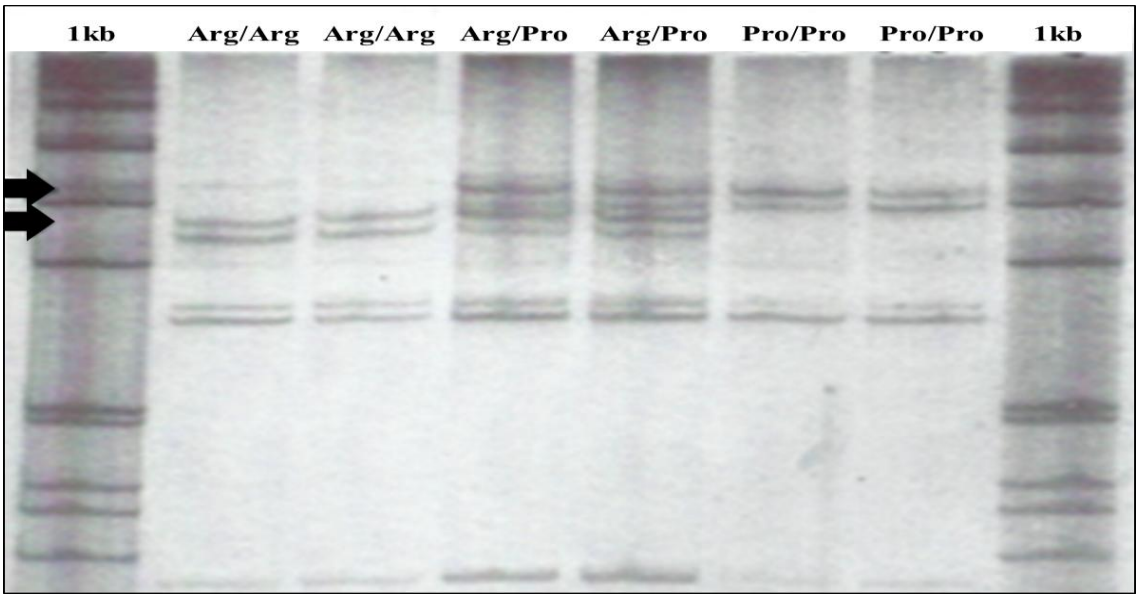

Fig. 1 C: SSCP analysis of same samples in B using PAGE and silver staining.

PCR-SSCP

The single-stranded conformational polymorphism (SSCP) bands were obtained by denaturing a sample mixture (10 µL) of 5 µL PCR amplified DNA and 5 µL of 90 % formamide at 100˚C for 4 minutes then immediately chilling on ice prior to running on a 8 % polyacrylamide gel electrophoresis (PAGE). This was then silver stained by fixing in 40 % ethanol for 30 minutes, then in 10 % ethanol for 30 minutes followed by soaking in impregnation solution (11 mM $AgNO_3$ in 20 mL $_{dd}H_20$) for 30 minutes and subsequently in developing solution (7.5g NaOH, 2 mL of 36 % formaldehyde in 250 mL of $_{dd}H_20$) until the SSCP bands discerned then immersed in 5 % acetic acid to stop reaction. The gels were then image on a UV transilluminator gel documentation system (Figure 1C) [16].

Detection and typing of HPV

The presence of HPV DNA was detected by nested PCR utilizing general primers GP5+ and GP6+ as initial primers and MY09 and MY11 as subsequent primers [17]. HPV type specific PCR for HPV-16 and HPV-18 were done utilizing the sense and antisense primers HPV16F/R and HPV18F/R respectively (Table 1). The extracted DNA were first amplified for detection by general primers in a reaction mixture (50 µL) consisting of 1.25 mM of each dNTP, 10 µM of GP5+ and GP6+ (primary) and later MY09 and MY11 (secondary), 35 mM of $MgCl_2$ in 10x PCR buffer and 0.2 µL *Taq* polymerase. The mixture was heated for 1 minute at 95°C prior to subjecting to 30 cycles

of primary PCR amplification at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds followed by elongation at 72°C for 7 minutes.

Table 1: The HPV forward and reverse primers of both the general and type-specific sequences for nested-PCR [17].

Primer	Sequences (5'→3')
	Consensus primers for HPV L1 region
MY09	CGTCCAAGAGGATACTGATC
MY11	GCACAGGGTCATAATAATGG
GP5+	TTTGTTACTGTGGTAGATACTAC
GP6+	GAAAAATAAACTGTAAATCATATTC
	Types specific HPV primers
HPV16F	ACCGAAACCGGTTAGTATAAA
HPV16R	GATCAGTTGTCTCTGGTTGCAAAT
HPV18F	CACACCACAATACTATGGCGCGCT
HPV18R	CTGCTGGATTCAACGGTTTCTGGC

Secondary amplification involved preheating at 95°C for 1 minutes then 30 cycles at 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 30 seconds and ending with elongation period for 7 minutes at 72°C. The PCR products were analyzed as previous stated. For HPV type specific detection the secondary amplification step involved type specific primers for the high risk HPV (hr HPV) subtypes investigated (Figure 2A).

RESULTS:

The study involved a total of 115 subjects; 62 histologically confirmed cervical cancer patients obtained from the Goroka Base Hospital and 53 healthy females from the School of Medicine and Health Sciences (SMHS), University of Papua New Guinea (UPNG). The types of cancers included pre-neoplastic lesions CIN I-III, squamous cell carcinoma and adenocarcinoma. Two highly sensitive tests were utilized to detect and confirm the genomic composition of the samples. RFLP and SSCP analysis followed the amplification of a 259 base pair (bp) sequence containing the codon 72 polymorphism which is a part of exon 4.

HPV association in cervical cancer samples

HPV was detected in 52 out of 62 (83.9 %) cervical cancer samples of which eight out of

the total HPV infected samples showed co-infection, while 10 samples were negative (Figure 2B). The high risk HPV types screened included HPV-6, 11, 16, 18, 31, 33 and 35. Type-specific determination of the high risk types showed HPV-11 accounting for 26.9 %, HPV-16 – 19.2 %, HPV-18 – 19.2 % and HPV-33 – 3.9 % while other HPV types accounted for the remaining 30.8% (Figure 2B and insert). HPV-6, -31 and -35 were not detected.

Figure 2. The general detection HPV and of high-risk type HPV in cervical cancer specimen

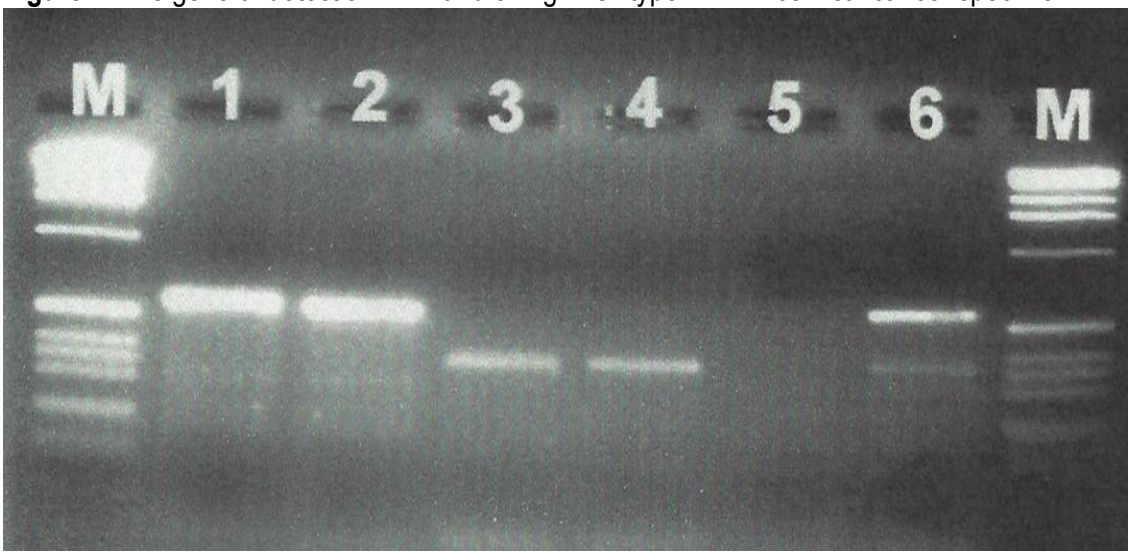

Fig. 2 A: Electrophoretic gel showing PCR amplification of HPV-16 (Lane 1 and 2), HPV-18 (Lanes 3 and 4), negative control (Lane 5), positive control for both HPV 16 and -18 (Lane 6) and 1 kb DNA ladder (M).

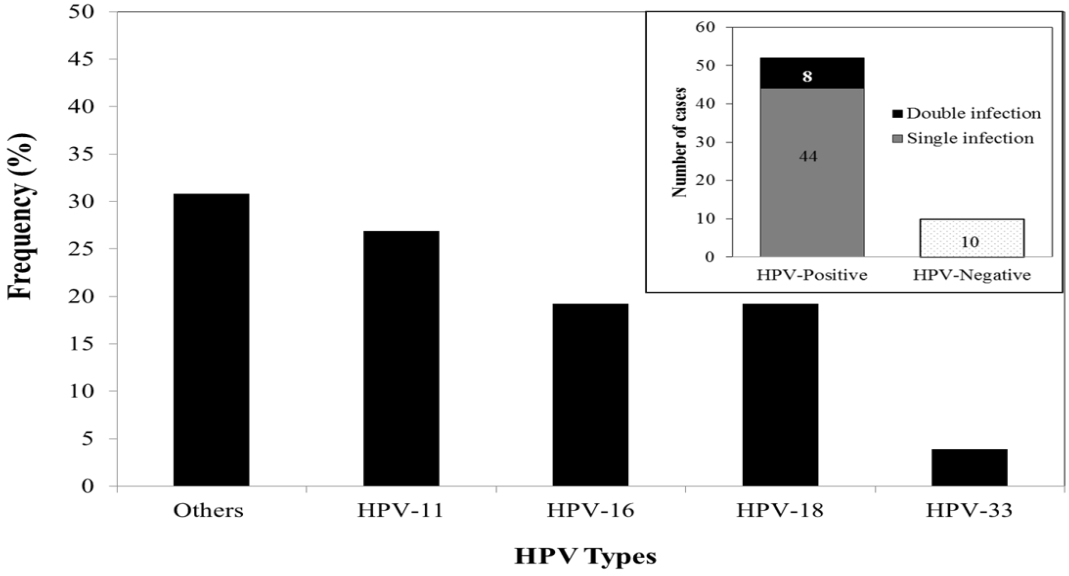

Fig. 2 B: The frequency of HPV subtypes in the population studies. (**B insert**) Represents the total number of HPV positive and negative cases and also includes cases of double infection.

p53 Codon 72 allelic frequencies

The allelic frequencies were calculated using the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$), where frequency of genotypes *Arg/Arg* = p^2, *Arg/Pro* = 2pq and *Pro/Pro* = q^2. On the other hand, looking at the results separately, the 53 normal subjects showed genotypic frequencies of *Arg/Arg* as 3.8 %, *Arg/Pro* represented 37.7 % and Pro/Pro 58.5 %, while, on the other hand, genotypic frequencies of the 62 cancer samples showed *Arg/Arg* genotype as 14.5 %, *Arg/Pro* represented 30.7% and *Pro/Pro* 54.8 % (Figure 3).

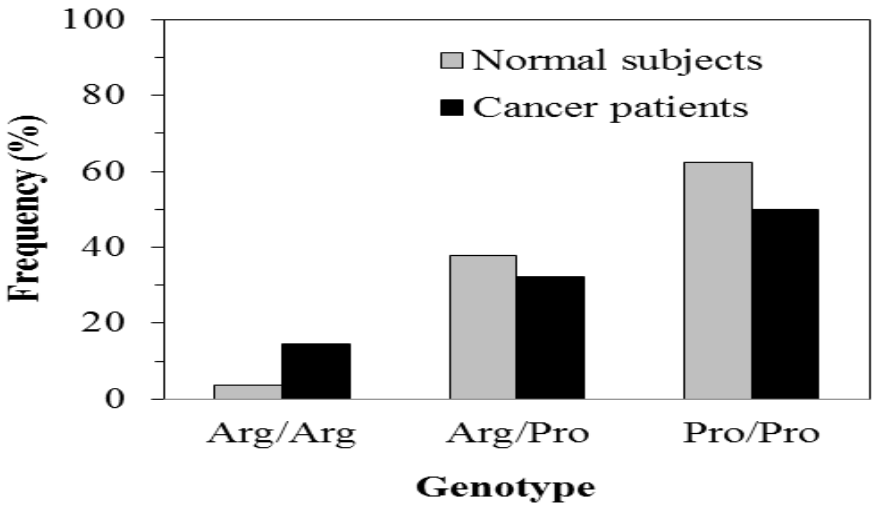

Fig. 3: Graph showing genotypic frequency distribution of the p53 codon 72 polymorphism in the studied population.

DISCUSSION:

It has been postulated that the frequencies of p53 codon 72 genotypes vary according to different ethnic groups [8, 18]. On the contrary, the polymorphic allelic frequency distribution of Arginine and Proline has demonstrated a distinct geographical pattern where the prevalence of the Proline allele across multiple genetically unrelated populations increases as one nears the equator. An impression that the Proline allele confers a protective effect against certain forms of UV damage [19]. The findings in this study are consistent with this observation revealing a higher frequency of the Proline allele (77.4 % and 70.2 % for normal and cancer subjects, respectively (Table 2)) in the Melanesian population. On the other hand, the frequencies of the p53 Arginine alleles are

generally lower around the equatorial region and increases with latitude, which was also noted in this study. The relationship between the allelic and genotypic susceptibility of the p53 codon 72 polymorphism and cervical cancer development could not be elucidated due to the lack of statistical power, despite the *Arg/Arg* homozygous genotype been established as the susceptible genotype. The trend observed in this study leans towards this association as the frequency of the patients with *Arg/Arg* genotype (9/62 – 14.5 %; Table 2) was relatively high for a small sample size. As expected the frequency of the *Pro/Pro* genotype was high in both the normal and the cancer samples. The observed heterozygote (*Arg/Pro*) distribution of the patients was slightly lower than that of the control population; an observation that favours the 'loss of heterozygosity' theory preferred for HPV mediated p53 degradation.

Table 2: The frequency distribution of p53 codon 72 and the HPV status of cervical cancer patients in Papua New Guinean women

Genotype frequencies

Genotype	**Normal (%)**	**Patients (%)**
Arg/Arg	2/53 (3.8)	9/62 (14.5)
Arg/Pro	20/53 (37.7)	19/62 (30.7)
Pro/Pro	31/53 (58.5)	34/62 (54.8)

Allelic frequencies

Allele	Frequencies (%)
P 53 Arginine	29.8
P 53 Proline	70.2

The introduction of the bivalent HPV vaccine [20] in recent years is a major breakthrough in the proactive management of cervical cancer. However, in developing countries like PNG where vaccination has not yet being routinely administered and thus herd immunity is yet to be achieved, cervical cancer is still a major problem. As such, the battle against this preventable and curable disease is still raging in all fronts. The basic approach of Papaniculou (PAP) smear done routinely along with adequate education on early symptom recognition and clinical presentation is still the gold standard of management, but again coverage is minimal.

HPV infection and persistence are necessary for the initiation and progression of cervical cancer [8, 21]. The HPV prevalence in PNG in general is not known but a small study done at the PNG Institute of Medical Research (PNGIMR) of 114 females attending the Gynaecology clinic at the Goroka Base hospital revealed a 52 % overall incidence with high-risk types HPV-16 and 18 accounting for 33 % [22]. In this study, 83.9 % (52/62) of cervical cancer samples analyzed contained HPV genome (Table 3), a finding that is consistent with literatures which have shown detection in up to 99.7 % of cases [23].

Table 3: HPV status of cervical cancer patients in Papua New Guinea

General detection	HPV-11	HPV-16	HPV-18	HPV-33	Others
52/62 (83.9 %)	14/52 (26.9 %)	10/52 (19.2 %)	10/52 (19.2 %)	2/52 (3.9 %)	16/62 (30.8 %)

The high risk types HPV-16 and 18 together accounted for 38.4 % of all cervical cancer cases while HPV-11 genome was detected in 26.9 %. Furthermore, the presence of co-infection of these high risk HPV types was detected in 15.38 % (8/62), a potential scenario where cancer initiation and progression is likely to be rapid. The data shows females as young as 19 years developing HPV-positive cervical cancer. This further adds to the fact that the HPV oncoproteins not only degrade p53 gene (E6) but the E6-E7 fusion protein degrades RB1 genes [24], both proteins that play vital roles in the maintenance of cellular genome integrity.

CONCLUSIONS:

The allelic and genotypic frequency distribution of the p53 codon 72 polymorphism in the Papua New Guinean population is shown to incline towards the general global occurrence. The results obtained in this study provide a glimpse of the amount of work required to establish the extent of the HPV-mediated cervical cancer in PNG, starting with the determination of the incidence and prevalence of high-risk HPV types within the population. This may lead to the recommendation for compulsory vaccination schedules to be incorporated into the national immunization programs. And finally, the high incidence of

HPV in cervical cancer specimen highlights the rationale for HPV testing in addition to cervical cytology in routine cervical screening.

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