ASSOCIATION OF HUMAN PAPILOMA VIRUS (HPV) 16 AND 18 IN BREAST CANCER BIOPSIES IN NAMIBIA

Davis R. Mumbengegwi[9], Samuelia van Kent[10] and Munyaradzi Tambo[11]

ABSTRACT

Cancer is a growing global health concern due to increased exposure to risk factors including infection by viruses such as human papillomavirus (HPV). HPV is associated with several cancers and may be an etiological agent contributing to increasing breast cancer cases in Namibia. This study investigated the association between HPV infection and breast cancer cases in Namibia. DNA was isolated from 47 breast tumour biopsies, (22 breast cancer positive and 25 negative) and analysed for HPV 16 and 18 sequences using PCR. HPV 16 and 18 were detected in 86.3 % and 81.8 % respectively, of breast cancer positive samples, whilst only 36 % and 48 % respectively, were found in breast cancer negative samples. In total 95.5 % of breast cancer positive samples were infected by at least either of HPV 16 or 18 compared to only 52 % of breast cancer negative samples. Infection with HPV 16 or 18 increases the risk of cervical cancer and possibly breast cancer, hence the results suggest that HPV may contribute to the increasing breast cancer statistics in Namibia. This is the first study in Namibia linking HPV and breast cancer, but a larger sample size will be required to power the study to make the findings statistically significant.

Keywords: Human papillomavirus (HPV), breast cancer, Namibia

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[9] Davis Mumbengegwi is an Associate Research Professor at Multidisciplinary Research Centre, University of Namibia. email: dmumbengegwi@unam.na

[10] Samuelia van Kent is a medical student at the School of Medicine, University of Namibia

[11] Munyaradzi Tambo is a PhD research student at Multidisciplinary Research Centre, University of Namibia

Cancer is a broad term for a class of genetic diseases characterised by abnormal cell proliferation and often involves the invasion of healthy cells in the body (National Cancer Institute, 2015). It is a global health concern with tens of millions of people diagnosed each year all around the world largely due to an increased adoption of cancer-causing behaviours in economically developing countries (World Health Organization [WHO], 2015). These risk factors include age, genetics, and environmental such as the increasing adoption of western diets and culture. Reducing the risk of several cancers may be possible through dietary and other lifestyle changes (Efstathiou et al., 2014).

There were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within five years of diagnosis) in 2012 worldwide. Of these cases, 57 % (8 million) of new cancer cases, 65 % (5.3 million) of cancer deaths, and 48 % (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions. Breast cancer was the most common cancer worldwide in women contributing more than 25 % of the total number of new cases diagnosed in 2012 (World Cancer Research Fund International, 2015). Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females accounting for about 23 % of the total cancer cases and 14 % of cancer deaths. It is the leading cause of death among females in economically developing countries; a shift from the previous decade during which the most common cause of cancer death in females was cervical cancer before the introduction of the vaccine (Jemal, Bray, & Ferlay, 1999).

World Health Rankings show that in Namibia, breast cancer has been ranked 22nd in the top 50 causes of deaths and it was responsible for approximately 0.35 % of deaths. Risk factors for up to 80 % of patients diagnosed with breast cancer may be less obvious and this increases the interest in identifying new risk factors for the disease. Cancer is on the increase in Namibia with the National Cancer Registry reporting cancer incidences between 2010-2014 at 11 248 new cases (Hansen, 2017) up 43.4% from the period 2006-2009. The registry lacks completeness as some cancer cases are missed or not formally diagnosed.

Prostate cancer (23.6 %) and Kaposi sarcoma (18.4%) are the most prevalent among males while breast (27.4%) and cervical (19.4%) cancers are the most prominent in females (Hansen, 2017). In addition, scholarly studies have linked breast cancer to viral infections including human papillomavirus [HPV], (Alibek, Kakpenova, Mussabekova, Sypabekova, & Karatayeva, 2013). Viral infections such as HBV/HCV and HPV are responsible for up to 25 % of

cancer deaths in low and middle-income countries by causing liver cancer and cervical cancer respectively (Forman et al., 2012). General or consensus PCR primers have been developed to detect a broad spectrum of HPV genotypes in a single PCR.

Papillomaviruses are members of the papovaviridae family which infect the basal epithelial cells. Over 40 HPV types can be easily spread through diet, sexual contact as well as from skin and mucous membranes of infected people to that of their partners (Hansen, 2017). Two of the proteins made by high-risk HPVs (E6 and E7) interfere with cell functions that normally prevent excessive growth thus helping the cell to grow in an uncontrolled manner hence avoiding cell death (Hansen, 2017). HPV was found to be associated with several types of cancer such as vulvar, vaginal, penile, oropharyngeal and anal. It was also established that high-risk HPVs are important risk factors for human cervical cancers with 96 % of these cancers found to be HPV positive with the most frequent HPV types being 16 and 18 worldwide (Ribeiro et al., 2015).

Furthermore, Al Moustafa et al. (2014a) reported that high-risk HPVs were found in 95.45 % of the samples for cervical cancer in Syria. These scholars also point out that vaccines against the high-risk HPV types, namely type 16 and 18 which are effective in the prevention of genital warts, pre-cancers and cancers in females have been developed (Al Moustafa et al., 2014b). In fact, a number of studies have identified HPV DNA in breast tissue and specimens (Delgado-García et al., 2017; Heng et al., 2009; Salman et al., 2017; Zhang et al., 2016). While etiological evidence has been suggested for the role of HPV in breast cancer, no clear explanation has been presented for the causative mechanism other than the high rate of HPV positivity as the primary reason (Ohba et al., 2014; Wang, Chang, Wang, Yan, & Cao, 2012).

The main aim of this study was to investigate the presence of HPV in a selected number of breast cancer cases. The study objectives were: To determine the absence or presence of HPV in the breast biopsies and to determine if there is a correlation between the presence of HPV in breast cancer samples and increasing statistics of breast cancer in Namibia.

Methodology

A sample of 47 formalin-fixed paraffin-embedded biopsies of breast tumour tissue were obtained from the Namibian Institute of Pathology (NIP). Twenty two breast cancer positive samples and 25 breast cancer negative samples were collected without a pre-selection criterion to randomise the samples to be analysed in terms of factors such as age, race and health status.

Ethical approval for the study was granted by the Ministry of Health and Social services.

Tissue preparation was guided by the literature (Manzouri, Salehi, Shariatpanahi, & rezaie, 2014): 6µm sections of tissue were cut with a standard microtome from every formalin-fixed, paraffin-embedded tissue block. To prevent samples' cross contamination, the microtome blade was washed with xylene and ethanol after sectioning each block. DNA extraction was performed on the 6µm section of paraffin-embedded tissues as follows: The following solutions were added to each microtube containing tissue: 600µl of 1% SDS**,** 0.1M NaOH solution (pH 12.7)**,** 10-20 beads of Chelex 20.

The tubes were then heated at 100 degree Celsius in a water bath for 45 minutes. The tubes were allowed to cool for 5 minutes. To withdraw the DNA solution, the top solidified wax layer was pierced by a micropipette tip. The solution below the wax layer was withdrawn and transferred to a clean 1.5ml micro tube. The chelex beads were not removed from the solution, and further extraction was performed according to Shi et al. (2002)**.**

Further extraction and purification procedures were performed through the following steps: 500µl phenol: chloroform and isopropanol alcohol was added at 25:24:1 ratio to the dewaxed tissue. Sample was mixed by vortex, and centrifuged at room temperature, 12,000 g for 10 min. The supernatant fluid was transferred to an autoclaved micro tube using a 100µl-pipette, and one volume of chloroform was added.

The solution was again mixed by vortexing, and centrifuged at 12,000 × g for 5 minutes. The upper aqueous supernatant was carefully transferred to another sterile micro tube. 0.1 volume of 3 M sodium acetate was added and the sample again mixed by vortexing after which 1 volume of isopropanol was added. The samples were then incubated at -20 ℃ overnight. The precipitated DNA was centrifuged at 12,000 × g at 4 ℃. The supernatant fluid was discarded, and the precipitate washed once with 75 % ethanol. The extracted DNA was collected after further centrifugation. The final yield of DNA was dissolved in 50 ml distilled water after drying completely in a hood and stored at -20 ℃.

Polymerase Chain Reaction (PCR) for HPV Detection (Hedau et al., 2011)– the presence of HPV DNA sequences was first verified by amplification with two sets of primers. A first round was performed with degenerated primers MY09, 5'-GGTCCMARRGGAWACT-3' and MY11, 5'-GCMCAGGGWCATAAYAATGG-3' which amplified a 450 bp long fragment in highly conserved regions of the L1 gene. A second round was then performed with consensus primers GP5+, 5'-TTTGTTACTGTGGTAGATACTAC-3', and

GP6+, 5'-CTTATACTAAATGTCAAATAAAAA-3' to generate 140 and 150bp long fragments in the L1 region of the virus. Both sets of primers (MY09, MY11 and GP5+, GP6+) targeted the detection of a broad spectrum of both oncogenic and non-oncogenic types of HPV. HPV-type specific amplification was also performed using primers designed to amplify the E6 gene of HPV-types 16 and 18, respectively. HPV PCR products were separated by electrophoresis with 2 % agarose gel stained with GRGreen Nucleic Acid gel stain and purified. Table 1 below illustrates the primers and primer sequences used for PCR.

Table 1

Primers and Primer Sequences Used for PCR

Primer Name	Primer Sequence
MY09	5'-GGTCCMARRGGWACT-3'
MY11	5'-GCMCAGGGWCATAAYAATGG-3'
GP5+	5'-TTTGTTACTGTGGTAGATACTAC-3'
GP6+	5'-CTTATACTAAATGTCAAATAAAAA-3'
HPV-16 (E6)	(Forward) 5'-CACAGTTATGCACAGAGCTGC-3'
	(Reverse) 5'-CATATATTCATGCAATGTATGGTGTA'-3
HPV-18 (E6)	(Forward) 5'-CACTTCACTGCAAGACATAGA-3'
	(Reverse) 5'-GTTGTGAAATCGTCGTTTTTCA-3'

Table 2

Reaction Conditions for PCR Primer Sets

General HPV amplification primers	Initial denaturation	Denaturation	Annealing	Extension	Cycles
MY09/MY11	95°C for 5mins	95°C for 30sec	55°C for 30sec	72°C for 30sec	30
GP5+/GP6+	95°C for 3mins	95°C for 20sec	40°C for 15sec	72°C for 30sec	45

E6 gene specific primers for HPV types 16 and 18	Initial denaturation	Denaturation	Annealing	Extension	Cycles
HPV-16 (E6)	95°C for 5mins	95°C for 1min	55°C for 1min 30sec	72°C for 1min	40
HPV-18 (E6)	95°C for 5mins	95°C for 5min	55°C for 1min 30sec	72°C for 1min 30sec	40

Results and Discussion

The results showed less presence of HPV16 E6 in breast cancer negative samples (Figure 1) compared to breast cancer positive samples. The same was observed for HPV 18 E6 (Figure 2) with the DNA from the virus being found in both breast cancer negative samples and breast cancer positive samples.

Figure 1

HPV 16 E6 DNA bands obtained in breast cancer positive samples, lanes, 1, 2, 3, 4, 5, 6, 7 & 8 are HPV 16 E6 positive samples; lane 9 is an HPV 16 E6 positive control and lane 10 is an HPV 16 E6 negative control.
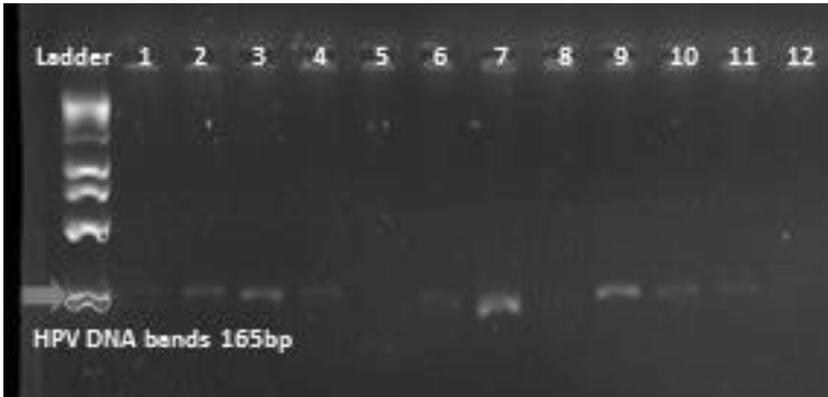

Figure 2

HPV 18 E6 DNA bands obtained in breast cancer negative samples, lane 1 is an HPV 18 E6 negative sample; lanes 2, 3, 4, 5, 6, 7, 8, 10 & 11 are HPV 18 E6 positive samples; lane 9 is an HPV 18 E6 positive control and lane 12 is an HPV 18 E6 negative control.

HPV 16 E6 was detected in 86.3% (19 out of 22) of breast cancer positive samples whilst only 36% (9 out of 25) of breast cancer negative samples were positive for HPV 16 E6 (Table 3). Breast cancer positive samples also showed that 81.8% (18 out of 22) were positive for HPV18 E6 whilst 48% (12 out of 25) of breast cancer negative samples had HPV 18 E6 (Figure 5; Table 3). An interesting observation was that some of the samples contained DNA from both high-risk types; 86.6% of the breast cancer (+) samples were co-infected with both HPV 16 E6 & 18 E6. In comparison, only 32% of breast (-) samples were also co-infected with both high-risk types. Thus, infection with HPV 16 E6 or 18 E6 increases risk of cervical cancer.

Table 3

Frequency of HPV Positive (16 E6 and 18 E6) by Breast Tumour Tissue

	Breast Tissue		
	Breast Cancer (+) N=22	Breast Cancer (-) N=25	Total N=47
HPV16 E6 Present	19 (86.3%)	9 (36%)	28 (59.5%)
HPV16 E6 Absent	3	16	19
HPV18 E6 Present	18 (81.8%)	12 (48%)	30 (63.8%)
HPV18 E6 Absent	4	13	17
HPV16/18 E6 Present*			
HPV16/18 E6 Absent	21 (95.5%)	13 (52%)	32 (68%)
	1	12	15

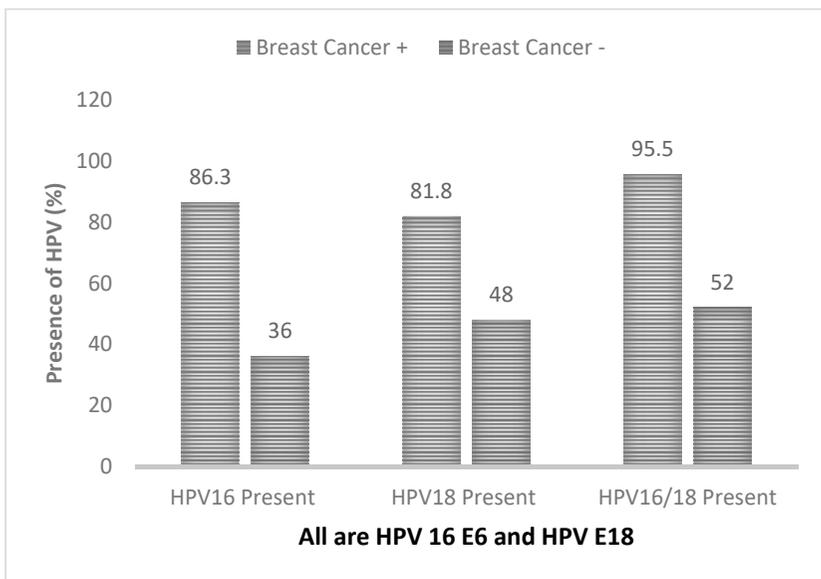*Positive samples contain either HPV 16 E6, 18 E6 OR both

Figure 3: *Presence of HPV 16 E6 and HPV 18 E6 in breast tumour biopsy samples. All samples were detected by PCR and visualized on a gel using both positive and negative controls for determination of true positives and true negatives*

There is a significant difference between the level of HPV DNA detected in breast cancer positive and breast cancer negative (Chi square test

p-value at ά = 0.05, p = 3.841), with more HPV 16 E6 and HPV 18 E6 found in breast cancer positive samples (Figure 3).

Breast cancer is the second most common cancer with 1.67 million new cases per year in 2012 (Ferlay et al., 2014). It is the most common cause of death from cancer among women in less developed regions [324 000 deaths annually] (Cumber, Nchanji, & Tsoka-Gwegweni, 2017). Several risk factors have been identified including sex, age, dense breast tissue, susceptibility genes, family history, ethnicity, hormones, and alcohol consumption. However, for most cases the initiating cause remains unexplained (Dumitrescu. & Cotarla., 2007).

Recently, with improvements in techniques and some encouraging results, there has been a resurgence of interest in the possibility that a significant proportion of human BCs may be caused by viral infections (Amarante & Watanabe, 2009; Joshi & Buehring, 2012) including EBV (Glenn, Heng, Delprado Iacopetta, Whitaker, & Lawson, 2012], and HPV (Damin, Karam, Zettler, Caleffi, & Alexandre, 2004; De Villiers, Sandstrom, Zur Hausen, & Buck, 2005; Huo, Zhang, & Yang, 2012). This study was based on the hypothesis that if HPV increases the risk of breast cancer, then breast tumours positive for cancer will be associated more with HPV 16 and 18 than breast tumours which are negative for cancer. This means that HPVs should be present in some human breast cancers and in some normal or pre-cancerous tissue however, at a lower proportion.

Paraffin-embedded tissues have been used for pathology studies for decades because they are suitable for storage over a number of years and they represent the largest available source of biological materials such as DNA (Kallio, Syrjänen, Tervahauta, & Syrjänen, 1991; Sengüven, Baris, Oygur, & Berktas, 2014). The increasing importance of molecular studies in determining the genetic basis of diseases has increased their use in conjunction with PCR (Sengüven et al., 2014). The results obtained were consistent with this hypothesis with the detection of HPV 16 in breast cancer positive samples; 86.3 % (19 out of 22) while in breast cancer negative samples, 36% (9 out of 25) were HPV 16 positive. Similar results were reported for HPV 18 where 81.8 % (18 out of 22) breast cancer positive samples had HPV18 compared to 48 % (12 out of 25) for breast cancer negative samples.

Almost all breast cancer positive biopsies were positive for either HPV 16 or 18 or both; 95.5 % (21 out of 22 samples) compared to 52 % (15 out of 25) of breast cancer negative tumours. A study in Algeria also concluded that there was an association between the presence of viral DNA

and aggressive breast cancer phenotypes (Gyenwali et al., 2014). A similar study by Salman et al. (2017) reported the presence of high risk HPVs in 42 % of breast tissues although viral activity was only confirmed in several invasive carcinomas. An even larger study in Spain by Delgado-Garcia (2017) with 251 cases (breast cancer) and 186 controls (benign breast tumours) revealed HPV DNA (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 84 and 89) in 51.8 % of the cases and in 26.3 % of the controls (p < 0.001). HPV-16 was the most prevalent serotype.

In this study infection by high risk HPV in breast cancer cases was very high (95 %) and even in breast cancer negative samples (52 %). This is cause for concern since HPVs are said to be the most common sexually transmitted infections. The results may be suggestive that HPV may be a contributing factor to the increasing breast cancer statistics in Namibia and may be considered a risk factor for the initiation and progression of not only cervical or head and neck cancer, but also breast cancer. The main limitation of this study was the small sample size of 47 breast tumour biopsies (25 benign breast tumour tissue samples and 22 breast cancer positive tumour samples) although the findings are similar to larger more in-depth studies. A meta-analysis of twenty-two case-control studies with 1897 case and 948 control group concluded that HPV infection increased the risk of breast cancer (Kim et al., 2016).

The findings of this pilot study on the viral etiology in breast cancer should be conducted in much larger samples perhaps with matched case control pairs based on factors such as age and ethnicity to shed more light on the risk of HPV infection related breast cancer. This is especially true since an HPV vaccine is already commercially available and may be used in groups at high risk of breast cancer.

CONCLUSION AND RECOMMENDATIONS

This study is the first of its kind to be conducted within the Namibian borders and its findings are of paramount importance in establishing a link between HPV and breast cancer in Namibia and Southern Africa at large. It is recommended that a larger study be conducted to confirm this high level of association between HPV and breast cancer and possible risk factors such as age or ethnicity. Furthermore, such knowledge can be used to put in place preventive measures for groups at risk of viral infection linked to breast cancer through the use of prophylactic HPV vaccines as these vaccines will

help to decrease the likelihood of many HPV-associated cancers. Risky sexual behaviour at an early age may also be a contributing factor to an increased risk of contracting HPV earlier in life and results from a larger study can provide evidence for health messaging on prevention of HPV infections.

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