P16 EXPRESSION IN SUBSETS OF HEAD AND NECK SQUAMOUS CELL CARCINOMA IN KENYATTA NATIONAL HOSPITAL

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DECLARATION

This is my original work and has not been presented for a degree in any other institution to the best of my knowledge.

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DEDICATION

I dedicate this work to my father Githaiga Kairu and my mother Wanjiru Githaiga for their unwavering belief in me and unconditional support they gave me.

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LIST OF ABBREVIATIONS

CEA: Carcinoembryonic antigen

DNA: Deoxyribonucleic Acid

EMA: Epithelial membrane Antigen

FFPET: Formalin fixed Paraffin wax embedded tissue

HNSCC: Head and neck squamous cell carcinoma

HPV: Human Papilloma Virus

H&E: Hematoxylin & Eosin

IHC: Immunohistochemistry

ISH: In Situ Hybridisation

KNH: Kenyatta National Hospital

PCR: Polymerase Reaction Chain

RB: Retinoblastoma gene

SOP: Standard operating procedures

SPSS: Statistical Package for the Social Sciences

SCC: Squamous cell carcinoma

U.O.N: University of Nairobi

OPERATIONAL DEFINATIONS

Immunohistochemistry or IHC refers to the process of detecting antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or apoptosis.

P16INK4A is a member of the INK4 protein family. Members of the INK4 protein family specifically inhibit cyclin-dependent kinase 4 (cdk4) and cdk6-mediated phosphorylation of the retinoblastoma susceptibility gene product (Rb). Has been identified as a tumor suppressor in many human cancers. Inactivation of p16INK4A in tumors expressing wild-type Rb is thought to be required in order for many malignant cell types to enter S phase efficiently or to escape senescence

ABSTRACT

Background: Traditionally head and neck squamous cell carcinoma (HNSCC) has been regarded as a homogenous group of tumours that differ only by anatomic site. However, numerous studies have indicated that this is not the case. Recognition of distinct molecular profiles now allows further classification of HNSCC into distinct subgroups that differ with respect to risk factors, pathogenesis, and clinical behavior. Among these subgroups is the Human papilloma virus (HPV) associated HNSCC which have been found to differ from the non HPV-associated HNSCC in that HPV-HNSCC affects younger patients, respond better to chemotherapy and radiotherapy and have a better 5 year survival rate independent of tumour stage. These subgroups cannot be readily differentiated morphologically on routine Hematoxylin and Eosin (H/E).Cellular tumour markers such as P16 Immunohistochemistry may be used to separate these groups of tumours. This study set out to identify the HPV associated HNSCC by P16 immunohistochemistry. This is due to the over expression of P16 tumour suppressor protein P16INK4A in these tumours. Several studies have found P16 immunohistochemistry has high sensitivity, specificity, positive and negative predictive value of P16 expression in relation to HPV status. P16 immunohistochemistry has been found to be a more cost effective method and it has been proposed to be used as a 1st line marker

Objective: To determine P16 expression and the prevalence of HPV- associated tumours in subsets of Head and Neck Squamous cell carcinoma (Oral, Oropharyngeal, Laryngeal carcinomas) reported at KNH.

Design: Laboratory-based, descriptive cross sectional study.

Setting: The University of Nairobi (UON), Department of Human Pathology, and the Kenyatta National Hospital (KNH) Nairobi, Kenya

Study population: A hundred and three cases reported as HNSCC (Oral, Oropharyngeal, Laryngeal carcinomas)

Methods: A hundred and three previously diagnosed HNSCC from 2008-2013 were analyzed for P16 expression by Immunohistochemistry on formalin fixed paraffin wax embedded tumor tissue blocks. P16 expression was correlated with age, sex, anatomic site of tumor and histological differentiation.

Results

The majority were males 76(74%) while females accounted for 27(26%). M: F was 2.8:1. Mean age was 57.4 with (SD 14).Most of the tumours were from the oral cavity 45(43.7%) followed by larynx 39(37.9%) and pharynx 19(18.4%). Majority of the tumours were well differentiated 65(63 %) followed by the moderately well differentiated tumours 29 (28%), while the poorly differentiated tumours were 8 (8%).Immunohistochemistry was carried out in all the cases, 15 (15%) were found to be positive for P16. The oral cavity had the highest frequency 7(46.67%), followed by larynx 4(26.67%) and lastly pharynx 4(26.67%).Majority of p16 positive HNSCC were found in males 10(67%). With regard to age, the highest frequency of p16 HNSCC occurred in those more than 60years 6 (40%), followed by 51-60 age group 5 (33%).However when cases are reclassified to above 60 years and below 60 years of note is the below 60 year cases are the majority (60%). Majority of the p16 positive HNSSC were well differentiated 9 (60%) followed by the moderately well differentiated carcinomas 4(26%). The poorly differentiated tumours had an increased likelihood of having HPV (OR 2.1, 95% C.I 0.4-11.9).

Conclusion

1. The majority of tumours were from the oral cavity and the overall HPV prevalence was 15%.
2. P16 HNSCC expression is most prevalent in patients below 60years.
3. Poorly differentiated HNSCC have increased likelihood of being associated with HPV.

Recommendations

1. Routine staining for P16 immunohistochemistry should be considered especially for the poorly differentiated carcinomas to inform clinical decisions and for prognostication.
2. Further studies with more sensitive techniques such as PCR are recommended to measure the real burden of HPV associated HNSCC and to further characterize the HPV subtypes.

1 INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) (oral, oropharyngeal, and laryngeal carcinomas) is an aggressive epithelial malignancy that is the sixth most common neoplasm worldwide today[1]. Apart from the skin, brain, and thyroid gland, more than 90% of head and neck cancers are squamous cell (epidermoid) carcinomas.

It is an acquired disease, mainly caused by environmental factors such as smoking and alcohol consumption[2], Smoking and alcohol act synergistically and the incidence increases with the amount and duration consumed[3]. Other risk factors include poor oral hygiene, chewing tobacco and betel nuts. Recent studies have shown that HPV infection especially the high risk typesoncogenic are responsible for a subset of HNSCC[4].

1.0.1HUMAN PAPILOMVIRUS IN HNSCC

Human papilloma viruses are 8-kb, circular Deoxyribonucleic Acid (DNA) viruses that specifically target the basal cells of the epithelial mucosa. Their importance in human carcinogenesis was first noted in the 1970s when it was discovered to be the causative agent of cervical cancer.

Since that time, more than 150 HPV sub types have been described and are grouped into low and high risk categories depending on their propensity to immortalize human keratinocyte cell lines and override cell cycle control mechanisms[5].Low-risk types are associated with benign lesions such as warts, while infections with high-risk types progress to malignant lesions. Among the strains that are considered high-risk, HPV-16 is associated with more than90% of HPV-related head and neck cancers. HPV-18 is the second most common[6].

The HPV genome is comprises several early and late genes, as well as a non-coding region, all of which play roles in viral replication, transcription, and carcinogenesis. The late (L) open reading frames encode the L1 and L2 capsid proteins and are transcribed only in productively infected cells. The early (E) open reading frames encode the E1, E2, E5, E6, and E7 proteins[7]. The E1 and E2 proteins regulate viral replication as well as the expression of the other early viral genes. At least 3 proteins (E5, E6, and E7) coded by the high-risk HPVs are considered oncogenic due to their transforming and growth stimulating properties. These proteins have the ability to deregulate tumor suppressor function by binding to and

abrogating the functions of the p21, p53, and pRb proteins, resulting in defects in apoptosis, DNA repair, cell cycle control, and eventually leading to cellular immortalization. The non-coding long control region (LCR) contains binding sites for the E2and E1gene products, located just upstream of the P97 promoter sequence, which controls the transcription of the E6 and E7oncogenes. There is a dose-dependent regulation of E6and E7expression by E2. High levels of E2 protein result in there pression of E6 and E7expression[8]

It is hypothesized that unique properties of the reticulated squamous epithelium within the head and neck region render the tissues susceptible to desquamation and infection by HPV. On entry into a host cell, viral oncoproteins, such asE6 and E7, are expressed early in the infection and can override cell cycle control mechanisms to facilitate viral replication and, inadvertently, cellular transformation. TheE6 and E7 proteins bind to p53 and pRb, respectively. The E6 protein induces degradation of P53 through ubiquitin-mediated proteolysis, leading to substantial loss of P53 activity. The usual function of P53 is to arrest cells in G1 or induce apoptosis to allow host DNA to be repaired. E6-expressing cells are not capable of this P53-mediated
response to DNA damage and, hence, are susceptible to genomic instability[9]. PRb plays an important role by inhibiting downstream expression of S-phase genes, which can block progression through the cell cycle. Thus by inhibiting pRb, HPV promotes unchecked cellular replication. Moreover, in HPV-infected cells, inhibition of wild-type pRb results in over expression of p16INK4A, an upstream tumor suppressor protein[10]. Due to this the keratinoctyes are transformed and become immortalized leading to malignant transformation

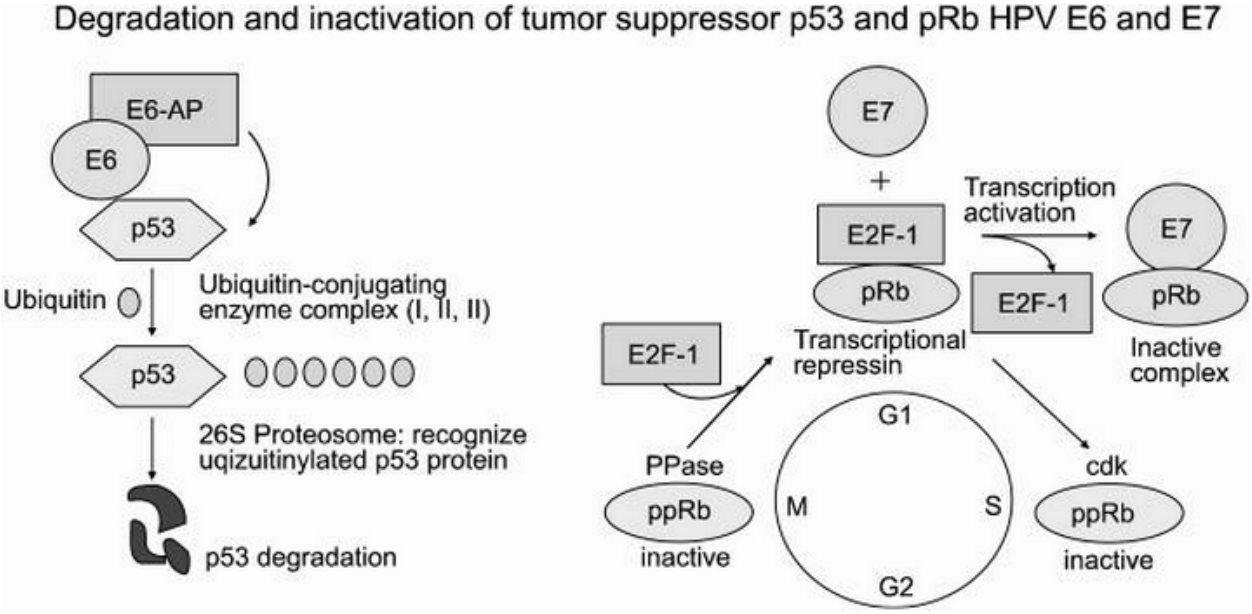


Figure 1;Degradation and inactivation of tumour suppressor p53 pRb HPV E6 and E7(9)

1.0.2. EPIDEMIOLOGY OF HNSCC

Head and neck cancer is the sixth most common cancer worldwide, with an annual incidence of 550,319 incident cases (specifically 263,020 oral cavity cancers, 136,622 oropharyngeal cancers, 150,677 laryngeal cancers) and 305,096 deaths(1).A study carried out by Gathere *et al*, for the period 2000-2003, head and neck cancers comprised over 12.8% (697) of all the 5462 cancers reported from all cancer sites within Nairobi, Kenya. There was a male to female ratio of 2:1. Among the head and neck sub-sites, oral cancers were the most frequent at 40.6%, followed by nasopharynx and laryngeal cancers with 20.8% and 13.8 % respectively. The commonest histology was squamous cell carcinoma(10).This study was based from data of the Nairobi Cancer Registry which gets its data from all of the hospitals within Nairobi that diagnose and treat cancer, the hospitals have a catchment area of approximately 3 million people.

The prevalence of HNSCC- HPV associated tumours in the world varies according to geographic location and the method of detection. In 2005, Kreimer *et al* did a meta-analysis of 60 published studies on HNSCC biopsies that employed PCR-based methods to detect and genotype HPV to describe the prevalence and type distribution of HPV by anatomic cancer site. In the 5,046 HNSCC cancer specimens from 60 studies, the overall HPV prevalence was 25.9% (95%CI), 24.7-27.2] (4). HPV prevalence was significantly higher inoropharyngeal

SCCs (35.6% of 969; 95% CI, 32.6-38.7) thanoral SCCs (23.5% of 2,642; 95% CI, 21.9-25.1) or laryngeal SCCs (24.0% of 1,435; 95% CI, 21.8-26.3). HPV16 accounted for a larger majority of HPV-positive oropharyngeal SCCs(86.7%; 95% CI, 82.6-90.1) compared with HPV-positive oral SCCs (68.2%; 95% CI, 64.4-71.9) and laryngeal SCCs (69.2%;95% CI, 64.0-74.0).(4) Conversely, HPV18 was rare in HPV positive oropharyngeal SCCs (2.8%; 95% CI, 1.3-5.3) compared with other head and neck sites [34.1% (95% CI, 30.4-38.0) of oral SCCs and 17.0% (95% CI, 13.0-21.6) oflaryngeal SCCs]. Aside from HPV16 and HPV18, otheroncogenic HPVs were rarely detected in HNSCC.[4]

1.0.3. CLINICAL FEATURES OF HNSCC

Clinical presentation of HNSCC will depend on the site, stage and time of presentation. HNSCC may remain subclinical for a long time and will not appear until the disease is well advanced. Patients are usually slow to come to the consultation and show symptoms similar to the usual benign processes such as catarrhal or discomfort in the oral cavity secondary to dentures. In many cases patients are treated with antibiotics and analgesics, thus delaying diagnosis and being detected in advanced stages, hindering the total eradication of tumor. When the tumor cells begin to grow, tend to spread easily to neighboring areas so it is important to make an early diagnosis. The main feature of the head and neck tumors is they easily spreading to regional lymph nodes[11].

Hypopharyngeal, laryngeal and supraglottic tumors may be responsible for dysphagia, change in quality of voice, foreign body sensation in the throat, haemoptysis, and odynophagia. Glottic SCC most commonly presents with hoarseness. In case of subglottic tumor, dyspnea and stridor are frequent clinical features [11].

In Oral and oropharyngeal cancers most patients display at the time of diagnosis signs and symptoms of locally advanced disease. Clinical features vary according to the exact site of the lesion. The most common presenting features are ulceration, pain, referred pain to the ear, difficulty with speaking, opening the mouth or chewing, difficulty and pain with swallowing, bleeding, weight loss, and neck swelling. Cancer of the buccal mucosa may present as an ulcer with indurated raised margins or as an exophytic growth. SCC of the floor of the mouth may arise as a red or ulcerated lesion or as a papillary growth. Cancer of the gingiva usually presents as an ulceroproliferative growth. Cancer of the tongue may appear as an ulcer infiltrating deeply and reducing the mobility of the tongue. SCC of the base of the tongue usually presents at a locally advanced stage as an ulcerated, painful, indurated growth. Cancer

of the hard palate often presents as a papillary or exophytic growth rather than a flat or ulcerated lesion. Cancer of soft palate and uvula often appears as an ulcerative lesion with raised margins or as a fungating mass. Occasionally, patients harbor enlarged cervical lymph nodes with no identifiable oral or oropharyngeal lesion. In very advanced disease, patients may present an ulceroproliferative lesion with areas of necrosis and extension to surrounding structures, such bone, muscle and skin[(11)].

1.0.4. LABORATORY DIAGNOSIS

The gold standard for diagnosis of HNSCC is histology. Tissue for diagnosis is usually acquired via conventional biopsies or from surgical resections. Further characterization can be done in difficult cases using immunohistochemistry markers such as Cytokeratins, Epithelial membrane Antigen (EMA) and Carcinoembryonic antigen (CEA). Current methods for diagnosing HPV-HNSCC include consensus and type-specific polymerase chain reaction (PCR) techniques, real-time PCR assays to quantify viral load, DNA In Situ Hybridisation (ISH), and immunohistochemical detection of surrogate biomarkers (e.g., p16 protein)[(12)]

Gross features

Depending on the stage of the tumour, HNSCC presents as flat plaque with well defined, raised edge, or exhibits a polypoid exophytic appearance which may relate to prognosis, sometimes the surface is ulcerated[(13)]

Histological characterization and subtypes

The 2005 WHO classification of head and neck tumours distinguishes SCC into 8 different histological subtypes;

Conventional Squamous cell carcinoma (SCC) is characterized by squamous differentiation (often seen as keratinization, sometimes with keratin pearl formation) and invasive growth with disruption of the basement membrane. Extension into the underlying tissue is often accompanied by a desmoplastic stromal reaction and a dense inflammatory infiltrate, mainly comprised of lymphocytes and plasma cells[(13)]. Angiolymphatic and perineural invasion may be seen. SCC is graded into well-, moderately-, and poorly-differentiated. Well-differentiated SCC closely resembles normal squamous mucosa whereas moderately-differentiated SCC displays distinct nuclear pleomorphism, mitoses (including atypical forms), and usually less keratinization. In poorly-differentiated SCC, immature cells

predominate, with numerous typical and atypical mitoses, minimal keratinization, and sometimes necrosis. Most SCCs are moderately-differentiated[13].

Verrucous carcinoma (VC) also known as **Ackermantumour**; is a non-metastasizing variant of well-differentiated SCC characterized by an exophytic, warty, slowly-growing tumor with pushing rather than infiltrative margins. The larynx is the second most common site of VC in the head and neck region after the oral cavity[13]. VC consists of thickened club-shaped papillae and blunt intrastromal invaginations of well-differentiated squamous epithelium with marked keratinization (church-spire keratosis) and thin fibro vascular cores. The squamous epithelium lacks cytological criteria of malignancy, and by morphometry, the cells are larger than those seen in SCC. Mitoses are rare, and observed in the basal layers[13]. DNA synthesis (S phase) is also limited primarily to the basal layers. VC invades the stroma with a pushing, rather than infiltrating border. Dense lymphoplasmacytic host response is common. Intraepithelial micro abscesses are seen, and the abundant keratin may evoke a foreign body reaction[13]. The surrounding mucosa shows progressive transition from hyperplasia to VC. A downward dipping of epithelium often “cups” the VC periphery, and is the ideal site for deep biopsy. Differential diagnosis includes verrucous hyperplasia and very well-differentiated SCC. Distinguishing these entities from verrucous carcinoma can be delicate. Analysis of a sample of sufficient size which has been accurately oriented is necessary before rendering a definitive diagnosis[13,14].

Basaloid squamous cell carcinoma; is a high-grade variant of SCC composed of both basaloid and squamous components. It is an aggressive, rapidly growing tumor characterized by an advanced stage at the time of diagnosis (cervical lymph node metastases) and a poor prognosis.BSCC has two components, i.e. basaloid and squamous cells. Basaloid cells are small, with hyperchromatic nuclei without nucleoli, and scant cytoplasm. They are closely packed; growing in a solid pattern with a lobular configuration, and in some cases, there is prominent peripheral palisading. Comedo-type necrosis is frequent[13]. Distinctive features of BSCC, not found in SCC, are small cystic spaces containing PAS- and Alcian bluepositive material, and stromal hyalinization. BSCC is always associated with a SCC component which can be either in-situ carcinoma, or invasive keratinizing SCC. The latter is usually located superficially; it may also present as a focal squamous differentiation within the basaloid tumour islands. The junction between the squamous and basaloid cells may be abrupt. Rarely, BSCC is associated with a spindle cell component. Metastases may demonstrate basaloid carcinoma, squamous carcinoma, or both[13].

Papillary squamous cell carcinoma (PSCC); is a distinct variant of SCC characterized by an exophytic, papillary growth, and a favorable prognosis. The tumour is characterized by a predominant papillary growth pattern. These papillae have thin fibrovascular cores covered by neoplastic, immature basaloid cells or more pleomorphic cells. Commonly, there is minimal keratosis. Foci of necrosis and haemorrhage are frequent(13). Multiple PSCC or precursor lesions may occur. Stromal invasion consists of a single or multiple nests of tumour cells with dense lymphoplasmacytic inflammation at the tumour-stromal interface. If no stromal invasion is found, the lesion should be called atypical papillary hyperplasia or PSCC in-situ[(13)].

Spindle cell carcinoma; is a biphasic tumor composed of a squamous cell carcinoma, either in situ and/or invasive, and a malignant spindle cell component with a mesenchymal appearance, but of epithelial origin. The spindle cell component usually forms the bulk of the tumour, which can assume several patterns. Resemblance to fibrosarcoma or malignant fibrous histiocytoma is most common .Occasional cases can appear less malignant and resemble a reactive fibroblastic proliferation or radiation induced stromal atypia. Foci of osteosarcomatous, chondrosarcomatous, or rhabdosarcomatous differentiation may be present, particularly in patients with previous radiotherapy[(13)]. Evidence for squamous epithelial derivation can be seen as either in-situ carcinoma or as invasive SCC. Carcinoma-in-situ can be obscured by extensive ulceration. Infiltrating SCC may be focal, requiring multiple sections for demonstration. Sometimes,only spindle cells are present; in such cases, SPCC can be mistaken for a true sarcoma. Metastases usually contain SCC alone or both SCC and spindle cell component, and rarely, only the spindle cell component[(13)].

Acantholytic squamous cell carcinoma; this is an uncommon histopathologic variant of squamous cell carcinoma;this neoplasm is composed of SCC, but with foci of acantholysis in tumour nests, creating the appearance of glandular differentiation. The pseudolumina usually contain acantholytic and dyskeratotic cells, or cellular debris, but they may be empty. They are more frequent in the deeper portions of the tumour. There is no evidence of true glandular differentiation or mucin production. The SCC component predominates, and is usually moderately differentiated. Clear and spindle cells may also be present. The stroma is usually desmoplastic, with a lymphoplasmacytic response .The acantholysis may also form anastomosing spaces and channels mimicking angiosarcoma[(13)].

Adenosquamous carcinoma; is rare aggressive neoplasm originates from the surface epithelium and is characterized by both squamous cell carcinoma and true adenocarcinoma. The larynx is the most frequent site of occurrence. The two components occur in close proximity, but they tend to be distinct and separate, not intermingled as in mucoepidermoid carcinoma. The SCC component can present either as in-situ or as an invasive SCC. The adenocarcinomatous component tends to occur in the deeper parts of the tumour. It consists of tubular structures that give rise to "glands within glands". Mucin production is typically present, either intraluminal or intracellular, and can appear as signet ring cells. However, mucin is not a requirement for the diagnosis in the presence of true glanduloductal formation. Metastases may display both components; one usually predominates[(13)].

Carcinoma cuniculatum; is a rare variant of oral cancer displaying similarities with lesions more commonly described in the foot in which the tumor infiltrates deeply into the bone. There is proliferation of stratified squamous epithelium in broad processes with keratin cores and keratin-filled crypts which seem to burrow into bone tissue, but lack obvious cytological features of malignancy. Clinical-pathological correlation is often needed to make the diagnosis(13).

1.0.5. TREATMENT

Patients with HNSCC are categorized into three clinical groups: those with localized disease, those with locally or regionally advanced disease, and those with recurrent and/or metastatic disease. Co-morbidities associated with tobacco and alcohol abuse can affect treatment outcome and define long-term risks for patients who are cured of their disease[(15)]. Currently HPV related tumours receive the same therapy as HPV unrelated tumours.

Patients with localized disease that is, T1 or T2 (stage I or stage II) lesions without detectable lymph node involvement or distant metastases are treated with curative intent by surgery or radiation therapy. The choice of modality differs according to anatomic location and institutional expertise. Radiation therapy is often preferred for laryngeal cancer to preserve voice function, and surgery is preferred for small lesions in the oral cavity to avoid the long-term complications of radiation, such as xerostomia and dental decay[(15)].

Locally or regionally advanced disease—disease with a large primary tumor and/or lymph node metastases—. Such patients can also be treated with curative intent, but not with surgery

or radiation therapy alone. Combined modality therapy including surgery, radiation therapy, and chemotherapy is most successful. Concomitant chemotherapy and radiation therapy appears to be the most effective approach. It can be administered either as a primary treatment for patients with un-resectable disease, to pursue an organ preserving approach, or in the postoperative setting for intermediate-stage resectable tumors[15].

Monoclonal antibody to the EGFR (cetuximab) increases survival rates when administered during radiotherapy. EGFR blockade results in radiation sensitization and has milder side effects than traditional chemotherapy agents. The integration of cetuximab into current standard chemoradiotherapy regimens is under investigation [15].

Patients with recurrent and/or metastatic disease are, with few exceptions, treated with palliative intent. Some patients may require local or regional radiation therapy for pain control, but most are given chemotherapy. Response rates to chemotherapy average only 30–50%; the duration of response averages only 3 months, and the median survival time is 6–8 months. Therefore, chemotherapy provides transient symptomatic benefit. Drugs with single-agent activity in this setting include methotrexate, 5-FU, cisplatin, paclitaxel, and docetaxel. Combinations of cisplatin with 5-FU, carboplatin with 5-FU, and cisplatin or carboplatin with paclitaxel or docetaxel are frequently used[15].

1.0.6. PROGNOSIS

The prognosis for patients with HNSCC is determined by the stage at presentation, established based on the extent of the tumor, as well as the presence of lymph-node metastases and distant metastases. Poorly differentiated tumors have a worse prognosis than well-differentiated tumours. In localized disease, overall 5-year survival is 60–90%. Most recurrences occur within the first 2 years following diagnosis and are usually local. In locally or regionally advanced disease five-year survival is 34–50% and in recurrent and/or metastatic disease median survival time is 6–8 months [15].

Another prognostic factor is HPV status of the tumour , A study reviewed all published reports and conducted a meta-analysis on the relationship between HPV and overall survival (OS) and disease-free survival (DFS) in HNSCC (16). Patients with HPV-positive HNSCC had a lower risk of dying (meta HR 0.85; 95% CI 0.7–1.0), and a lower risk of recurrence (meta HR 0.62; 95% CI 0.5–0.8) than HPV-negative HNSCC patients[16]. Site-specific

analyses showed that patients with HPV-positive oropharyngeal tumours had a 28% reduced risk of death (meta HR 0.72; 95% CI 0.5–1.0) in comparison with patients with HPV-negative tumours, Several other studies also confirmed that HPV-positive HNSCC have a better prognosis than HPV-negative HNSCC [17–19].

1.1 LITERATURE REVIEW

The association between HPV and HNSCC was initially noted in the early 1980s [20,21], where similarities in the morphological features between genital and oral squamous cell carcinoma lesions seemed to indicate that HPV might be involved in oral and laryngeal squamous cell carcinoma. Since then more research has been done and studies have shown that there is a causal relationship between HNSCC and HPV[22]

Initial studies done on HPV associated HNSCC were mainly Polymerase chain reaction (PCR) and In situ hybridization (ISH) based [22,23]. P16 immunohistochemistry methods were incorporated later after it was found that there was over expression of p16 tumour suppressor protein p16INK4A in these tumour[s][24].

It has been shown that p16 is a suitable surrogate and screening marker for detection of HPV –related HNSCC. This is due to the over expression of p16 tumour suppressor protein p16INK4A in these tumours, In a study by Singh *et al*. a sensitivity, specificity, positive and negative predictive value of p16 expression in relation to HPV status of 100%, 74%, 91% and 100%, respectively was found[12]. A similar study by Kian Ang *et al*. found sensitivity, specificity, positive and negative predictive values were 96%, 81%, 90% and 93%, respectively[25]. P16 immunohistochemistry has been found to be a more cost effective method and it has been proposed to be used as a 1st line marker [26].

Several studies have found p16 positive tumours tend to have a characteristic basaloid squamous or poorly differentiated squamous cell carcinoma morphology on histology [22,27]. A similar study done in Japan by Fujimaki *et al* found that non keratinizing squamous cell carcinoma and hybrid squamous cell carcinoma were highly predictive of being HPV associated, also noted was the presence of comedo necrosis among non maturing islands and abrupt keratinisation were characteristic histological features of HPV associated tumours [28].

P16 positive HNSCC have been noted to affect patients of a younger demographic, have better response to therapy, lower risk of dying and lower risk of recurrence when compared

p16 negative HNSCC[29].In a study carried out by Smith et al found that, compared to p16+ HNC cases, those who did not express p16 had significantly worse disease-specific (DS) survival (Hazards Ratio, adj.HR=2.0. 1.0-3.9) and recurrence (adj.HR=3.6, 1.6-8.2); [19].

1.2. RATIONALE AND JUSTIFICATION

Head and Neck Squamous cell Carcinoma (HNSCC) has long been regarded as a uniform group of tumours that differed only by anatomic site, however ongoing studies have indicated that this is not true. Recognition of distinct molecular profiles now allows finer resolution of HNSCC into distinct subgroups that differ with respect to risk factors, pathogenesis, and clinical behavior and response to therapy.

Among these subgroups is the P16 positive HNSCC which have been found to differ from the P16 negative HNSCC in that, it affects patients of a younger demographic, they respond better to chemotherapy and radiotherapy and they have a better 5 year survival rate independent of age, tumour size and lymphnode status[16].

It has also been found that in p16 positive HNSCC, p16 is over expressed in keratinocytes that are infected with high-risk HPV types, and due to this we can use P16 immunohistochemistry to identify these tumours. P16 based assays are ideal in our set up because they are more cost effective when compared to the other methods, also they have high sensitivity, specificity, positive and negative predictive value of p16 expression in relation to HPV status., P16 IHC expression shows good concordance with high-risk HPV-ISH and can be used as a first-line marker[26,30,31].

Among the strains that are considered high risk/oncogenic, HPV-16 is associated with 90% of HPV related HNSCC, HPV-18 is second most common. Currently two preventative HPV vaccines have been approved and are in use for prevention of cervical and HPV related anogenital cancer worldwide. Gardasil is a quadrivalent vaccine against HPV types 6, 11, 16, and 18, while a bivalent vaccine Cervarix prevents infection from only the oncogenic HPV types (types 16 and 18). There has been noclinical trial to investigate the efficacy of these vaccines in preventing HNSCC, but, in theory, they are promising since they have been found to elicit systemic high titers of neutralizing antibodies against HPV-16 and may reduce the burden of HPV infection within the population. The vaccine would have more impact on HPV- related HNSCC than it has had on carcinoma of the cervix because HPV type 16 is responsible for more than 90% of HPV-associated HNSCC as opposed to 50% in carcinoma of the Cervix.

Prevalence of HPV related HNSCC varies from region to region and according to the method of detection used, several studies have shown that incidence HPV related HNSCC are on the increase while HPV unrelated HNSCC are reducing or are stable, data from sub Saharan region is lacking, so this study will contribute to the knowledge on the prevalence of HNSCC in our region.

The aim of this study was to determine the p16 expression in HNSCC and the prevalence of HPV related HNSCC which in turn will guide clinicians on management of patients and prognosis. In addition it will guide policy on whether there is a need to use HPV vaccine in prevention of HPV associated HNSCC.

1.3. RESEARCH QUESTION AND OBJECTIVES

1.3.1 RESEARCH QUESTION

What is the pattern ofP16 expression in subsets of Head and Neck Squamous cell carcinoma (Oral, Oropharengeal and Laryngeal carcinomas) in KNH?

1.3.2 BROAD OBJECTIVE

To determine P16 expression and hence the prevalence of HPV- associated tumours in subsets of Head and Neck Squamous cell carcinoma reported at KNH between 2008-2013

1.3.3 SPECIFIC OBJECTIVES

1. To describe the histo-morphology of all cases previously diagnosed as HNSCC on light microscopy from 2008 to 2013.
2. To determine P16 expression of these tumours using immunohistochemistry and re-classify as HPV-related or non-HPV related tumours

1.3.4 SECONDARY OBJECTIVES

1. To compare light microscopy features and socio-demographic factors.
2. Compare Hematoxylin and Eosin (H&E) features with P16 expression

2 METHODOLOGY

2.1STUDY DESIGN

2.1.14.1 TYPE OF STUDY

This was a Laboratory based descriptive cross sectional study carried out on selected cases previously diagnosed as HNSCC in KNH

2.1.24.2 STUDY AREA DESCRIPTION

Blocks stored in KNH histology archive were retrieved. Sectioning and staining was carried out at the UON histology laboratory. The laboratory is located in the same building as the histology archive. The blocks were then re-examined in the anatomic pathology department to confirm the diagnosis of HNSCC. A hundred and ten cases were initially retrieved and re-examined however only a hundred and three cases were included in the study.

2.1.3 STUDY POPULATION

Cases consisted of one hundred and three FFPET seen and diagnosed as HNSCC (Oral, Oropharyngeal and Laryngeal carcinomas) at the Kenyatta National Hospital from 2008 to 2013.

2.1.4STUDY ELIGIBILITY CRITERIA

INCLUSION CRITERIA

All cases diagnosed as HNSCC between 2008 to 2013 (Oral, Oropharyngeal and Laryngeal carcinomas)

EXCLUSION CRITERIA.

1. FFPET found unsuitable for sectioning due to physical disintegration.
2. Poorly processed tissues.
3. Squamous cell carcinomas metastatic to head and neck region

2.1.5 SAMPLE SIZE DETERMINATION

The standard statistical approach to determination sample size for a cross-sectional study was used. This required the prevalence, the desired level of confidence and a tolerance error margin or width of the confidence interval.

The sample size formula below was used to estimate the sample size.

$$n = \frac{z^2_{1-\alpha/2} P(1-P)}{D^2}$$

Where:

n = is the required sample size

p = expected prevalence or proportion or estimated proportion of HPV related HNSCC. In this study a prevalence of 25.9% was used.

D= degree of precision or a tolerance error margin or width of the confidence interval

(A measure of precision of the estimate which ranges from 1%- 20%), this study used 9%(32).

Z= Z statistic for a level of confidence or is the normal distribution critical value for a probability of /2 in each tail.

For a 95% CI, z=1.96

Using this formula

$$n = \frac{(1.96)^2 (0.259) (0.841)}{(0.09)^2}$$

$$= 103.2623407407$$

N= 103

2.1.6 SAMPLING METHOD

Purposive sampling method was used.

2.1.7 RECRUITMENT PROCEDURE

The files containing histology reports at the Kenyatta National Hospital were perused to identify all the cases that met inclusion criteria.

A hundred and three cases were identified. The name, sex, patients' hospital number and laboratory number were noted from the histology reports as the cases were identified .The information was used to retrieve the complete case notes and the archival specimen.

2.2 SAMPLE RETRIEVAL

Formalin fixed Paraffin wax embedded tissue blocks were retrieved from the histopathology archive using the laboratory numbers on the pathology reports. Some blocks were missing from the archive while others while others did not have enough tissue to be sectioned. An initial a hundred and ten cases were re-examined but only a hundred and three cases were included in the study.

The slides were labeled with study numbers as follows; S001/2013, S002/2013 up to S103/2013. The same number appeared on the corresponding data sheet together with laboratory number of the block. In addition the test name were indicated on the slide as S001/2013/H&E or S/001/2013/p16

The routine H&E histological preparation and P16 Immunohistochemistry was done at the University of Nairobi histology laboratory as shown in **appendix 2** and **appendix 3**respectively.

2.3 MICROSCOPY

Five micron sections were taken from each of the formalin-fixed paraffin-embedded tissue blocks of cases that met the inclusion criteria and these were stained with H&E for review of morphology; this was done according to protocol in **appendix 2**. The slides were then reviewed to ascertain that the histo-morphology was compatible with a diagnosis of squamous cell carcinoma and to establish that there was sufficient tumor tissue on the slide. The tumours were classified into the different histological subtypes. The information was then tallied onto the data sheet. The slides were initially reported by the principal investigator (P.I) then reviewed together with two supervisors who are qualified senior pathologists.

2.4 IMMUNOHISTOCHEMISTRY& INTERPRETATION

Five micron sections were fixed from the formalin-fixed paraffin-embedded archival tissues of cases meeting inclusion criteria and mounted on to Poly-L-lysine treated slides. The slides were stained in batches of 20 using a manual system described in **appendix 3**. Invasive cervical squamous cell carcinoma was used as positive controls in every batch. The slides were dried and labeled with study numbers as follows from P16001 up to P16103. All the slides were independently reviewed by the principal investigator and the two supervisors. They were then scored and entered into the data entry sheet.

2.4.1 Interpretation of IHC

Reactions were scored as follows;

Negative: represented less than 10% reactive cells;

2. **+1**: focal/scattered positivity represented greater than 10% and less than or equal to 80% reactivity;

3. **+2**: diffuse positivity, represented greater than 80% reactivity).

2.5Quality assurance

All reagents were prepared according to the manufacturer's instructions. Standard operating procedures (SOPs) were adhered to during all the procedures. The reagents were physically checked for expiry date, turbidity, odor and precipitates. The recommended storage for all reagents was observed. Positive controls were used for immunohistochemistry staining interpretation. The slides were well labeled before mounting the sections and then arranged serially in order to avoid mix up of slides. All the scores were independently reviewed by the supervisors who are qualified senior pathologists. The two pathologists were blinded and each reviewed the slides independently

2.6. Ethical consideration

Permission for records and specimen retrieval and use in this study was obtained from the KNH/UON Scientific & Ethics and Research Committee. All patient identifiers were protected to maintain confidentiality. Study numbers were used instead of original laboratory numbers to maintain confidentiality. Any discrepancies or additional information that has been found in this study has been conveyed as an addendum to the primary care doctor wherever possible. The findings of this research will also be disseminated to KNH for use in the management of HPV-associated HNSCC patients in future. The findings will also be submitted to scientific journals for publication.

2.7. DATA MANAGEMENT AND STATISTICAL ANALYSIS

2.7.1.1 DATA COLLECTION AND STORAGE

Data was first entered in a collection sheet (**Appendix 1**).

2.7.2DATA ANALYSIS AND PRESENTATION

Data was then entered in Microsoft Excel (Ms 2007) on a password protected laptop and analyzed using SPSS version 17.0 for Windows (SPSS Inc).The study population was described using age and sex which was analyzed and presented as a mean and percentages respectively. Previous and review morphology were presented as percentages and comparison was tested using Wilcoxon rank test. P16 expression was analyzed and presented as a percentage and the proportion stratified according to the site of the tumor. Light microscopy features were associated with socio-demographic characteristics and P16 expression using Chi-square test. Both 95 % confidence intervals (95 % CI) and P-value<0.05 were used to test the statistical significance of results. Study findings were presented in form of tables, graphs and description photomicrographs.

RESULTS

A total of 103 cases seen and diagnosed as HNSCC (Oral, Oropharyngeal and Laryngeal carcinomas) at the Kenyatta National Hospital from 2008 to 2013 were recruited

SOCIO-DEMOGRAPHIC DATA

Ages of the Study participants

The age of the participants ranged between 07 to 91 years with a mean age of 57.4 years. The males were significantly more than female at 76%. The age distribution is as shown in table 1

Table 1: Socio-demographic factors

Variable	**Frequency (%)** **N=103**
Age, mean (SD)	57.4 (14.0)
Category	
<30	4 (3.9)
31-40	7 (6.8)
41-50	19 (18.4)
51-60	33 (32.0)
61-70	26 (25.2)
>70	14 (13.6)
Sex	
Male	76 (73.8)
Female	27 (26.2)

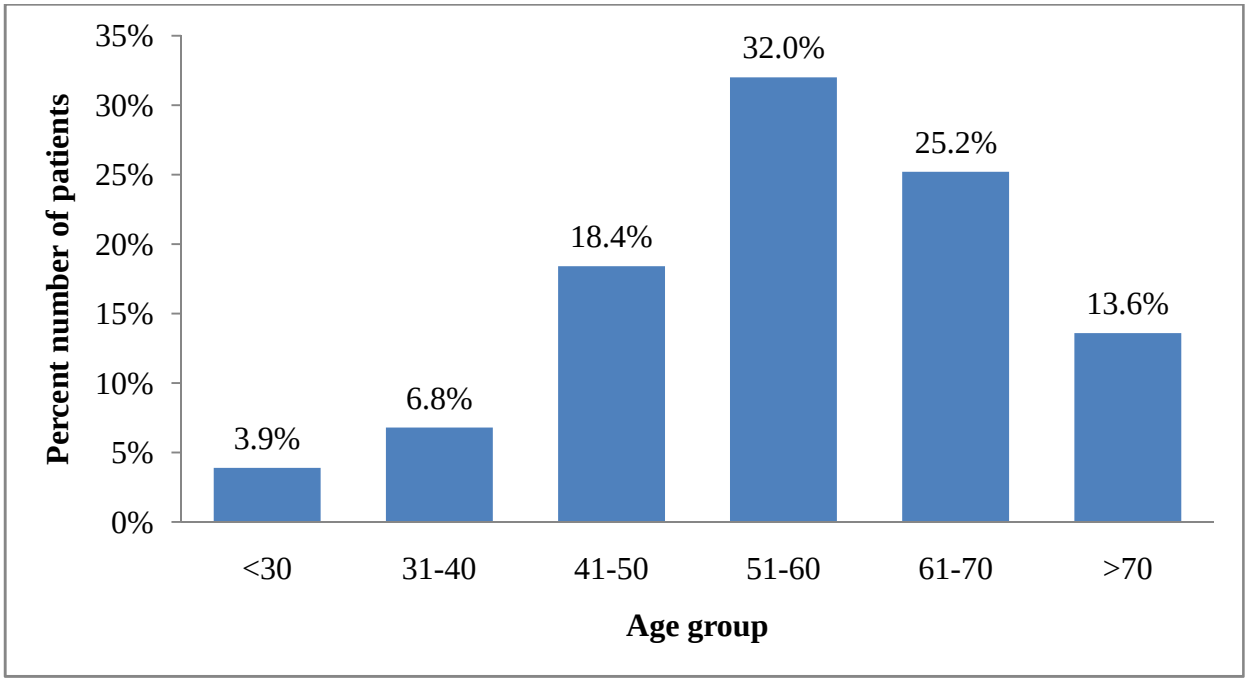


Figure 2: Age distribution

The majority of the tumours were found within the oral cavity 43.7% followed closely by the larynx 37.9% and pharynx 18.4%. (Table 2.)

Table 2: Site of the tumor

Site	**Frequency (%)**
Larynx	39 (37.9)
Pharynx	19 (18.4)
Oral	45 (43.7)
Total	**103 (100.0)**

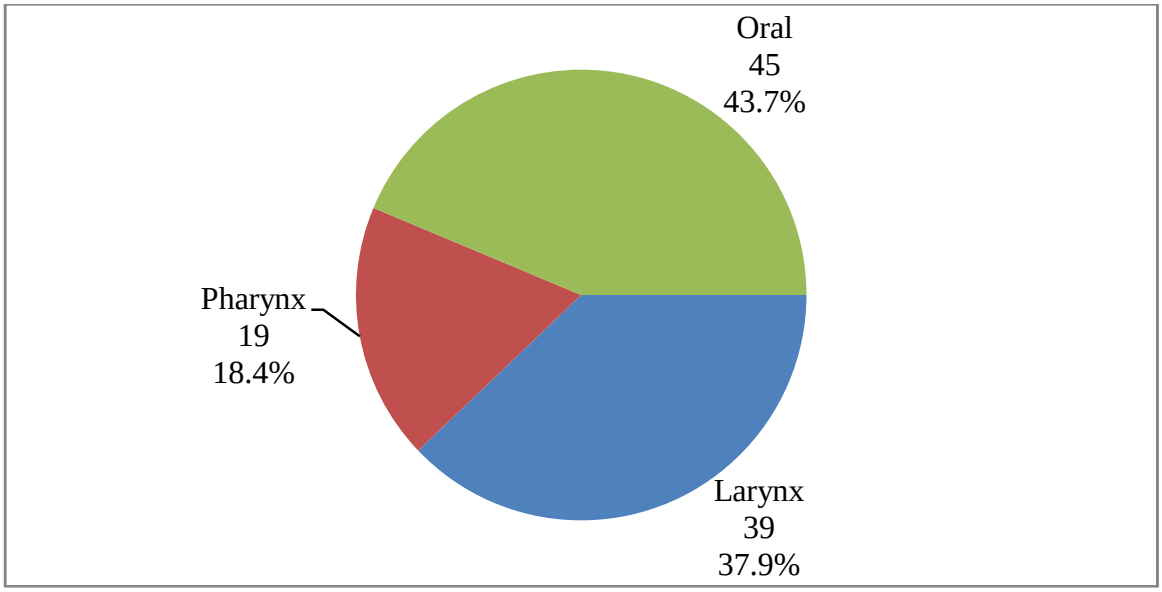


Figure 3: Site of tumour

Most of the tumours were found to be well differentiated 65/103 (63.1%) followed by the moderately well differentiated tumours 29/103 (28.2%), while the poorly differentiated tumours were 8/103 (8%). (Table 3)

Table 3: Tumour differentiation

	Diagnosis		**P value**
	Initial	**Present**	**(Wilcoxon signed rank test)**
Diagnosis			
Well differentiated	69 (67.0)	65 (63.1)	0.718
Moderately differentiated	22 (21.4)	29 (28.2)	
Poorly differentiated	8 (7.8)	8 (7.8)	
Other	4 (3.9)	1 (1.0)	
Total	**103 (100.0)**	**103 (100.0)**	

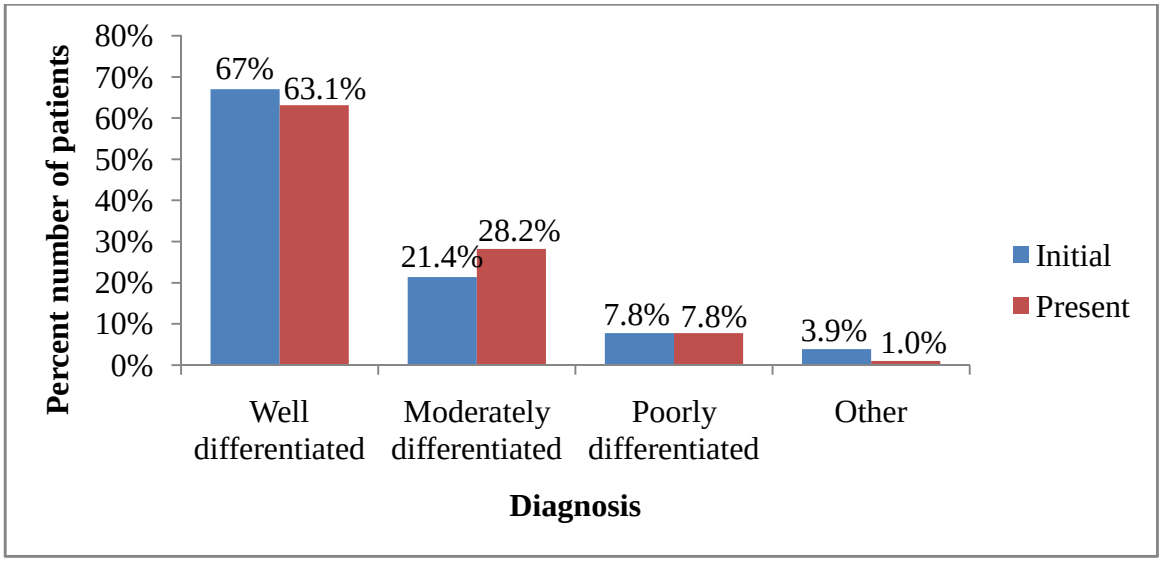


Figure 4: Tumour differentiation

Of the 103 tested HNSCC for P16, 15/103 (14.6%) were found to be positive (**Table 4).**

The oral cavity had the highest frequency at 7/15 (46.67%), followed by larynx 4/15 (26.67%) and pharynx 4/15 (26.67%). **(Table 5)**

Table 4: P16 expression distribution

Variable	**Frequency (%)**	**95% CI**
HNSCC		
P16 positive	15 (14.6)	8.4-22.9
P16 negative	88 (85.4)	77.1-91.6
Total	**103 (100.0)**	

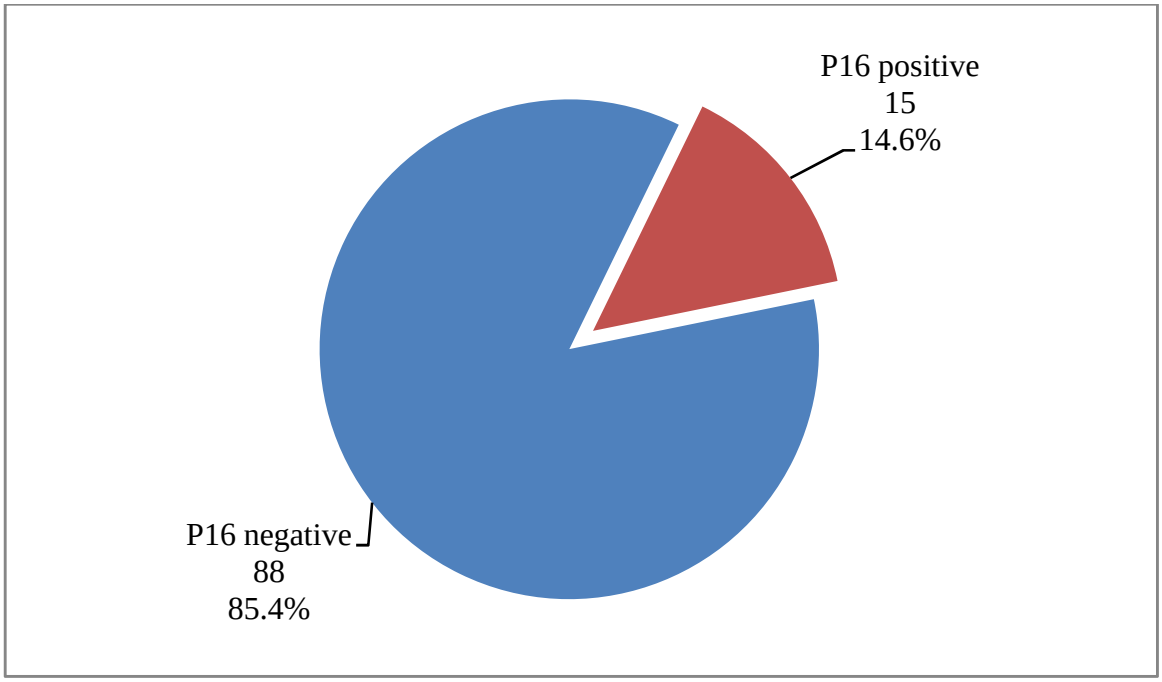


Figure 5: P16 expression distribution

Table 5: P16 expression by anatomical site

Variable	**Oral**		**Oropharyngeal**		**Laryngeal**	
	n (%)	**95% CI**	**n (%)**	**95% CI**	**n (%)**	**95% CI**
HNSCC						
P16 positive	7 (15.6)	6.5-29.5	4 (21.1)	6.0-45.6	4 (10.3)	2.9-24.2
P16 negative	38 (84.4)	70.5-93.5	15 (78.9)	54.4-94.0	35 (89.7)	75.8-97.1
Total	**45 (100)**		**29 (100)**		**39 (100)**	

Table 6: Correlation of histopathological grade and demographic features

	Well differentiated **n=65**	**Moderately differentiated** **n=29**	**Poorly differentiated** **n=8**	**P value**
Age group				
40 and below	7 (10.8%)	4 (13.8%)	0 (0.0%)	0.256
41-50	12 (18.5%)	3 (10.3%)	3 (37.5%)	
51-60	22 (33.8%)	7 (24.1%)	4 (50.0%)	
Above 60	24 (36.9%)	15 (51.7%)	1 (12.5%)	
Sex				
Male	44 (67.7%)	24 (82.8%)	7 (87.5%)	0.253
Female	21 (32.3%)	5 (17.2%)	1 (12.5%)	

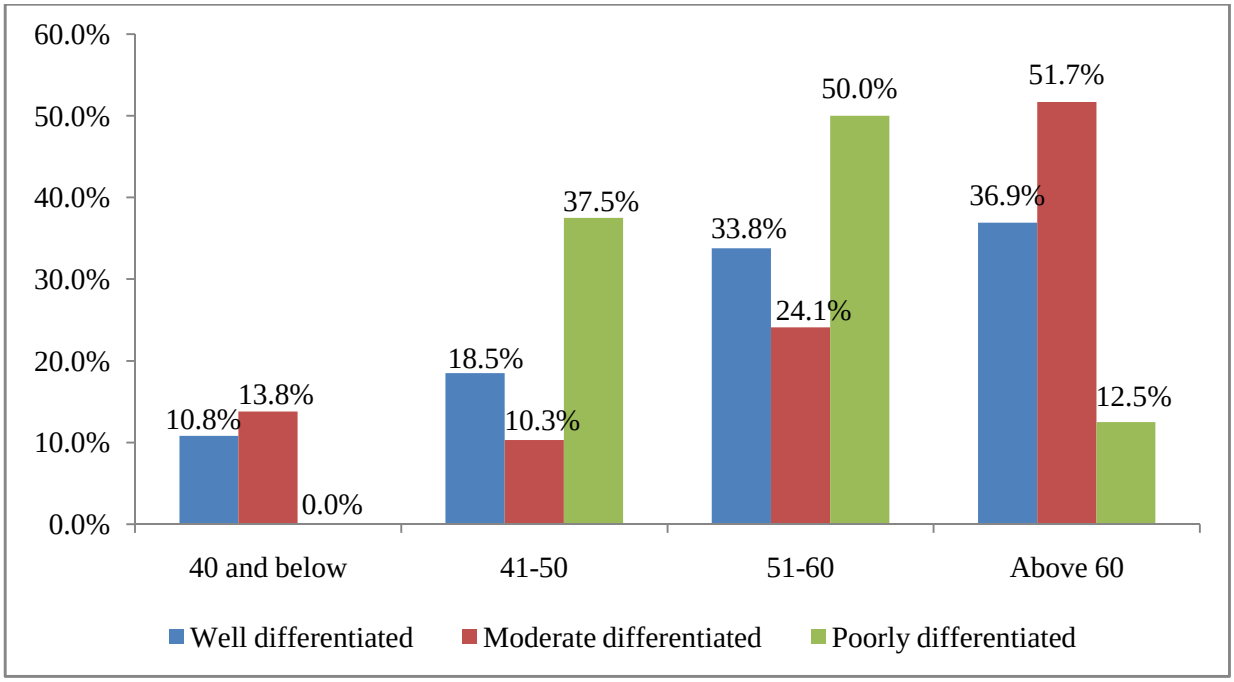


Figure 6: Tumour differentiation categorized by age

The majority of the participants less than 60 years 63/103, however the highest overall age group affected were those above 60 years 40/103, while the least affected age group were those below 40 10/103 (table 6, figure 6)

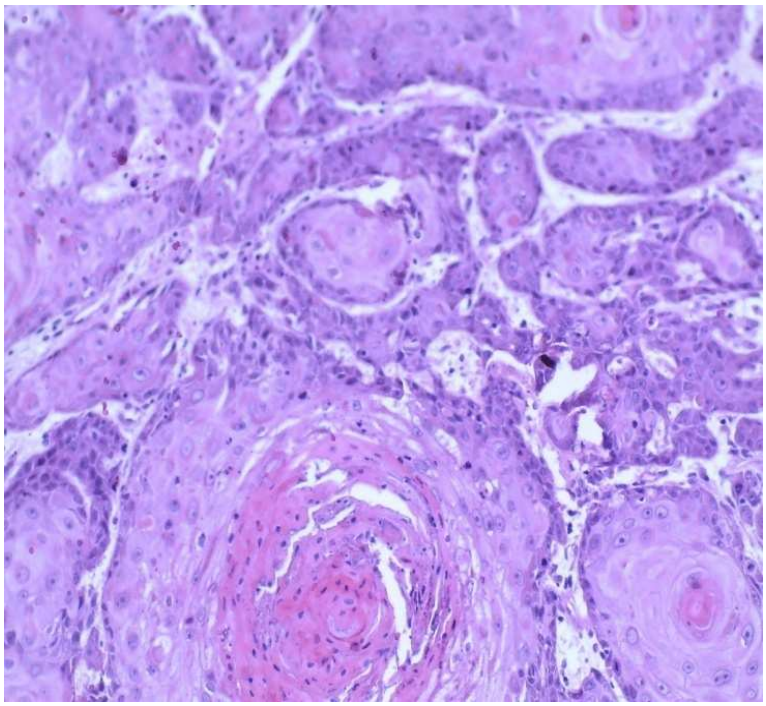
Majority of P16 positive HNSCC were found amongst males 10/15(66.7%). In regards to age, the highest frequency of p16 HNSCC occurred in the age group of 60 and above 6/15 (40%), followed by 51-60 age group 5/15 (33.3%). Most of the p16 positive HNSSC were well differentiated 9/15 (60%) followed by the moderately differentiated carcinomas 4/15 (26%). However after the poorly differentiated HNSCC were found to have increased likelihood to be HPV associated (OR 2.1).

Table 7: correlation of age, sex and grade with P16

Variable	**P16 Status**		**OR (95% CI)**	**P value**
	Positive (n=15)	**Negative (n=88)**		
Sex				
Male	10 (13.2)	66 (86.8)	0.7 (0.2-2.2)	0.532
Female	5 (18.5)	22 (81.5)	1.0	
Age	57.1 (16.7)	57.5 (13.6)	-	0.913
Age group				
40 and below	2 (18.2)	9 (81.8)	1.0	
41-50	2 (10.5)	17 (89.5)	0.5 (0.1-4.4)	0.470
51-60	5 (15.2)	28 (84.8)	0.8 (0.1-4.9)	0.811
Above 60	6 (15.0)	34 (85.0)	0.8 (0.1-4.6)	0.797
Age group				
60 and below	9 (14.6)	54 (85.4)	1.0	
Above 60	6 (15.0)	34 (85.0)	1.1 (0.3-3.2)	0.920
Present Diagnosis				
Well differentiated	9 (13.8)	56 (86.2)	1.0	
Moderate differentiated	4 (13.8)	25 (86.2)	1.0 (0.3-3.5)	0.995
Poorly differentiated	**2 (25.0**)	**6 (75.0)**	**2.1 (0.4-11.9)**	0.405
Other	0 (0.0)	1 (100.0)	-	1.000

Figure 7: Histomorphology of the various Head and Neck Squamous cell Carcinoma

A



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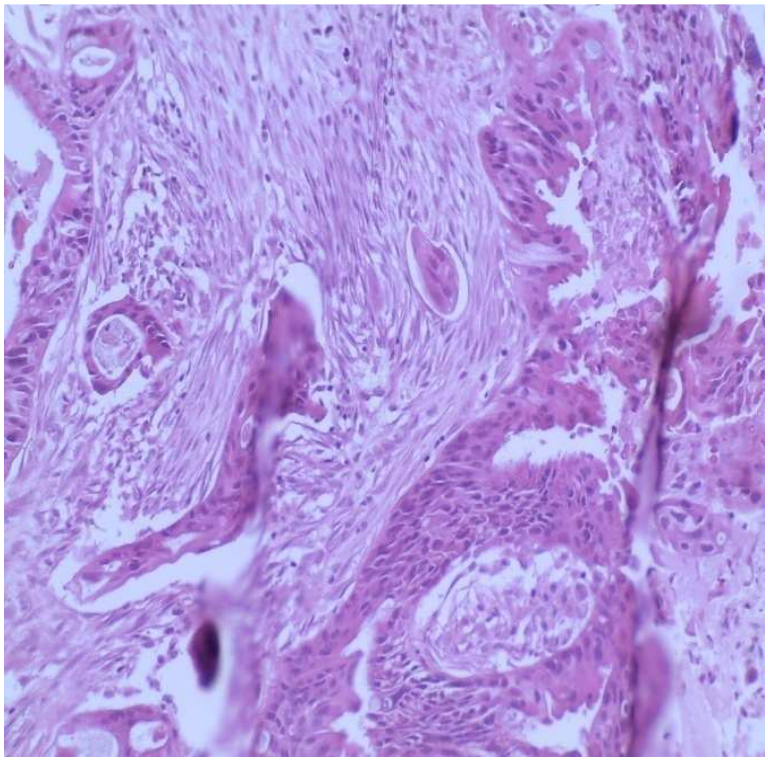

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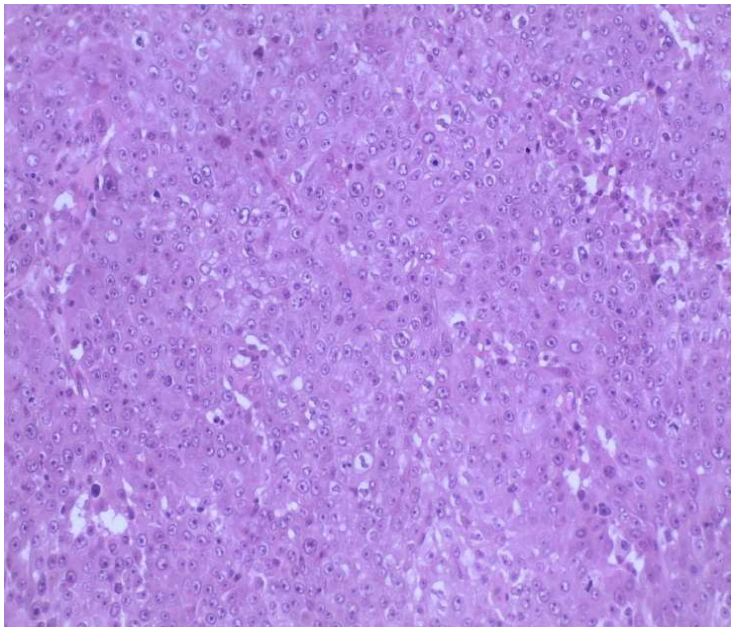

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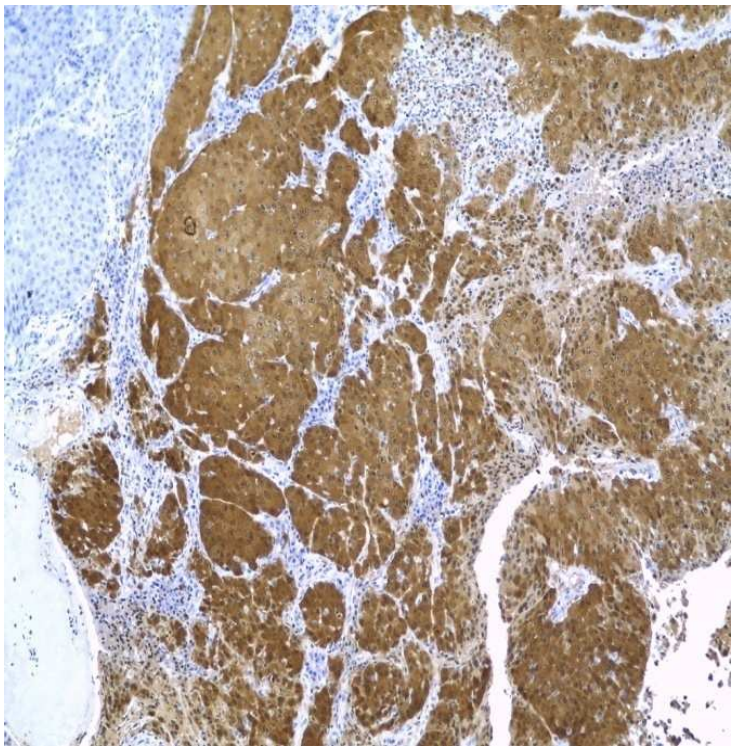

Figure 7. (A) Well differentiated squamous cell carcinoma of oral cavity (40x); (B) poorly differentiated squamous cell carcinoma of the larynx ((40x)); (C) moderately differentiated squamous cell carcinoma of the oropharynx (40x); (D) moderately differentiated carcinoma of the oropharynx showing strong nuclear and cytoplasmic staining for $P16^{ink4A}$ (40x)

DISCUSSION

To our knowledge this is the first study to be carried out in Kenya to determine the prevalence of HPV associated HNSCC by P16 immunohistochemistry. P16 positive HNSCC represents an important subgroup of head and neck cancers that are characterized by a distinct risk factor profile, clinical behavior, response to therapy and a favorable prognosis when compared to the p16 negative tumours[29]. Majority of existing information on HPV and HNSCC is derived from studies in Europe and North America, data from sub-Saharan Africa is very limited.

A total of 103 cases seen and diagnosed as Head and Neck squamous cell carcinoma (Oral, Oropharyngeal and Laryngeal carcinomas) at the Kenyatta National Hospital from 2008 to 2013 were included in the study. The HNSCC were more common in males than females at a ratio of 2.9:1. The age range was 07-91 years. The mean age was 57.4 years with SD of 14.0.The age group most affected was 60-70, followed by 50-60 age groups. These results are similar to findings from a previous local study carried out by Gathere *et al*, which reported that HNSCC was more common in males than females at a ratio of 2:1, it was also noted that the most affected age group was 50-54, followed by the 60-64 age group[10].A similar study carried out in Sudan by Ahmed *et al* also reported HNSCC was more common in males at a ratio of 1.5:1, and the mean age was 54 years[33]. Majority of the tumours were from the oral cavity followed by those from the larynx and pharynx. These findings are similar to those reported by Gathere *et al*, who found that the most frequent tumour were the oral cancers followed by the nasopharynx, larynx and oropharynx respectively.[10].

With regard to histological grade the majority of the tumours were well differentiated followed by the moderately well differentiated tumours, and the poorly differentiated. These findings differ from what has been reported in other regions of Africa. In Ghana and Nigeria Abdulai *et al* and Effiom *et al* respectively found that poorly differentiated tumours were the majority followed by the well differentiated tumours [34,35]. The differences could be due to the different geographic sites, HPV infection rates, smoking, alcohol & other environmental factors. Multiple studies on the association between histological grade and prognosis have shown that there is a strong association between histological grade and survival in patients with early oral cavity SCC. High histological grade in early stage oral cavity cancer is associated with poorer survival and carries independent prognostic value in addition to tumor size, node status, and presence of distant metastasis. [36–39].

Of the 103 tested HNSCC for P16, (14.6%) were found to be positive for P16. The oral cavity had the highest frequency (46.7%), followed by larynx and pharynx. These findings are similar to a study done in Sudan by Ahmed *et al*, where P16 positivity was 15% [(40)]. Another study also by Ahmed et al on HNSCC found an overall positivity of 20.7%. However, this second study included tumours from the Esophagus and other sites. When these extra sites were excluded the positivity reduced to 14% which is similar to the present study[(33)]. A study carried out in Senegal by Ndiaye *et al* on HNSCC found an overall positivity of only 3.4% indicating prevalence of HPV associated HNSCC was very low in that country (41). When compared to a meta- analysis study done by Kreimer *et al* on prevalence and type distribution of HPV by anatomic cancer site the overall positivity was 25.9%. The differences between these studies could be due to the different methodologies used and geographic sites. The meta analysis study done by Kreimer *et al* employed PCR based methods and the geographic locations were mainly in North America, Europe and Asia. It is well known that prevalence of HPV associated HNSCC varies according to methodologies used and geographic locations(4). Another possible reason for the difference could be due to ethnicity, some studies have shown that HPV associated tumours are less prevalent in people of African descent than in Caucasians[(42,43)].Majority of P16 positive HNSCC were found amongst males (67%). With regards to age, about 40% of cases were in the more than 60 year age group followed by 51-60 age group which accounted for about a third of cases. However when cases are reclassified to above 60 years and below 60 years of note is the below 60year cases are the majority (60%).These findings are similar to what has been reported in other studies [(29)]. More than two thirds of p16 positive HNSSC were well differentiated followed by the moderately differentiated carcinomas at about 26%. These findings differ from what has been reported in other studies, Gillison *et al* found that tend to have characteristic basaloid morphology (OR= 18.1, 95% C.I = 2.1-167)[(22)]. Mendelsohn *et al* found that HPV positive and P16 positive are highly predictive for poorly differentiated tumors and basaloid squamous carcinoma[(27)]. The differences between these studies could be due to the low numbers of poorly differentiated squamous carcinoma in the present study. In the present study the poorly differentiated HNSCC were found to have increased likelihood of being HPV associated, OR 2.1 (95% C.I 0.4-11.9). These findings are similar to other studies which reported HPV positive and P16 positive to be highly predictive for both poorly differentiated tumors and basaloid squamous carcinomas [(22,27)].

CONCLUSIONS AND RECOMENDATIONS

Conclusion

1. The majority of tumours were from the oral cavity and the overall HPV prevalence was about 15%.
2. P16 HNSCC expression is most prevalent in patients less than 60 years

3. The poorly differentiated HNSCC have increased likelihood of being HPV- associated

Limitations

1. The study findings were not strengthen with molecular techniques, like ISH or PCR
2. The lack of individual data on risk factors such as smoking and alcohol intake

Recommendations

1. Routine staining for P16 immunohistochemistry should be considered especially for the poorly differentiated carcinomas since they are more likely to be HPV associated.
2. Further studies with more sensitive techniques such as PCR are recommended for measuring the real burden of HPV associated HNSCC and to characterize the HPV subtypes affecting the Kenyan population

CONFLICT OF INTEREST STATEMENT

The author declares that there is no potential conflict of interest relevant to this study

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APPENDICES

APPENDIX 1: DATA CAPTURE SHEET.

PROJECT TITLE:

P16 EXPRESSION IN SUBSETS OF HEAD AND NECK SQUAMOUS CELL CARCINOMA REPORTED IN KNH

DATE……

1. IN PATIENT NUMBER……

2. LABORATORY NUMBER……..

3. STUDY NUMBER……

4. STUDY SITE: THE KENYATTA NATIONAL HOSPITAL

5. SEX 1. M

2. F

6. AGE …….

7. ANATOMIC SITE OF TUMOUR

   1. ORAL

   2. OROPHARYNGEAL

   3. LARYNGEAL

8. PREVIOUS MORPHOLOGY REPORT

9. REVIEW OF MORPHOLOGY REPORT

10. IMMUNO-HISTOCHEMISTRY.

$p16^{INK4a}$EXPRESSION

1 NEGATIVE

2 POSITIVE +1

3 POSITIVE +2

p16^{INK4a}EXPESSION SCORE:

Each lesion will be graded according to a 3-tier system: brown nuclear and cytoplasmic reactivity was scored from negative to 2+:

1. **Negative**: representing less than 10% reactive cells;

2. **+1**: focal/scattered positivity, representing greater than 10% and less than or equal to 80% reactivity;

3. **+2**: diffuse positivity, representing greater than 80% reactivity).

APPENDIX 2-HARRIS HAEMATOXYLIN AND EOSIN STAINING PROCEDURE

Principle of the stain

The mordant forms a lake on the tissue. It is on the lake that the stain gets attached thus colouring the cell nuclei. The nuclei having an affinity for the basic radical in the dye retains the colour even after treatment with 1%acid alcohol. Eosin stains the cytoplasm as a counter stain

Staining technique

1. Bring section to water
2. Stain in Harris haematoxylin for 5 minutes
3. Rinse in tap water
4. Differentiate in 1%acid alcohol, 3dips
5. Rinse in tap water
6. Blue in Scotts tap water for 30 seconds or in running tap water for 10 minutes
7. Counter stain in Eosin for 5 minutes
8. Rinse in tap water to remove excess eosin followed by 70%ethanol to obtain the desired shades of red and pink.
9. Dehydrate in the 3 changes of absolute alcohol
10. Clear in 3 changes of Xylene
11. Mount with D.P.X

APPENDIX 3-P16INK4A IHC PROCEDURE

Manual immuno- staining procedure will be performed. After mounting the sections on poly –L –lysine treated slides and loading them into the machine, IHC staining for p16INK4a consisting of a series of the following steps will be carried out.

1. Rinse slide twice with Bond wash solution and twice with tris EDTA buffer.

2. Incubate with tris EDTA buffer for 20 minutes at 100 ^{0}C.

3. Incubate further with the tris EDTA buffer for another 12 minutes at room temperature. Steps 2&3 are also referred to as heat induced epitope retrieval (HIER). HIER describes a process of heating formalin-fixed paraffin-embedded tissue sections for improved immunoreactivity of tissue antigens with their specific antibodies.

Following antigen retrieval;

4. Rinse three times with bond wash solution.

5. Wash with bond wash solution for three minutes.

6. Block with peroxide for 5 minute

7. Rinse three times using bond wash solution at 35^{0}C.

8. Incubate

9. Rinse once with bond wash solution.

10. Apply post primary antibody for 8 minutes and wash with bond wash solution thrice each wash taking 2 minutes.

11. Apply Polymer for 8 minutes and wash with bond wash solution twice each wash taking 2 minutes.

12. Rinse with deionized water.

13. Rinse with mixed DAB refine then incubate the sections with mixed DAB refine for 10 minutes; DAB acts as the chromate.

14. Rinse with deionized water.

15 Stain with hematoxylin for 5 minutes.

16. Rinse with deionized water,

17. Rinse with bond wash solution,

18. Rinse with deionized water

19. Air-dry.

20. Visualize P16INK4a expression with a light microscope.

21. Score P16INK4a expression on the data sheet

APPENDIX 4: KNH/ERC ETHICAL APPROVAL

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254 020) 2726300 Ext 44355

KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: www.uonbi.ac.ke
Link:www.uonbi.ac.ke/activities/KNHUoN

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

KENYATTA NATIONAL HOSPITAL
APPROVED
ETHICS & RESEARCH COMMITTEE

Ref: KNH ERC/A/277

9th September, 2013

Dr. Boniface Kalru Githaiga
Dept.of Human Pathology
School of Medicine
University of Nairobi

Dear Dr. Githaiga

RESEARCH PROPOSAL: P16 EXPRESSION IN SUBSETS OF HEAD AND NECK SQUAMOUS CELL CARCINOMA REPORTED IN KENYATTA NATIONAL HOSPITAL (P367/07/2013)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and approved your above proposal. The approval periods are 9th September, 2013 to 8th September 2014.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN.**

Yours sincerely

PROF. M. L. CHINDIA
SECRETARY, KNH/UON-ERC

c.c. Prof. A.N. Guantai, Chairperson, KNH/UoN-ERC
The Deputy Director CS, KNH
The Principal, College of Health Sciences, UoN
The Dean, School of Medicine, UoN
The Chairman, Dept.of Human Pathology, UoN
AD/Health Information, KNH
Supervisors: Dr. L.W. Muchiri Dr. E.A. Rogena