THE CORRELATION BETWEEN p16 OVER EXPRESSION AND HISTOLOGICAL GRADING OF OROPHARYNGEAL SQUAMOUS CELL CARCINOMA AT MUHIMBILI NATIONAL HOSPITAL

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MMed (Anatomical Pathology) dissertation
Muhimbili University of Health and Allied Sciences
October 2020

Muhimbili University of Health and Allied Sciences

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By

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A dissertation research report submitted for partial fulfilment of the requirements for the Degree of MMed (Anatomical Pathology)

Muhimbili University of Health and Allied Sciences
October 2020

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled "***The correlation between p16 over expression and histological grading of oropharyngeal squamous cell carcinoma at Muhimbili National Hospital****"* in fulfilment of the requirements for the degree of Master of Medicine in Anatomical Pathology of Muhimbili University of Health and Allied Sciences.

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EddaVuhahula, DDS, PhD, FC-Path ECSA

_____

Date

DECLARATION AND COPYRIGHT

I, **Dammy A. Shimbo**, declare that this **dissertation** is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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ACKNOWLEDGEMENT

Many people have contributed towards the accomplishment of my research work, but I am beholden to the following:

Dr EddaVuhahula, for spending her valuable time in sharpening the research ideas, reading and commenting on the manuscript, and her advice as the main supervisor.

Prof Amos Mwakigonja, for sparing his valuable time as well as his advice and administrative support as Head of the Department.

Mr Erick Magorosa of Muhimbili National Hospital (MNH), for his assistance in laboratory testing.

The staff at the Central Pathology Laboratory at MNH and the Departments of Pathology at MUHAS for their support and cooperation

My Husband, Christopher S. Mwansasu and Father Abdulrahman Amir Shimbo for the financial support for my MMed Degree at Muhimbili University of Health and Allied Science (MUHAS).

Last but not the least to my beloved husband, my children, Caroline Sia Christopher Mwansasu, Clarissa Christopher Mwansasu, Collins Christopher Mwansasu and mother Margaret Emmanuel Samiji for their patience and understanding while I was working on my research.

To Almighty God for giving me strength throughout the study period.

DEDICATION

I dedicate this hard work to my beloved husband and children, Caroline, Collins and Clarissa.

I dedicate my work to my parents.

I dedicate my work to my sibling Godfrey Shimbo.

ABSTRACT

Background: Oropharyngeal squamous cell carcinoma (OPSCC) is the most common type of cancer in the oropharynx and is commonly associated with high risk human papilloma virus (rHPV) infection predominantly HPV 16 and 18. HPV oncoproteins E6 and E7 which are encoded by (rHPV) DNA are primarily viral factors which are responsible for initiation and progression of human papilloma virus (HPV) related cancers by inactivation of p53 and Rb proteins, which results in a cascade of molecular events that induce the expression of a p16-related transcript. Subsequently, p16 have been used as a surrogate marker for high risk HPV lesions.

About 50-70% HPV related OPSCC demonstratep16 immunostaining overexpression of which 71-90% are grade II &III (non-keratinizing) sub-types. Despite of a tendency to present at high histological grades, management outcome of HPV-related OPSCC is associated with better patient survival compared to the HPV negative and p16 overexpression appear to have implication in the diagnosis, prognosis and treatment of OPSCC. In economic constrained settings like in Tanzania where detection of rHPV and/or IHC assessment for p16 status cannot be ensured in every OPSCC case, it is important to identify the most likely rHPV related cases that can be subjected for testing in order to be practically cost effective.

Objective: The aim of this study was to find out whether there is a correlation between histological grading of OPSCC and rHPV using p16 immunohistochemistry (IHC) overexpression.

Methodology: The study was a retrospective hospital based study of OPSCC cases diagnosed at Histopathology Department of Muhimbili National hospital for a period of 2 years from January 2016 to December 2017. The H&E slides of oropharyngeal carcinoma were reviewed to confirm the diagnosis and assigned to histological grading according to Broder's grading system. p16 IHC was performed in all cases using formalin fixed paraffin embedded tissue blocks. A case was considered to have p16 (IHC) overexpression if there was diffuse nuclear and cytoplasm stain in more than 75% of the cells. Data were recorded and analyzed by SPSS version 20 and demographic data were analyzed by frequency and percentages. The association between

variables was calculated using chi square where the association was regarded to be significant if p value of <0.05 was obtained.

Results: Eighty five (85) cases of OPSCC were retrieved composed of57(67.1%) malesand28(32.9%) females, with male to female ratio of 2:1.Their age range was between 22 and 92 years with median age of 54 years and mean age of 54.98±14.73 years. Majority (61.2%) of cases were between the age of 31-60 years old. Histologically, about 49.4% (42/85) of the cases were grade II, whereas 35.3% (30/85) were grade I and only 15.3% (15/85) were grade III. The non-keratinizing tumors comprised of 64.7% (55/85). In overall p16 overexpression was observed in 27/85 (31.8%) among which 15(35.7 %) and 9(69.2%) were grade II and grade III respectively and only 10% (3/32) of cases in grade I OPSCC had p16 overexpression. About 59.3% (16/27) of male patient showed p16 overexpression however no association was noted between p16 status with sex, age and site p value=0.3, 0.1 and 0.4 respectively. There was a highly significant association between histological grading of OPSCC and p16 status (p=0.000).

Conclusion: Tumor histology correlated well with p16 overexpression (p< .001), furthermore about one third of OPSCC in this study showed p16 over-expression predominantly grade II and III (non keratinizing type of OPSCC) indicating good response after treatment therefore better prognosis in such group of patients.

Recommendation: Routine p16 IHC should be adopted in our setting especially in lesions presenting with grade II and III OPSCC. This will assist in the management and prognostication of OPSCC. Furthermore, the results of this study recommend inclusion of both gender in preventive programs against HPV related tumors to reduce the rising trend of HPV related malignancies.

CONTENTS

CERTIFICATION i
DECLARATION AND COPYRIGHT ii
ACKNOWLEDGEMENT iii
DEDICATION iv
ABSTRACT v
LIST OF TABLES x
LIST OF FIGURES x
ABBREVIATION: xi
DEFINITION OF TERMS xii
CHAPTER ONE 1
1.0 INTRODUCTION 1
1.1 BACKGROUND 1
1.2 PROBLEM STATEMENT 4
1.3 RATIONALE 5
1.4 HYPOTHESIS/OBJECTIVES 6
1.4.1 Research question 6
1.4.2 Broad objective 6
1.4.3 Specific objectives 6
1.5 LITERATURE REVIEW 7
1.5.1 Prevalence of oropharyngeal carcinoma 7
1.5.2 Risks factors for Oropharyngeal squamous cell carcinoma 7
1.5.3 p16 IHC overexpression in oropharyngeal squamous cell carcinoma 9
1.5.4 Histological grading of OPSCC 9
CHAPTER TWO 11
2.0 MATERIALS AND METHODS 11
2.1 Study design 11
2.2 Study settings 11
2.3Study population 11
2.3.1 Eligibility criteria 11
2.3.1.1 Inclusion criteria 11

2.3.1.2 Exclusion criteria 11
2.3.2 Sampling procedure 12
2.3.2.1 Simple random sampling 12
2.3.2.2 Sample size estimation 12
2.5.1 Sampling method 12
2.4 Laboratory techniques 13
2.4.1 H&E staining 13
2.4.2 p16 antibody staining 13
2.5 Data collection techniques 14
2.6 Quality assurance 15
2.6.1 Sample collection and processing 15
2.7 Variable 16
2.8 Statistical analysis 16
2.9 Ethical considerations and approval 16
CHAPTER THREE 17
3.0 RESULTS 17
3.1 Clinical demographic characteristic of the study population 17
3.2 Histopathological grading of OPSCC 17
3.3 p16 IHC expression 19
3.4 Association of histological grading of OPSCC with p16 IHC status 23
3.5 Association between histological grading of OPSCC and p16 expression 23
CHAPTER FOUR 25
4.0 DISCUSSION 25
CHAPTER FIVE 30
5.0 CONCLUSION AND RECOMMENDATION 30
5.1 Conclusion 30
5.2 Recommendation 30
REFERENCE 31
APPENDICES 37
APPENDIX 1 37
Data capture sheet 37

APPENDIX 2 38
APPENDIX 3 39
H&E staining 39
Interpretation of the results 39
APPENDIX 4 40
p16 antibody staining 40
Interpretation of results 40
APPENDIX 5 41
APPENDIX 6 42

LIST OF TABLES

Table 1: Clinical demographic characteristics 15

Table 2: Association between site, sex and Age andp16 status 19

Figure 3: Association between histological grading and tumor differentition of OPSCCC with p16 status 20

Table 4: Association between histological grading of OPSCC and staining intensity 21

LIST OF FIGURES

Figure 1: Photomicrograph showing p16 IHC staining intensity (A: Negative B: staining in <25% of tumor cells C: Staining in 26%-75% of tumor cells D: Strong diffuse staining >75% tumor cells).....

Figure2: Flow chart showing how cases were collected and stained with p16 IHC 16

Figure 3: Age of participants 17

Figure 4: Histological grading of OPSCC 17

Figure 5Hematoxylin and Eosin stained photomicrograph showing histological grading of OPSCC 18

Figure 6: p16 of OPSCC 19

Figure 7: A:Hematoxylin and Eosin stained photomicrograph showing grade III OPSCC; B: p16 IHC showing diffuse stainin of both mucleus and cytoplasm stain in grade III OPSCCC 20

ABBREVIATION:

CDKN2A	Cyclin Dependent Kinase inhibitor
CKD	Cyclin Dependent Kinase
DAB	Diaminobenzidine
ENT	EarNose Throat
G1	Growth phase 1
H &E	Hematoxylin and Eosin stain
HOD	Head of Department
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
IHC	Immunohistochemistry
MNH	Muhimbili National Hospital
MUHAS	Muhimbili University of Health and Allied Sciences
OPSCC	Oropharyngeal squamous cell Carcinoma
OSCC	Oral Squamous Cell Carcinoma
RB	Retinoblastoma
rHPV	High risk Human Papilloma Virus
SOP	Standard Operation Procedure
SRM	Sakura Model Microtomy
TBS	Tris buffer solution
USA	United States of America
WHO	World Health Organization

DEFINITION OF TERMS

Oropharyngeal squamous cell carcinoma is a malignant tumor arising from the oropharyngeal region of squamous epithelium in origin.

Immunohistochemistry is the method of detection and localization of protein antigens in cells based on the principle of antigen-antibody reaction and is then visualized and interpreted by microscopy.

p16 is a tumor suppressor protein that slows cell division by slowing progression of cell cycle from G1 phase to S phase.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Oropharyngeal squamous cell carcinoma (OPSCC) is the most common type of cancer in the oropharynx. The OPSCC may originate from the base of the tongue, tonsil and oropharyngeal wall and is commonly associated with high risk HPV infection predominantly Human papilloma virus (HPV) 16 and 18. It commonly affects male with the highest incidence seen in France, Slovakia, and Switzerland(1). HPV-associated OPSCCs are commonly related to sexual behavior, particularly the lifetime number of oral sex partners(1).In the study that was done by C.E Faggons et al assessing pattern of head and neck squamous cell carcinoma in relation to risk factors in sub Saharan Africa showed the oral and oropharynx were the commonest affected site by SCC but its association with HPV was not established(2).

Human papilloma viruses (HPVs) are a group of host-specific Deoxyribonucleic acid(DNA) viruses, with remarkable epithelial cell specificity and they are commonly found in the ano-genital tract, urethra, skin, larynx, tracheo-bronchial and oral mucosa(3). There are more than 100 different HPV types that have been identified and classified as high risks such as HPV 16, 18, 31 and low risk including HPV 6, 11, 42, 36(3). HPVs are characterized by special tropisms for squamous epithelial cells, keratinocytes. The synthesis of viral DNA and the expression of viral genes (especially for those codifying capsid proteins) are linked to the keratinocyte level of differentiation (3).It is well established that non keratinizing squamous cell carcinoma (SCC) of the oropharynx is closely related to transcriptionally active human papilloma virus (HPV) and has better survival as compared to carcinomas with a keratinizing phenotype (KSCC)(4), hence rHPV have more affinity to squamous epithelium causing squamous cell carcinoma which may range from grade I to grade III type (well to poorly differentiated) (4)

The molecular mechanism of HPV induced carcinogenesis includes HPV oncoproteins E6 and E7 encoded by high risk HPV DNA. These are primarily viral factors that are responsible for initiation and progression of HPV related cancer by inactivation of p53 and Rb protein which are tumor suppressor genes of the cell cycle(5). E6 promotes cell proliferation by stimulating degradation of p53 protein via cellular ubiquitination, this affects the control of cell cycle hence increase tumor growth. E7 bind Rb protein causing inactivation of Rb protein which affects its

interaction with E2F resulting in the release of E2F in transcriptional active forms. The active E2F stimulates replication and cell division hence inhibiting cell apoptosis. This allows mutated genes to pass through the cell cycle (6).

p16is a tumor suppressor gene and a member of INK4 class of cell cycle inhibitors located on chromosome 9p21 and is involved in G1 checkpoint. It binds to CDK-4,-6 inhibiting phosphorylation of retinoblastoma protein which in turn inhibits transition of cell cycle from G1 to S phase. In normal cells p16 expression is epigenetically silenced by polycomb repressive complexes. In activation of Rb protein by E7 causes accumulation of hypo phosphorylated Rb, which in turn signal cell cycle arrest, by releasing p16 hence induces the overexpression of a p16-related transcript(5–7). p16 is used as a surrogate marker for high risk HPV lesions and the findings have been used to determine prognostic as well as developing preventive and treatment guidelines(8).

Some lesion which are not associated with rHPV infection i.e. epithelial ovarian cancer have been known to overexpression p16 protein(9,10). In epithelial ovarian cancer, expression of p16 cells leads to downregulation of the expression of RB. Thus the Cyclin dependent kinase inhibitors and Rb may be functionally linked but this p16 expression is not associated with rHPV oncoproteins E6 and E7; furthermorep16 protein may not be detected in 34% primary ovarian cancers, and loss of p16 is significantly more common in cases with wild-type p53(9,10). In pancreatic ductal adenocarcinoma (PDAC) more than 50% of the tumor have loss of p16 protein due to inactivation of p16 which is induced by mutation, homozygous deletion, and promoter methylation associated transcriptional silencing(11). Hence most of the pancreatic ductal adenocarcinoma does not overexpress p16 protein.

Studies done among Western and African countries have shown that HPV related oropharyngeal carcinoma predominantly squamous cell carcinoma show p16 overexpression and are associated with high risk HPV predominantly HPV 16(4,12,13). HPV positive OPSCC have shown to have a better diagnosis compared to the HPV negative OPSCC and the two entities differ in etiological factors as well molecular carcinogenesis. This knowledge helps in predicting patient survival rate between the two entities.

The World Health Organization (WHO) characterizes the OPSCC as HPV positive squamous cell carcinoma and HPV negative SCC. Studies that were done in African countries including Mozambique and Egypt demonstrated low prevalence of rHPV related OPSCC. The prevalence was observed to be high among middle aged population predominantly male. With HPV related OPSCC showing good patient survival rate(13,14). On the other hand risk factors of oropharyngeal carcinoma such as tobacco smoking, tobacco chewing and alcohol play a major role in the carcinogenesis of HPV negative OPSCC. It has been observed that chewing betel quid and areca nut also increase the risk and play a carcinogenetic role in acquiring HPV negative OPSCC in Asia(1,12,15,16)

Oropharyngeal carcinoma lesions are not infrequent encountered at Muhimbili National Hospital as well as other hospitals in Tanzania(17,18). However, studies on these lesions in relationship to HPV subtypes are scanty in our region, partly because HPV detection requires use of fresh tissue specimens and advanced laboratory methods such as PCR. On the other hand, researchers have found that p16 immunohistochemistry overexpression on paraffin embedded sections can be used as a surrogate marker for high risk HPV.(4,19)

The aim of this study was to find out whether there is correlation between histological grading of OPSCC and rHPV using p16 immunohistochemistry (IHC) overexpression in Tanzanian patients. The results will assist in proving baseline data for more studies targeting primary prevention of OPSCC as well as predict the prognosis that can guide in the treatment plan of OPSCC patients.

1.2 PROBLEM STATEMENT

Squamous cell carcinoma (SCC) constitutes more than 90% of the malignant neoplasms of the oral cavity and oropharynx. Global trend show increasing incidence of HPV positive OPSCC in male and young to middle aged adults especially in economically developed countries(39).It has been clearly documented that there is a causal link between HPV infection and OPSCC. The number of oral sexual partners has strongly been linked to HPV related OPSCC(4,40).

HPV related OPSCC demonstrate pathologies ranging from well differentiated to poor differentiated (Grade I- III) with grade II and III being the predominant pattern. Unlike HPV negative oropharyngeal carcinoma, HPV positive OPSCC with p16 overexpression is known to have better prognosis and good patient survival rate(4,41).

In Tanzania there are several studies on pattern and clinical pathological characteristics of head and neck cancer including OPSCC(18,42,43) however its association with HPV has not been elucidated. Most patients with OPSSC in Tanzania have advanced stage of tumor presentation with possible lymph node metastasis and because there is no characterization of the tumor in relation to HPV infection these patients are traditionally labeled with poor prognosis which would have been different if these lesion would have p16 over expression. Recently the Nation has established HPV vaccination program targeting young girls from 9 years old in order to decrease the incident of HPV related cervical cancer but males are excluded. Since both females and males have risk of developing OPSCC vaccinating both of them would have prevented all rHPV related disorders including OPSCC

Lack of data on HPV related OPSCC in our settings, denies the opportunity for our country to study feasibility and establishment of a potential prevention programs and management protocol narrowed for HPV related OPSCC as seen in some of the countries like china and Colombia

Understanding the burden of HPV related OPSCC worldwide and in our setting has important implications in management of the disease and a potential prevention through prophylactic HPV vaccination also introducing male vaccination since they are the major risk group. p16 IHC being a surrogate marker for HPV it is overexpressed in almost all tumors caused by rHPV.

1.3 RATIONALE

This study aimed at determining whether there is a correlation between histological grading of OPSCC and rHPV using p16 immunohistochemistry (IHC) overexpression. The findings of this study will provide a basis for routine use of p16 IHC in our settings especially in the cases present with grade II and grade III OPSCC. This may help in predicting the prognosis as well as providing baseline data for more studies aiming at establishing primary prevention of OPSCC through screening programs and vaccination. Formalin fixed paraffin embedded (FFPE) tissue can be used in p16 expression as a surrogate marker for high risk HPV. MNH has well established histopathology department where biopsies from dental and ENT (Ear Nose and Throat) clinics are processed, but p16 IHC is not routinely done.

1.4 HYPOTHESIS/OBJECTIVES

There is association between histological grading of OPSCC and p16 IHC overexpression.

1.4.1 Research question

1. Is there any association between histological grading of OPSCC and rHPV using p16 immunohistochemistry (IHC) overexpression at Muhimbili National Hospital?

1.4.2 Broad objective

1. To determine the association between histological grading of OPSCC and rHPV using p16 immunohistochemistry (IHC) overexpression at Muhimbili National Hospital.

1.4.3 Specific objectives

1. To determine the histological grade of each OPSCC using Broder's grading.

2. To determine the proportion of OPSCC lesions with rHPV using p16 immunohistochemistry (IHC) overexpressionp16.

3. To determine the association between histological subtypes OPSCC and rHPV using p16 immunohistochemistry (IHC) overexpression

1.5 LITERATURE REVIEW

1.5.1 Prevalence of oropharyngeal carcinoma

Globally there has been a significant increase in incident and mortality caused by OPSCC(20). Worldwide it is estimated that the incident of pharyngeal cancer to be about 48500 and 88000 cases in developed and developing countries respectively with high mortality among developing countries(15). The prevalence of HPV-positive OPSCC is higher among men than women and it is significantly highest among Caucasian population with low prevalence seen in black(21).The rising trend in oropharyngeal cancers has been observed in young adults and middle ages patients worldwide with mean age of 55 years(22).In Tanzania oropharyngeal tumor accounts 9.7% of all head and neck tumor with 8.8% being OPSCC(18) but its association with HPV not been established. In the study that was done in Mozambique to assess HPV related OPSCC, showed female predominance with the mean age of the female being below 59 years(14).HPV positive OPSCC are more predominant among Caucasians and have good patient survival rate compared to HPV negative OPSCC and in other hand HPV negative OPSCC are associated with lifestyle risk factors such as alcohol drinking, smoking and tobacco chewing(1).

1.5.2 Risks factors for Oropharyngeal squamous cell carcinoma

1.5.2.1 Non- HPV related risk factors

1.5.2.1 .1 Benign Oral Mucosa lesion

In the study assessing lifestyle risks of OPSCC and oral HPV infection has showed that leukoplakia and oral lichen planus are among the most common oral mucosa lesion. Leukoplakia was diagnosed in 16 out of 137 cancer patients (12%) where by 7 (5.3%) had homogenous leukoplakia. Oral lichen planus was diagnosed in six cases (4.5%) and six controls (1.9%).(1)Current lesions of herpes labialis were diagnosed in one case and one control. Reactivated herpes labialis was reported by 21 of the cases (16%) and 84 of the controls (26%). There was no association between a history of herpes labialis and HPV DNA positivity (1)

1.5.2.1.2 Alcohol and Tobacco use

Tobacco use and alcohol consumption are well-established risk factors for oral and oropharyngeal squamous cell carcinoma. The risks increases significantly corresponding to

duration and number of cigarettes smoked per day as well as increasing levels of alcohol consumption are considered together for all cancer sites (16,23). HPV negative OPSCC is commonly seen in patient with history of smoking .In the study assessing prevalence of oropharyngeal, tongue and lip squamous cell carcinoma among Egyptians showed that 76% of patient with history of smoking had OPSCC with negative p16 overexpression(13)

1.5.2.2 HPV related risk factors

1.5.2.2.1 Oral sex habits

Oral sexual behavior is the most common lifestyle risk factor for oral HPV infections though sexual habits differ across different geographical regions (16, 17). Most patient with oral and oropharyngeal carcinoma have reported to have oral sexual partners during their lifetime. Oral sex is most common in the low age group <45 years. The risk of Oral and Oropharyngeal squamous cell carcinoma has been associated with an increasing number of oral sexual partners(1,23). Presence of HPV in the oral cavity is associated with increased risk of oral cavity or oropharyngeal cancer. HPV-positive oropharyngeal cancers have a distinct molecular, clinical, and pathologic disease entity that is likely causally associated with HPV infection and has a markedly improved prognosis.(24). Several studies done in Africa have showed that oral sex seemed to be an uncommon practice in the region but oropharyngeal HPV was closely associated with multiple sexual partners (9,36)

1.5.2.2.2 High risk HPV infection

High-risk HPV (rHPV) infection is known to be significantly associated with oral sex (1). In the study assessing head and neck squamous cell carcinoma in South African patients rHPV predominantly HPV 16 were isolated in oropharynx and was associated with oropharyngeal squamous cell carcinoma (25). rHPV commonly HPV16 and HPV18 are known to be tumorigenic in human epithelial tissues. The viral oncoproteins of high-risk HPVs, E6 and E7, promote tumor progression by in- activating the TP53 gene (also known as p53) and retinoblastoma tumor suppressor gene products, respectively(26). HPV positive OPSCC are less likely to occur among moderate to heavy drinkers and smokers; patients with HPV-positive tumors are known to have reduced risk of death from cancer compared with HPV-negative HNSCC patients .(24)

1.5.3 p16 IHC overexpression in oropharyngeal squamous cell carcinoma

Globally about 50-70% OPSCC are HPV positive and demonstrate positive p16 immunostaining with 70-90% being grade II and III(non-keratinizing type)(19,27). The study which was done in Egypt it revealed 28 % the patients had HPV related OPSCC with positive p16 IHC (13) hence demonstrate low number of HPV positive OPSCC compared to HPV negative through both p16 IHC and HPV in situ hybridization**.** HPV positivity in OPSCC prevalence varies among regions and races; this may be contributed by lifestyle practices in the region(28,29). p16 expression has been established as a good surrogate marker for high-risk human papilloma virus (rHPV) infection in oropharyngeal squamous cell carcinoma (OPSCC) patients(30–32). Due to the rise of incident of HPV related cervical and oropharyngeal carcinoma several countries including China and Colombia have initiated HPV vaccination to both female and male population whereby significant decrease in oral HPV detection has been noticed among vaccinated population in Colombia(33,34)

1.5.4 Histological grading of OPSCC

Squamous cell carcinoma is one of the most common HPV related Oropharyngeal Carcinoma(19). There are various grading systems for oral and OPSCC including Anneroth, Broders' and the Bryne's invasive tumor front (ITF).In several studies that have been done OPSCC are commonly grouped as keratinizing, non-keratinizing and non-keratinizing with maturation(hybrid)(27,35) other studies have further classified OPSCC based on uncommon histological types i.e. as basaloid, papillary, lymphoepithelial, adenosquamous as well as OPSCC with verrucous features. The classification is largely based on the immature, non-keratinized and basaloid appearance of its tumor cells(27,36).

HPV-positive OPSCCs are widely perceived as poorly and can undergo small cells transformation(36).The majority of HPV positive OPSCC are non-keratinizing SCC with p16 overexpression (13,27). HPV-positive OPSCCs usually arise from the reticulated epithelium lining the tonsillar crypts, and they consistently retain the basaloid and non-keratinizing appearance of this specialized epithelium(36). Squamous cell carcinomas are also graded into Grade 1, 2, 3 and 4 according to broder's grading. Broder's grading system grades the squamous cell lesions based on tumour differentiation i.e. grade I squamous cell carcinoma are well differentiates and produce keratin and grade IV are poorly differentiated the cells are anaplastic

with no keratin formation (37), these parameter are important in the prognostic value of OPSCC(35). OPSCC with p16 overexpression are known to have favorable prognosis and globally the overall disease-specific survival rates in OPSCC variants are 46.6% NK SCC (grade III), 24.6% K SCC(grade I) and 28.8% (grade II)hybrid SCC(19,38). NK SCC occurs in middle aged patients commonly in male and it is more frequently presented with lymph node metastases.(38)

This study aimed to find out whether there is correlation between histological grading of OPSCC and rHPV using p16 immunohistochemistry (IHC) overexpression at MNH. The results will assist in primary prevention of OPSCC as well as predict the prognosis that can guide in the treatment plan of OPSCC patients.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study design

This was a retrospective cross sectional hospital based study, in which OPSCC cases diagnosed at the histopathological unit at MNH between January 2016 and Dec 2017were enrolled.Slides of each case were retrieved and reviewed to confirm the diagnosis of OPSCC and histologically graded using Broder's grading system by the investigator under the facilitation of the supervisor. Thereafter, sections from paraffin blocks were stained with p16 IHC and assessed according to College of American Pathology (CAP) criteria.

2.2 Study settings

The main stay of the study was the Department of Histopathology at Muhimbili National Hospital (MNH) at Central Pathology Laboratory (CPL) with a total biopsies received in a year ranging from 9700-10169. MNH is a National referral hospital, receiving patients from different parts of the country. It also serves as a University Teaching Hospital for MUHAS. Patients with oropharyngeal squamous cell carcinoma attend the Dental and Otorhinolaryngology department where endoscopy and open biopsy are performed and then sent to histopathology laboratory at MNH. Biopsies from other health facilities in the country are also attended at CPL-MNH.

2.3Study population

All OPSCC cases whose biopsies were submitted to histopathology unit at MNH from January 2016 to December 2017 MNH were enrolled.

2.3.1 Eligibility criteria

2.3.1.1 Inclusion criteria

All OPSCC cases diagnosed during the study period having investigation forms, tissue blocks with H&E slides.

2.3.1.2 Exclusion criteria

- All OPSCC cases with crushed artifacts
- Cases lacking clinical notes or paraffin embedded tissue blocks in the archive

2.3.2 Sampling procedure

2.3.2.1 Simple random sampling

All 120 cases with OPSCC submitted to histopathology unit at MNH from January 2016 to December 2017 were retrieved and assigned numbers and through simple random sampling the cases were picked to attain the estimated sample size of 87 cases.

2.3.2.2 Sample size estimation

The sample size was estimated to be 87 cases. This was based on a a pilot study I did and found 120 OPSCC cases in a period of 2 years and a 28% prevalence of HPV positive OPSCC in a study done at Egypt(13). Based on the finding the sample size was calculated by the formula

The sample size formula:

n = [DEFF*Np (1-p)]/ [($d^2/Z^2_{1-\alpha/2}$*(N-1) +p*(1-p)]

where:

N= 120estimated total number of cases with OPSCC diagnosed in the 2 years

Z = 1.96 at 95% Confidence Interval.

P = hypothesized percentage of frequency of outcome in the population is 28%

d =Confidence limits as % of 100(absolute +/- %)(d) as 5% in this study

Hence:

Required sample size at 95% confidence intervalwas estimated to be 87 cases

2.5.1 Sampling method

Cases were obtained through archival reports showing OPSCC as well as searching through the register book for all oropharyngeal tissue registered through the study period. All cases were given serial numbers and selected by simple random sampling to attain the required sample size. The selected cases were microscopically reviewed to confirm the OPSCC diagnosis and assigned histological grade by the investigator under supervision of a senior pathologist using Broder's grading system which grades squamous cell lesions based on tumor differentiation i.e grade I squamous cell carcinoma are well differentiates and producing keratin and grade IV are poorly differentiated, the cells are anaplastic with no keratin formation (Appendix 2). The representative

tissue blocks were retrieved, recut and stained with p16 IHC. Among 87 cases, 2 cases were excluded due to missing blocks.

2.4 Laboratory techniques

2.4.1 H&E staining

The regressive Hematoxylin and Eosin staining was done for the all the casesthat had missing H&E slides. Tissue blocks were retrieved for sectioning, sections of 3 micrometer were cut from iced paraffin embedded blocks using rotary microtome (Sakura model) SRM200 CW, mounted on slide labeled with ID number and were allowed to drain before being put on hot plate at 60 degrees centigrade for 15-30 minutes. Sections was dewaxed in two changes of xylene ten dips each and brought down to water through descending grades of ethyl alcohol (absolute, 95%, 80%, 70%).Sections were then stained with Harris hematoxylin for ten minutes and washed in tap water until water was clear and then was differentiated in 1% HCL in 70% ethanol for three seconds. Thereafter tissue sections were washed in running tap water and subsequently blued in running tap water for ten minutes. Sections were counter stained in 1% aqueous eosin for three minutes, and were washed in tap water until the water was clear. Sections were then dehydrated in ascending grades of ethanol (70%, 80%, 95% and absolute) 10 dips each. Clearing of section was done in two changes of xylene and then mounted in DPX and labeled. *(Histo SOP No.5*).

2.4.2 p16 antibody staining

Paraffin embedded blocks of 85 selected cases with confirmed diagnosis were retrieved and recut for p16 IHC procedure where 2 cases were excluded due to missing blocks. The sections of 3 micrometer were cut from iced paraffin embedded blocks using rotary microtome (Sakura model) SRM 200 CW. These sections were then floated in water bath at 45 degrees centigrade, mounted on standard frosted charged glass slides from Thermostat Company each labeled with ID number. Slides were allowed to drain before being put on hot plate at 60 degrees centigrade for 50 minutes. Sections were then dewaxed in two changes of xylene ten dips each and brought down to water through descending grades of alcohol (absolute, 95%, 80% and 70%).Sections were ringed with PAP pen and citrate buffer with pH 6.0 (from DAKO company) was heated in pressure cooker for 15 minutes which was followed by placing tissue sections in antigen retrieval solution in pressure cooker and closing the lid. Tissue sections were kept in for two minutes in

full pressure. Thereafter slides were removed from pressure cooker and placed in water. Slides were not allowed to dry from this point onwards. TBS was added for three minutes, drained off and 2 drops of peroxidase blocking solution (SM 8OIfrom DAKO Company) were added leaving it for 15minutes. Slides were placed in tap water for 3 minutes, and then covered with prediluted p16 monoclonal anti mouse antibody (G175-405) from Medaysis and incubated for 60 minutes. TBS was washed gently on the slide for five minutes, then drained off and covered with HRP (from DAKO Company) for 30 min. (this reagent was kept at room temperature before using it). Sections were washed gently with TBS for 5 minutes, drained, and applied1 drop of working DAB (dilution of 1 drop of DAB to 1 ml of substrate buffer) and incubated for 10 min. Slides were washed in water, counterstained with Harris hematoxylinfor 30 seconds, and then were dipped in 1%acid alcohol 2 dips, blued in tap water for 2 minutes, dehydrated, cleared in xylene, mounted with DPX and then cover-slipped. Finally, these slides were evaluated under light microscope model Olympus CX 41 and representative pictures were taken for the results by using Olympus Soft Image Solutions by USB SC camera (100pixels) (*Histo SOP No. 28*).

The stained slides were reviewed by the principal investigator and the supervisor who isalso a senior pathologist. A known tissue section with cervical carcinoma was used as positive control and adipose tissue was used as negative control.

2.5 Data collection techniques

A special designed checklist (appendix I) with various sections as per the objectives of the study was used to collect data from the selected cases which met the inclusion criteria. Socio demographic characteristics and histopathological diagnosis of lesions were filled .Slides were reviewed to confirm diagnosis of OPSCC and assigned histological grading according to Broder's grading then stained with p16 IHC.

p16 IHC slides were interpreted by the investigator and reviewed by the supervisor based a study by Lewis(19).

The interpretation of p16 status is as follows;

- 0= No stained cell (Negative)
- 0-25 = Nuclear and cytoplasm stain in < 25% f the cells(Negative)
- 25-75 =Nuclear and cytoplasm stain in 26-75% of the cells (Negative)
- >75 =Positive (Strong diffuse nuclear and cytoplasm stain in >75% of the cells

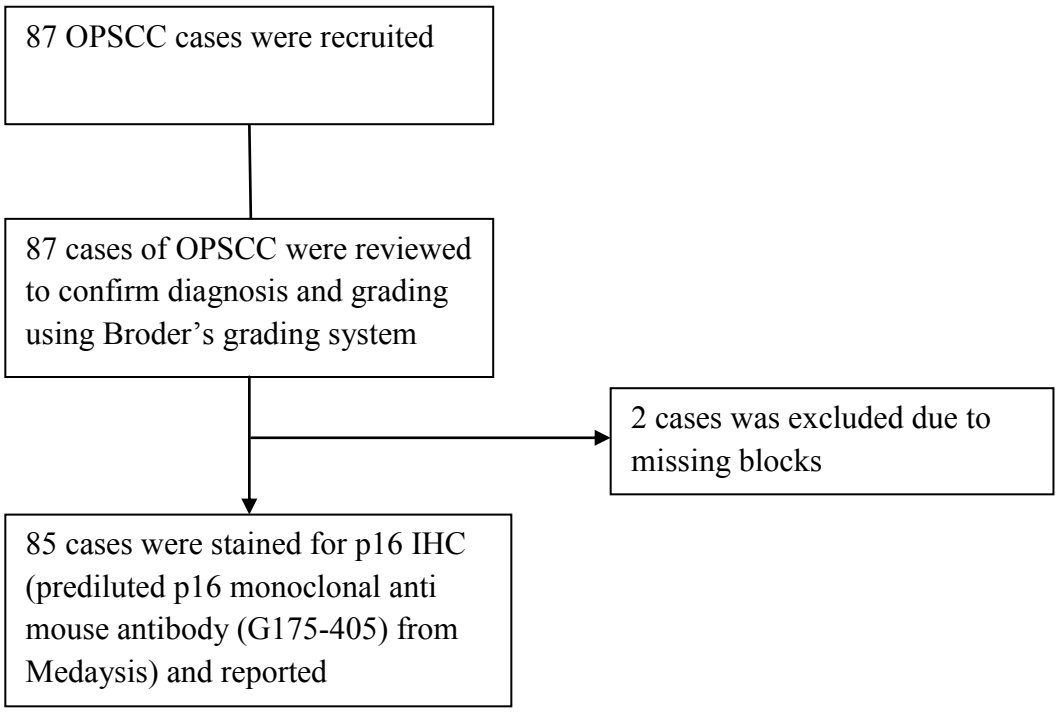


Figure 1: Flow chart showing how cases were collected and stained with p16 IHC

2.6 Quality assurance

2.6.1 Sample collection and processing

All H&E slides were processed as per MNH Central Pathology Laboratory routine protocol and all p16 IHC cases were stained per IHC protocol.

2.7 Variable

Independent variable included: Oropharyngeal squamous cell carcinoma, age sex and site, histological grading

Dependent variable: p16 Immunohistochemistry

2.8 Statistical analysis

The data were electronically transcribed from the data collection sheet to SPSS version 23 program and then analyzed. Data was summarized into proportion and frequency tables and summary statistics with number of cases and percentage were used to show the frequency of each histological grade OPSCC which was graded by Broder's grading system. OPSCC cases were further classified based on degree of keratinization where cases with >20% of the keratinized cells were called "Keratinizing" OPSCC while cases with cells showing <20% of keratinized cells were called "Non keratinizing". Also number of cases and percentage for categorical variables were used to describe the proportion of cases with and without p16 IHC overexpression. The relationship between p16 IHC expression with age, site and sex was performed using Chi square or Fisher exact test to assess the statistical significance**.** Furthermore summary statistics with number of cases and percentage for categorical variables were used to determine the association of p16 IHC overexpression with histological grading of OPSCC. The relationship between p16 IHC overexpression with OPSCC histological grading and tumour degree of keratinization was performed using Chi square or Fisher exact test to assess the statistical significance; the data was considered to be statistically significant when p value was <0.05.

2.9 Ethical considerations and approval

Prior to commencement of the research, the proposal was presented to the department of Pathology of Muhimbili University of Health and Allied Sciences. Ethical clearance was sought from the Research and Publication Committee of the School of Medicine and from the Senate Research and Publications Committee of Muhimbili University of Health and Allied Sciences. Administrative permission to conduct the study was obtained from Muhimbili National Hospital as per the hospital management protocols. Informed consent was not need because only archives tissue materials were used without disclosing the identity of the population.

CHAPTER THREE

3.0 RESULTS

3.1 Clinical demographic characteristic of the study population

During the study duration of 2 years, 87 OPSCC cases were enrolled. Among 87 cases, two were excluded because of missing blocks. Therefore, Broder's grading with consecutive p16 IHC were done in all 85 cases and were interpreted and compared.

Among 85 OPSCC cases,57(67.1%) were male, with male to female ratio of 2:1,age of the patients in the study ranged from 22-92 years with majority of the cases seen in patients between the age of 31-60 years old52(61.2%) however four cases were young adults between 20-30 years. The mean age of participants was 54.98 ± 14.73years with the mode and median of 45 and 54 years respectively *(Figure3*). OPSCC was commonly seen in the base of the tongue 43(50.6%) while oropharyngeal wall being the least affected site 12(14.1%). (*Table1*)

Table 1: Clinical demographic characteristics

Demographic data		**Frequency N=85**	**%**	**Mean age**	**Mode**	**STD deviation**
Sex	Males	57	67.1	54.91	50	15.94
	Female	28	32.9	55.10	48	12.31
Age	≤30 years	4	4.7	54.98	45	14.73
	31-60 years	52	61.2			
	≥61 years	29	34.1			
Site	Base of the tongue	43	50.6			
	Tonsil	30	35.3			
	Oropharyngeal wall	12	14.1			

3.2 Histopathological grading of OPSCC

Majority of OPSCC were Grade II 42(49.4%) with grade III being the least common 13(15.3%) (*Figure4*), no grade IV tumor was noted. In this study OPSCC was further classified based on degree of keratinization where 55(64.7%) were Non keratinizing and 30 (35.3%) keratinizing type.

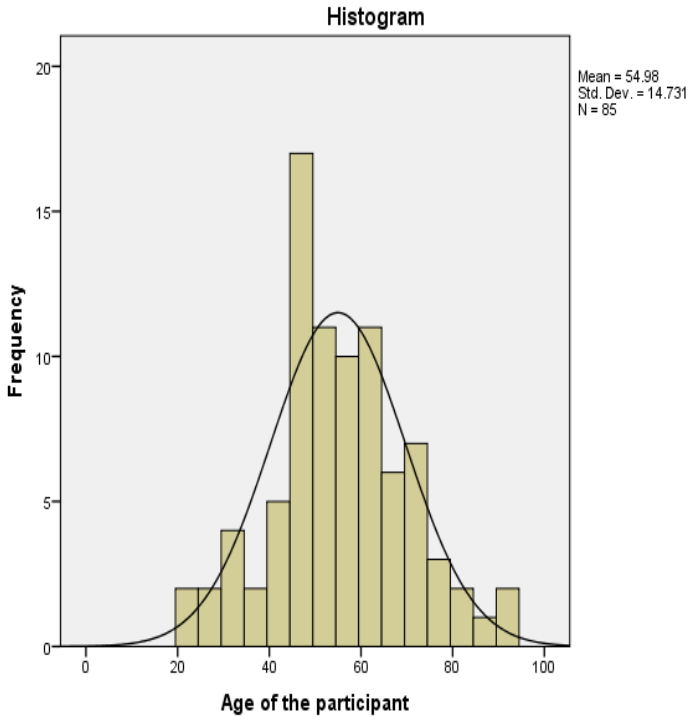


Figure 2: Age normal distribution curve of the study showing median of 54 years and peak age seen at 45 and 65 years.

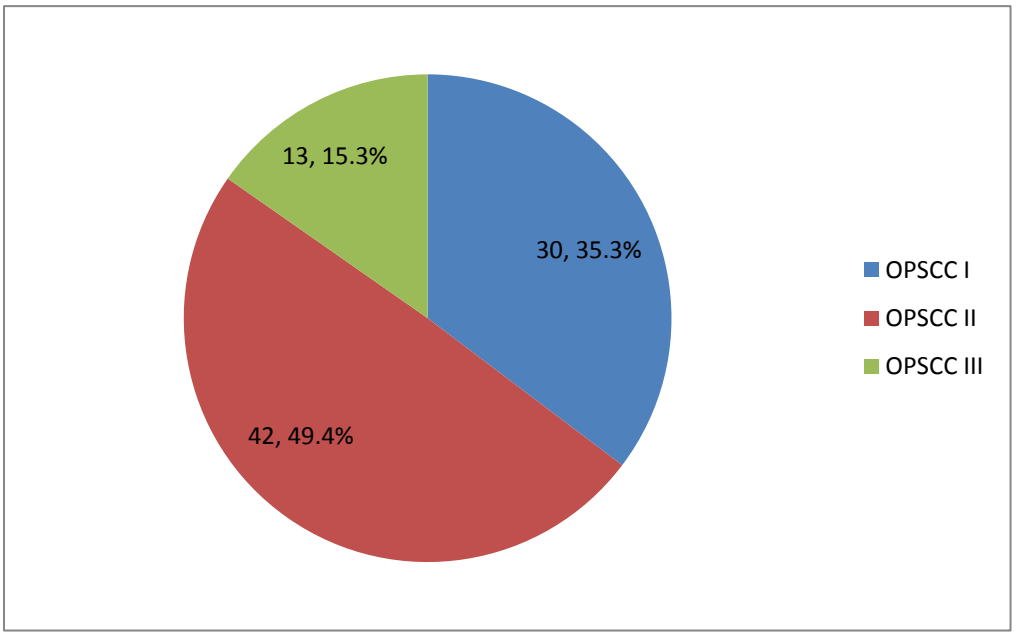


Figure 3: Histological grading of OPSCC

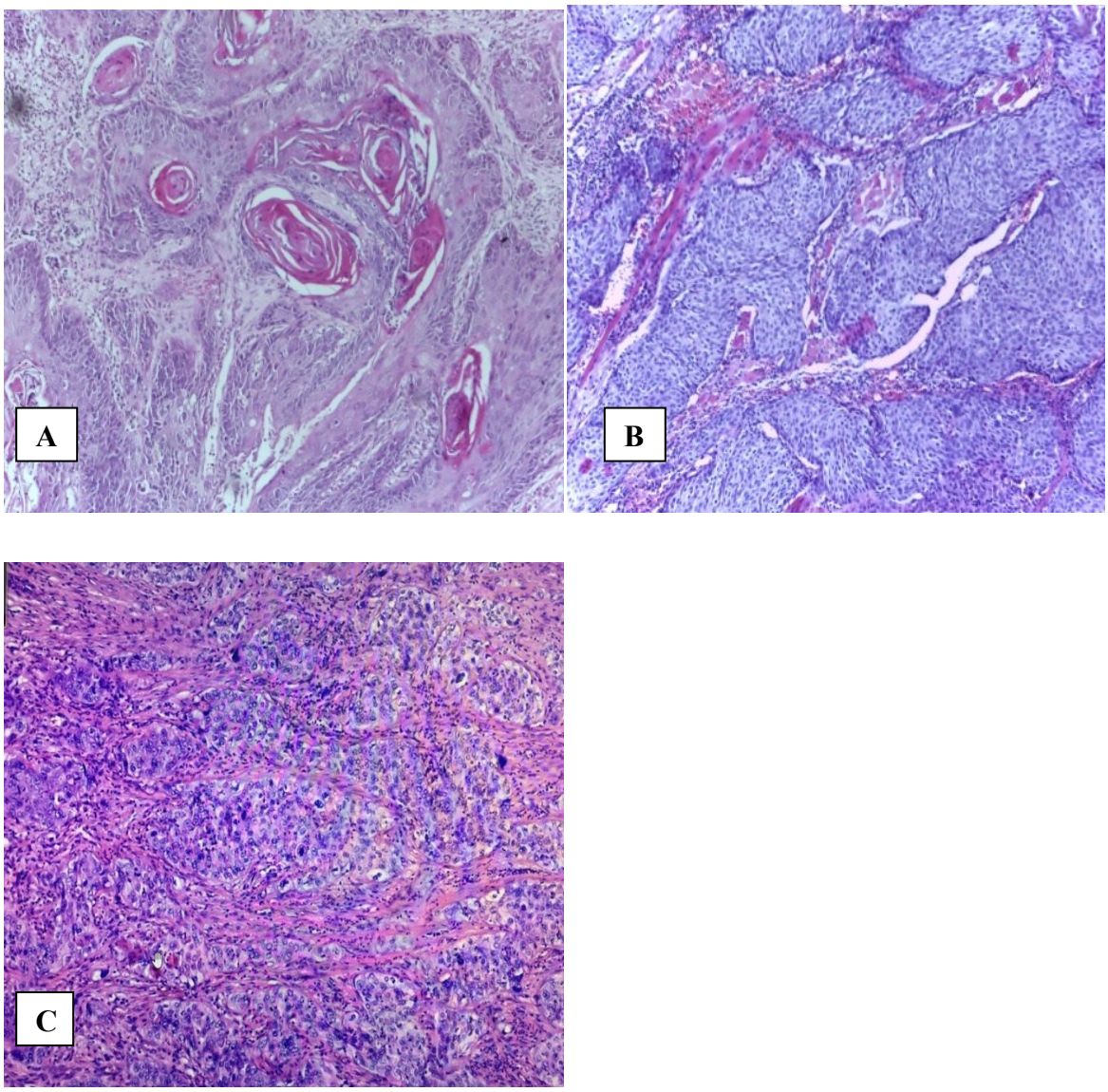


Figure 4: Hematoxylin and Eosin stained photomicrograph showing histological grading of OPSCC (A: Grade I(X10), B: Grade II (X10), C: Grade III (X10))

3.3 p16 IHC expression

Twenty-seven (27) cases out of 85 cases which accounted to 31.8% of OPSCC overexpressed p16by IHC(*figure 6*). Proportionally, females (39.3%), the patient below 30 years (75%) and tonsil (40%) as anatomical site were observed to overexpress p16 than their respective counterpart; however, the difference was not statistically significant. These findings with their

respective p value are summarized in Table 2. The study also noted that only four patients below the age of 30 years, of whom 3 had p16overexpression status.

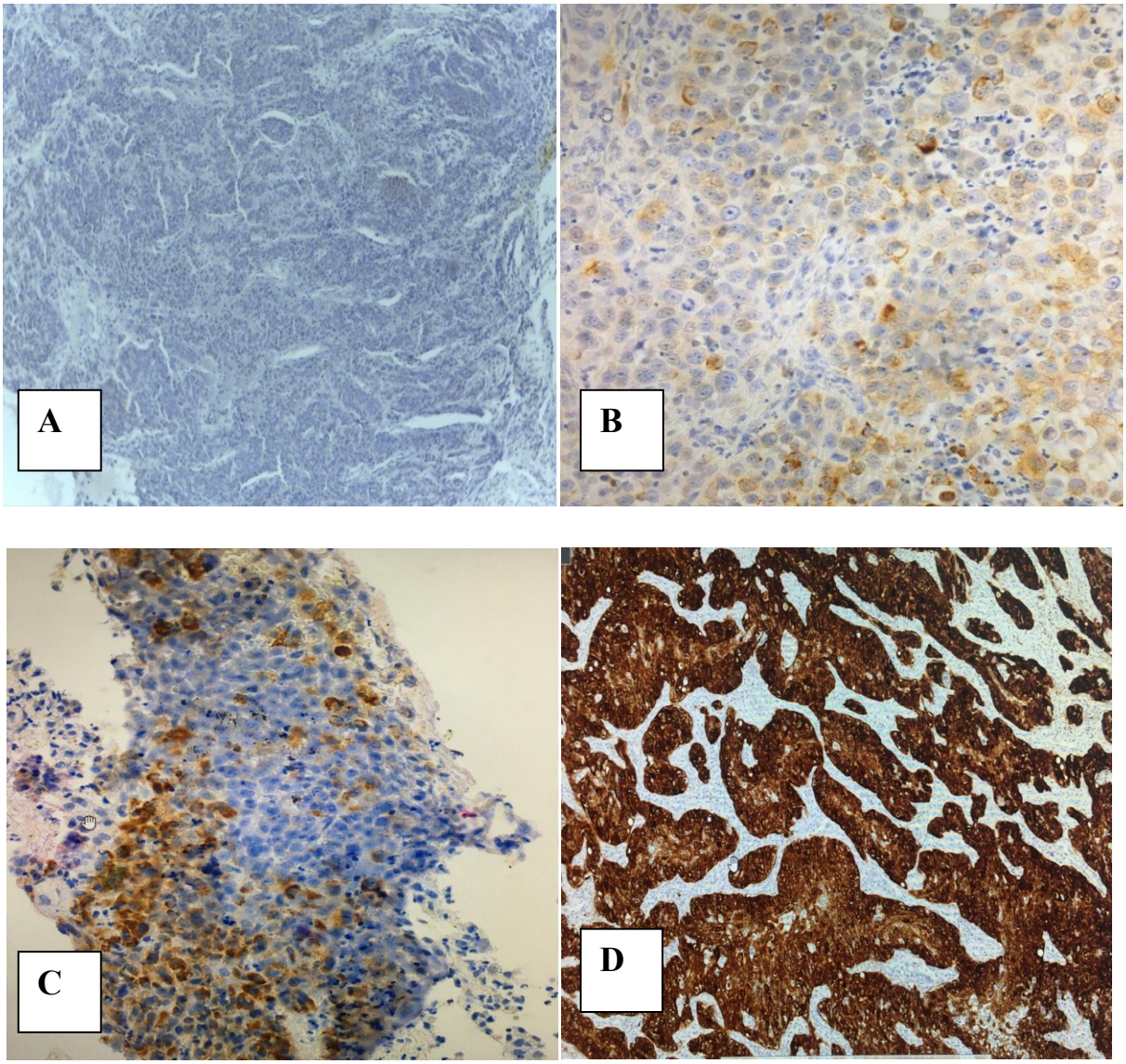


Figure 5: Photomicrograph showing p16 IHC staining intensity (A: X4 Negative B: X10 staining in <25% of tumor cells C: X 10 Staining in 26%-75% of tumor cells D: X10 Strong diffuse staining >75% tumor cells).

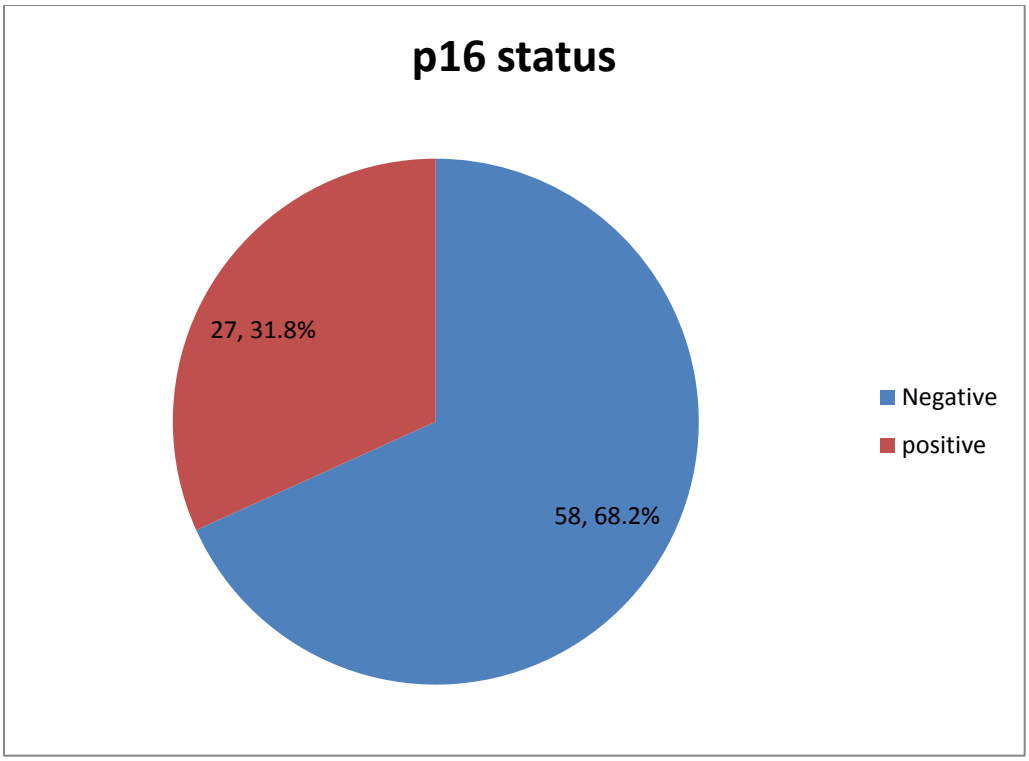


Figure 6: p16 of OPSCC

Table 2: Association between site, Sex and Ageandp16 status

Variable	Negative	**p16** Positive	**X^2**	**p value**
Sex				
Males	41 (71.9%)	16 (28.1%)		
Females	17(60.7%)	11 (39.3%)	1.090	0.31
Age in years				
≤30	1(25%)	3(75%)		
31-60	39(75%)	13(25%)		
≥61	18(62.1%)	11(37.9%)	5.05	0.07
Site				
Base of the tongue	32(74.4%)	11(25,6%)		
Tonsil	18(60%)	12((40%)		
Oropharyngeal wall	8(66.7%)	4(33.3%)	1.711	0.42

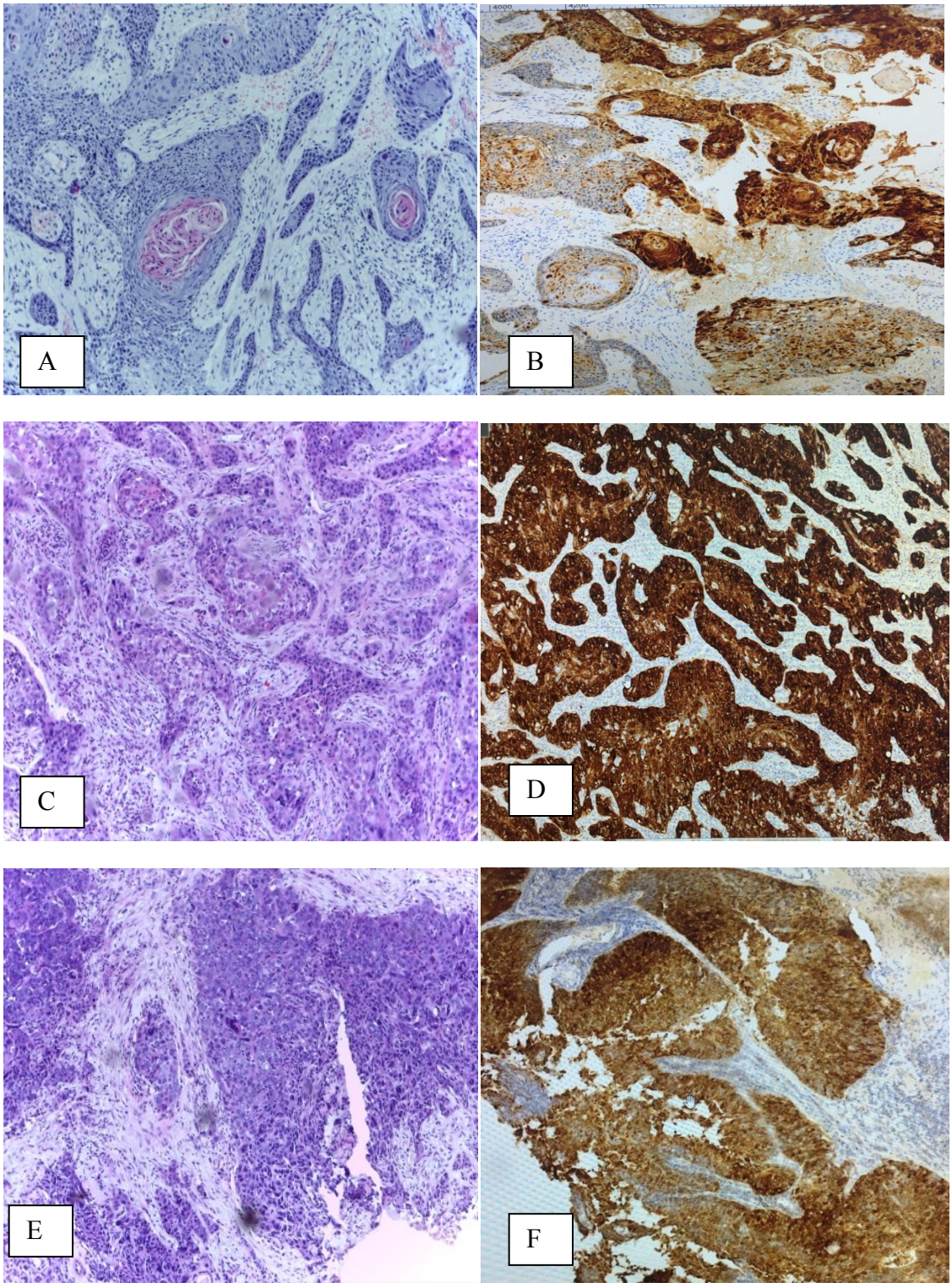


Figure 7: **H&E and p16 IHC photomicrography x10 showing grade I, II & III OPSCC; with their consecutive p16 IHC stain B: patchy cytoplasm &nucleusstaining while D and F: showing diffuse staining of both nucleus and cytoplasm stain**

3.4 Association of histological grading of OPSCC with p16 IHC status

Among grade I OPSCC only 3(10%) showed p16 overexpression; majority of the grade I cases 27(90%)did not show p16 IHC overexpression. In grade II OPSCC 15(35.7%) had p16 overexpression while despite grade III OPSCC having only 13(15%) p16 was overexpressed 9(69.2%) of all grade III OPSCC cases. A statistically significance association was found between histological grading of OPSCC and p16 expression (*fishers exact test p=0.00*).

OPSCC was further classified based on tumor differentiation (Keratinizing and non keratinizing) where among 55 (64.7%) ofnon keratinizing OPSCC 22(42.3%) demonstrated p16 overexpression and a statistically significant association was observed between the histological grading of OPSCC and tumor degree of keratinization (p value=0.01).

Table 3: Association between histological grading and tumor differentiation of OPSCC with p16 status

Variable		p16 status Positive	Negative	X^2	p value/f
Histological diagnosis	OPSCC I	3 (10%)	27(90.0%)		
	OPSCC II	15(35.7%)	27(64.3%)		
	OPSCC III	9(69.2%)	4(30.8%)	15.28	0.00
Degree of keratinization	Keratinizing	3(10%)	27(90%)		
	Non keratinizing	24(43.6%)	31 (56.4%)	6.87	0.01

3.5 Association between histological grading of OPSCC and p16 expression

The p16 IHC stain was interpreted based on percent of the cells that it has stained (Appendix 1). Among OPSCC I, almost half of the cases 16(53.3%) did not stain for p16 IHC. The rest of grade I 9(30%) cases stained less than 25% tumor cells and only 3(10.7%) had a p16overexpression (diffuse nuclear cytoplasm stain in more than 75% of tumor cells).Fifteen (35.7%) of grade II OPSCC showedp16 overexpression with a strong diffuse nuclear cytoplasm stain. However 13 (31%) of grade II cases had p16 stain in 25%-75% of the tumors while no p16

stained was noted in 15(34.1%) grade II. Despite few cases 13(15%) grade III OPSCC9 (69.2%) of the cases demonstrated p16 overexpression with a diffuse strong nuclear cytoplasm stain in more than 75% of the cells while three had a negative stain with no p16 staining in tumor cells.

Table 4: Association between histological grading of OPSCC and p16 IHC expression

		p16 expression					
		0	**+1**	**+2**	**+3**	**X^2**	**p value**
Histological grading	OPSCC I	16(53.3%)	9(30%)	2(6.7%)	3(10%)		
	OPSCCII	14(33.3%)	0(0%)	13(31%)	15(35.7%)		
	OPSCIII	3(23.1%)	0(0%)	1(7.7%)	9(62.9%)	39.96	0.00

CHAPTER FOUR

4.0 DISCUSSION

This study aimed at finding if there is a correlation between histological grading and rHPV using p16 immunohistochemistry (IHC) overexpression of OPSCC as a surrogate marker of rHPV. The age range of the whole study population was between 22-92 years with mean age of 54.98±14.73 years and majority of the cases were in patients between 31-60 years of age, the findings are similar to the three different studies done by J. Lewis et al and F.Faraj et al at United State of America and Blumberg et al at Mozambique where the mean age of the patient with OPSCC was55±9, 59.1±9.5and 59.3 years respectively(14,19,21). Furthermore study done by Aisha Hussein et al showed rising global incidence of OPSCC in patient below 45 years of age this may be due to early onset of risk factors i.e. poor diet and habitual use kola nuts and tobacco use (20).

Males were most affected by OPSCC compared to females in this study. Similar findings were noted in three different studies assessing HPV related OPSCC(19,21,44) this may be due to multiple lifestyle risk factors among men including cigarette smoking, alcohol intake, multiple sexual partners and sexual practices(23,45–48); however a different pattern was noted in a pilot study done at Maputo, Mozambique, whereby female were more affected by OPSCC compared to males, the different in the pattern may be due to a small sample size of the study done in Maputo.

The base of the tongue was the common affected site by the tumor, which accounted for 43(50.6%) of all OPSCC in this study, while in the study done at Brighton University UK by S. Rahim et al showed the, palatine tonsil was the most affected site(49). Most of the studies have not sub classify the tumor into oropharynx sub-sites but rather generalize all lesions as OPSCC (13,27,38,50) however the reason for the discrepancy seen in findings between this study and Rahim et al study could not be ascertained.

Broder's grading system grades squamous cell carcinoma into grade I, II, III based on tumour differentiation. In this study almost half of the OPSCC tumors were grade II 42(49.4%) while only 13(15.3%) grade III which was similar to the study by chernock et al were grade II(moderate differentiated OPSCC) were 43(79.6%) of all OPSCC(4)

When OPSCC was more classified based on tumor degree of keratinization more than half of the cases 55(64.7%) were non keratinizing OPSCC, these findings are similar to several studies done by J. Lewis et al and El mofty assessing histological typing of OPSCC were Non keratinizing type was a predominant feature (19,27,51,52).These findings may be the results of tumor cells emulating into immature appearing of the basal cells of reticulated tonsilar crypts epithelium of both lingual and palatine tonsil(27,53).

Tumor suppressor protein (p16) works at G1 phase of the cell cycle, this protein is overexpressed in rHPV related Squamous cell carcinoma. This is due to the reaction of HPV oncoprotein E6 and E7 on p53 and Rb tumor suppressor genes at the cell cycle hence p16 IHC is commonly used as a surrogate marker of rHPV. In this study p16 IHC was used as a surrogate marker for rHPV infection which occurs more frequent in Squamous cell carcinoma of the oropharynx than any other head and neck site(38). p16 positive OPSCC were about one third 27(31.8%) of the cases while majority of the cases did not overexpress p16 protein 58 (68.2%).This trend is also seen in the study done at Mozambique and Egypt where at Maputo, Mozambique, no HPV related oropharyngeal carcinoma was seen through both p16 IHC or PCR while in Egypt 9(28.1%) OPSCC cases had p16 IHC overexpression(13,14). However studies assessing HPV related OPSCC are scarce in the region. Similar trends was also noted in the study done at Japan where 58(39%) overexpressed p16 IHC and were HPV positive by PCR. Also the study done among patients of difference race in America prevalence of HPV positive OPSCC among African Americans 46.3% was low compared to other race however rising trends of OPSCC with p16 overexpression was noted among African American patients.(21)

Despite the low proportion of p16 overexpression in OPSCC among African countries and some Asian countries; the pattern is different from western countries where high proportion of HPV related OPSCC through p16 IHC and PCR has been noted(19,27,38,54). In the study done by Lewis et al among Americans, 187 (78%) of the study population with OPSCC overexpressed (19). This difference in proportion of p16 overexpression associated OPSCC among the regions may be due to race and lifestyle risks such as sexual practices as it was noted in the study done among South African men assessing HPV status where majority of the cases had negative HPV infection by PCR with only 7(5.6%) cases which presented with positive HPV and oral sex was uncommon practice among the population 41(40.8%)(12). Furthermore the difference in

proportion of OPSCC p16 overexpression in these studies may be due to variations of p16 primary antibody clones as well as dilution concentration which have been used in these studies but also interpretation criteria, since these factors may have affect on p16 antibody staining results(55). Additionally absence of p16 overexpression in OPSCC may have been contributed by p16 wild type staining pattern where there is a loss of p16 protein due to inactivation of p16 which is induced by mutation or due to Rb dysfunction causing p16 overexpression(9,11).

In this study no association was found between p16 overexpression of OPSCC and sex where by 28.1% and 39.3% of male and female respectively had p16 IHC overexpression respectively, the findings corresponds to the study done by J Lewis et al assessing HPV related OPSCC(19) were no association was seen between sex and p16 overexpression.

Oropharynx is a rich site with lymphoid tissue hence no marked difference was seen in terms of p16 overexpression in these sites; this study found no association between the site of oropharyngeal biopsy and p16 expression, despite the fact that among oropharyngeal sites about 12(40%) of the biopsy from the tonsil had p16 overexpression while only 4(33.3%) from the oropharyngeal wall demonstrate p16 overexpression. To the best of our knowledge this is the first study in our setting to assess p16 expression in oropharynx subsite. Most of the studies have not sub classify the site rather than using a general term of oropharynx.

Despite the fact that in this study most of the patient with OPSCC were found between 31-60 years only 13(25%) demonstratedp16 overexpression while 3(75%) of the patient below 30 years showed p16 overexpression however no association was found between age and p16 status p=0.18. These finding are support by two different studies assessing and staging of HPV related OPSCC where no association was seen between these two variables(19,41).

During histological grading of OPSCC using Broder's grading system majority of the case were grade II and few cases were grade III OPSCC; however one third of grade II and two third of grade III showed p16 overexpression, furthermore, this study found an association between histological grading of OPSCC and p16 expression (*fisher exact test p= 0.000*).OPSCC was further classified in relation tumor degree of keratinization where in this study most of the OPSCC where no keratinizing and a statistically significant association was noted between p16 expression and tumour degree of keratinization indicating good prognosis among these patients .

These finding were supported by studies done on OPSCC by lewis et al and R. Chernock et al assessing histological typing and prognosis of HPV related (19,27,38) were a statistically significant association was noted between histological grading of OPSCC and p16 expression. Many studies have noted that HPV positive OPSCC have a distinct histological feature (i.e. its association with grade II and grade III, the non keratinizing variant of OPSCC)(19,38). Although no clear molecular mechanism has been found regarding this association but these findings may be related to HPV viral oncogene reaction to oropharynx site where tumor cells may resemble a reticulated epithelium of the tonsil(27,53,56). Furthermore some studies have shown that OPSCC that overexpress p16 has good prognosis compared to those do not overexpress p16 despite commonly having poorly differentiated type(19,57,58) .

p16 IHC is a surrogate marker of rHPV, in the current study this test was used as a surrogate marker for rHPV in OPSCC where p16 (overexpression)was graded based on the percent of tumor cells. Majority of grade had p16 overexpression with a strong diffuse stain in 75% of the tumor cells and the study found an association between histological grading of OPSCC and p16 IHC overexpression (*fishers exact p=0.000*), however some of the grade II OPSCC cases had a positive stain in 25% to 75% of the cell. These finding are supported by a study done by Lewis et al assessing p16 expression in small biopsies (51) where a statistically significant association was noted.PCR is a highly specific test and is considered as gold standard test in diagnosis of HPV related OPSCC(59,60), in majority of the studies p16 IHC test was further supported by molecular test including PCR and ISH which helped in eliminating false positive and false negative from p16 IHC(4,54,59–61).The sensitivity and specificity of p16 immunostaining for detecting high-risk HPV in OPSCC patients has been in the range of 63-97% and 94% respectively compared with the RNAqPCR "gold standard(19,31,59). p16 expression of oropharyngeal SCC is defined as clearly strong and diffuse nuclear and cytoplasmic expression in >75% of tumor cells(4,19,27).

4.1 Study Limitation

This study had a small sample size but also two cases had missing blocks. This study has not assessed other the risk factors of OPSCC i.e. tobacco use and betel nut chewing, smoking, alcohol intake, sexual practices and pre malignant lesion among study population. In this study no molecular test (i.e. PCR and ISH) was done to support p16 results due financial constraints.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Tumor histology correlated well with p16 overexpression ($p < .001$), furthermore about one third of OPSCC in this study showed p16 over-expression predominantly grade II and III (non keratinizing type of OPSCC) indicating good response after treatment therefore better prognosis in such group of patients.

5.2 Recommendation

- Routine p16 IHC should be adopted in our setting especially in lesions presenting with grade II and III OPSCC. This will assist in the management and prognostication of OPSCC.
- More large-scale studies on HPV related OPSCC to assess prognosis and lifestyle risk factors (i.e. betel nuts and tobacoo chewing, smoking, alcohol in taking, sexual practices), involving molecular tests (i.e. PCR, ISH) are encouraged.
- The results of this study recommend inclusion of both gender in preventive programs against HPV related tumors to reduce the rising trend of HPV related malignancies

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APPENDICES

APPENDIX 1

Data capture sheet.
STUDY TITLE: CORRELATION BETWEEN HISTOLOGICAL GRADING OF OPSCC AND p16 OVEREXPRESSION

DATE……
1. PATIENT HP NUMBER……
2. LABORATORY NUMBER……..
3. STUDY NUMBER……
4. STUDY SITE: MUHIMBILI NATIONAL HOSPITAL
5. AGE (specify in completed years)…….
≤30
31-60
≥61

6. GENDER

DIAGNOSIS:

7. HISTOPATHOLOGICAL GRADING OF OROPHARYNGEAL SQUAMOUS CELL CARCINOMA
1) GRADE 1
2) GRADE 2
3) GRADE 3
4) Anaplastic/Pleomorphic

8. IMMUNO-HISTOCHEMISTRY.
p16 expession score:
The interpretation of p16 status is as follows;

- 0= No stained cell (Negative)
- 0-25 = Nuclear and cytoplasm stain in < 25% f the cells(Negative)
- 25-75 =Nuclear and cytoplasm stain in 26-75% of the cells (Negative)
- >75 =Positive (Strong diffuse nuclear and cytoplasm stain in >75% of the cells.(19)

APPENDIX 2
Broder's grading of SCC

- Well differentiated (Grade I) = 0-25% of undifferentiated cells
- Moderately differentiated (Grade II) 25-50% of undifferentiated cells
- Poorly differentiated (Grade III)= 50-75% of undifferentiated cells
- Anaplasic/Pleomorphic (Grade IV)= 75%-100 of undifferentiated cells

APPENDIX 3

H&E staining

1. This will be done to all selected cases.
2. Tissue blocks will be retrieved for sectioning. Sections of 3 micrometer will be cut from iced paraffin embedded blocks using rotary microtome (Sakura model) SRM200 CW, mounted on slide labeled with ID number and will be allowed to drain before being put on hot plate at 60 degrees centigrade for 15-30 minutes.
3. Sections will be dewaxed in two changes of xylene ten dips each and brought down to water through descending grades of ethyl alcohol(absolute, 95%, 80%, 70%),
4. The section will be stain with Harris hematoxylin for ten minutes and washed in tap water until water is clear and then will be differentiated in 1% HCL in 70% ethanol for three seconds.
5. Thereafter tissue sections will be washed in running tap water and subsequently blued in running tap water for ten minutes.
6. The sections will be counter stained in 1% aqueous eosin for three minutes, and will be washed in tap water until water is clear, then they will be dehydrated in ascending grades of ethanol(70%, 80%, 95% and absolute) 10 dips each,

Clearing of the section in two changes of xylene and then mounted in DPX and labeled. *(Histo SOP No.5*).

Interpretation of the results

H&E slides will be read by the investigator to confirm the diagnosis and histological grading according to broder's grading system and then reviewed by the pathologist.

APPENDIX 4

p16 antibody staining

7. Paraffin embedded blocks of 87 selected cases with confirmed diagnosis was retrieved and recut for p16 IHC procedure.
8. Sections of 3 micrometer were cut from iced paraffin embedded blocks using rotary microtome (Sakura model) SRM 200 CW.
9. These sections were floated in water bath at 45 degrees centigrade, mounted on standard frosted glass slides each labeled with ID number.
10. Slides were allowed to drain before being put on hot plate at 60 degrees centigrade for 50 minutes. Sections were dewaxed in two changes of xylene ten dips each and brought down to water through descending grades of alcohol (absolute, 95%, 80% and 70%).
11. Sections will ringed with PAP pen citrate buffer with pH 6.0 was heated in pressure cooker for 15 minutes was then followed by placing tissue sections in antigen retrieval solution in pressure cooker and closing the lid.
12. Tissue sections were kept in for two minutes in full pressure. Thereafter slides were removed from pressure cooker and placed in water. Slides were not be allowed to dry from this point onwards.
13. TBS was added for three minutes, drained off and 2 drops of peroxidase blocking solution (SM 8OI from DAKO Company) were added leaving it for 15minutes.
14. Slides were placed in tap water for 3 minutes,then were covered withprediluted p16 monoclonal anti mouse antibody (G175-405) from Medaysis and incubated for 60 minutes.
15. TBS was washed gently on the slide for five minutes, then drained off from the slide and covered with HRP for 30 min. (this reagent was kept at room temperature before using it).
16. Sections were washed gently with TBS for 5 minutes, drained, applied working DAB (1 drop of DAB to 1 ml of substrate buffer) and incubated for 5 min.
17. Slides then were washed in water, counterstained with hematoxylin for 30 seconds, and then dipped in 1%acid alcohol 2 dips, blued in tap water for 2 minutes, dehydrated, cleared in xylene, mounted with DPX and then coversliped.
18. Finally these slides were evaluated under light microscope model Olympus CX 41 and few pictures were taken from the results by using Olympus Soft Image Solutions by USB SC camera (100pixels).
19. Tissue section with cervical carcinoma was used as positive control and adipose tissue was used as negative control (*Histo SOP No. 28*).

Interpretation of results

Interpretation of p16 IHC: brown intensity reflected the antigen present. p16 stains both the nucleus and cytoplasm diffusely. Positive p16 will be regarded as a strong diffuse full thickness nucleus cytoplasmic stain in >75% of tumor cells according to the study done by Lewis Jr.(19,54)

APPENDIX 5

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES
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2nd September, 2019

Dr. Dammy A. Shimbo
MMed. Anatomical Pathology
MUHAS.

RE: APPROVAL OF ETHICAL CLEARANCE FOR A STUDY TITLED: "THE CORRELATION BETWEEN P16 EXPRESSION AND HISTOLOGICAL GRADING OF AROPHARYNGEAL SQUAMOUS CELL CARCINOMA AT MUHIMBILI NATIONAL HOSPITAL"

Reference is made to the above heading.

I am pleased to inform you that, the Chairman has, on behalf of the Senate, approved ethical clearance for the above-mentioned study. Hence you may proceed with the planned study.

The ethical clearance is valid for one year only, from **30th September, 2019 to 29th September, 2020.** In case you do not complete data analysis and dissertation report writing by **29th September, 2020,** you will have to apply for renewal of ethical clearance prior to the expiry date.

Dr. Emmanuel Balandya
ACTING: DIRECTOR OF POSTGRADUATE STUDIES

cc: Director of Research and Publications
cc: Dean, School of Medicine

APPENDIX 6

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In reply please quote: MNH/TRC/Permission /2019/250 10th December, 2019

Head,
CPL Department,
Muhimbili National Hospital

Head,
✓ Records Department
Muhimbili National Hospital.

RE: PERMISSION TO COLLECT DATA AT MNH

Name of Student	Dr. Dammy A. Shimbo
Title	**"The correlation between PI 6 expression and Histological granding of arophyryngeal squamous cell carcinoma at Muhimbili National Hospital".**
Institution	Muhimbili University of Health and Allied Sciences
Supervisor	Dr. Edda Vuhahula
Co-Supervisor	Dr. Leah Mnango
Period	10th December, 2019 to 10th June, 2020

Approval has been granted to the above mentioned student to collect data at MNH.

Named MNH based supervisor must ensure that the student abide to the ethical principles and other conditions of the research approval.

Sincerely,

Dr. Faraja S. Chiwanga
Head of Teaching, Research and Consultancy Unit

c.c. DCSS, DICT
c.c. Dr. Dammy A. Shimbo
c.c. Dr. Leah Mnango

All Correspondences to be addressed to the Executive Director