THE UTILITY OF TOUCH IMPRINT CYTOLOGY IN DIAGNOSIS OF ORAL SQUAMOUS CELL CARCINOMA –UNIVERSITY OF NAIROBI, SCHOOL OF DENTAL SCIENCES.

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2019

The utility of Touch Imprint Cytology in Diagnosis of Oral Squamous Cell Carcinoma –University of Nairobi, School of Dental Sciences.

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A Thesis Submitted in Partial Fulfillment of the Degree of Master of Medical Laboratory Sciences in the Jomo Kenyatta University of Agriculture and Technology

2019

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to my husband and beloved daughters for their support during my studies.

ACKNOWLEDGEMENT

I thank Lord Jesus Christ for helping me achieve in this study. I extend my sincere gratitude to those who contributed both directly and indirectly to enable me successfully complete this study. I extend my sincere gratitude to my supervisors, Dr. Dimba, Dr. Kaggia and Dr. Walong for their guidance throughout the study period. I thank Washington Ochieng for his valuable assistance provided. Finally I thank my fellow colleagues, students and family for their encouragement and moral support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AJCC	American Join Centre committee
CIS	Carcinoma Insitu
DPX	Distyrene Plasticizer and Xylene
EA	Eosin Azure Stain
EGF	Epidermal Growth Factor Receptor
E6	Papilloma Virus gene
E7	Papilloma virus gene
H & E	Hematoxylin and Eosin
HHV	Human herpes Virus
HPV	Human Papilloma Virus
IUCC	Union for international Cancer Control
KNH	Kenyatta National Hospital
OG-6	Orange G
OSCC	Oral Squamous Cell Carcinoma
MDSCC	Moderately differentiated squamous cell carcinoma
NPV	Negative predictive value

PPV	Positive predictive Value
PSCC	Poorly differentiated squamous cell carcinoma
Rb	Retinoblastoma
SIN	Squamous intraepithelial Neoplasia
SPSS	Statistical package for Social sciences
TIC	Touch Imprint Cytology
TNM	Tumour Nodes Metastasis
WDSCC	Well differentiated squamous cell carcinoma
UON	University of Nairobi
WHO	World Health Organization

ABSTRACT

Oral squamous cell carcinoma (OSCC) is an aggressive neoplasm with limited therapeutic options and poor survival rates. Assessment of surgical margins in OSCC during intra-operative procedure is important and influences its local recurrence, patient's survival rates, management and overall prognosis. Currently histopathology technique is being used as a gold standard; it is reliable but time consuming while touch imprint cytology is cheap and quick technique useful where rapid results are required. The objective of this study was to evaluate the utility of touch imprint cytology in assessing OSCC in comparison with histopathology. A descriptive cross-sectional study of 73 participants with suspected oral squamous cell carcinoma was conducted, whereby on analysis 69 of the samples were diagnostic. Touch imprints were done and results were compared with histopathology. Data was analyzed using SPSS version 17.0 and descriptive summary statistics done were presented as proportions and percentages in the form of tables and charts where relevant. The level of agreement between the diagnostic techniques was calculated using the chi-square test and kappa statistics. A p value less than 0.05 was considered statistically significant. The most participants were male (71%) and the highest age being 50-59 years. The most common site of lesion was tongue (43.5%) and cytomorphology pattern most demonstrated was a singly dispersed cell pattern. There were 53 true positives, 12 false negatives and 4 true negatives and no false positive. The sensitivity, specificity, positive predictive value, negative predictive value and the overall accuracy were 81.5%, 100%, 100% and 25% respectively with an overall accuracy of 90.75% and with a kappa level agreement of 53% between touch imprint cytology and histopathology and a p-value of 0.033 which is statistically significant. Touch imprint cytology is therefore a sensitive technique which can be useful in screening and reliable where rapid preliminary diagnosis is needed in the surgical management; it is also an affordable technique for developing countries with limited resource.. We recommend sampling from multiple sites should be applied while preparing touch imprints to increase harvesting of malignant cells from biopsies. Touch imprint cytology requires practice and experience and should be adopted in pathology routine work to enable technical staff develop and acquire expertise.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Squamous cell carcinoma (SCC) is a health condition involving the uncontrolled growth of abnormal cells in the skin outer squamous cells of the epidermis. Oral squamous cell carcinoma (OSCC) occurs in oral region and is an aggressive malignant neoplasm with high morbidity and mortality which when advanced requires disfiguring surgical procedures in order to completely excise the malignant lesion (Suttichai *et al*, 2012). Squamous cell carcinoma is the most common malignant neoplasm accounting for more than 90% of all oral malignancies (Silverman *et al*, 2003; Javaoivc *et al*, 1993). The diagnostic gold standard is histopathological analysis of stained tissue sections, which is necessary for further patient management. Although the diagnosis from biopsy specimens is considered definitive, it is labor intensive and time consuming and may not be useful for intra-operative consultation. Frozen sectioning is currently the most favored technique for providing rapid results in intra-operative consultation, however it employs expensive equipments which is not affordable especially in developing countries like Kenya.

Previous studies have been conducted on application of touch imprint preparation as an adjunct technique to histopathology for diagnosis of various cancers (Kolte *et al*, 2010; Creager et al, 2004; Hussein *et al*, 2005). These studies found cytological preparations to play an important role in the peri-operative assessment of malignant lesions and could be used for screening and diagnostic purposes. The commonly used cytological preparations for diagnosis analysis are touch imprint, scrape, squash and fine needle aspirates.

Several studies have been done using the different cytological preparations (Khunamornpong *et al,* 2003; Kochzan *et al*, 2006). Touch imprint cytology has an advantage due to the relative ease of performing the procedure, it is non-invasive, and is a rapid procedure that does not alter tissue or produce undesirable artifacts. It provides excellent cytological clarity (Kolte *et al*, 2010).

1.2 Problem statement

Intra-operative consultation is useful in providing information that can assist in immediate therapeutic decision during surgical procedure Wenig BM(2008).; it can also assist to assess adequacy of specimen and also the extent of spread of a tumor. Currently diagnosis is through incision biopsy and histopathology which is considered definitive (Kumar *et al*. 2010. Histopathology is time consuming and may not be useful in intra-operative consultation where quick decisions are required. The use of frozen section for intra operative consultation has been widely used however it employs expensive equipment's which is not affordable especially in developing countries like Kenya. There is need for cheaper and less time consuming technique which can aid in intra operative consultation. Cytology preparation does not affect normal histopathology processing since fresh surgical specimens are first used to make cytology preparation before the tissue is processed as paraffin embedded sections. It is possible to assess tumor cells using cytology preparation since tumor cells are generally characterized by reduced cohesiveness which makes them easier to be in tissue fluid thus the tissue surface may be selectively enriched with detached tumor groups giving a unique source of cytological analysis (Mannweiler *et al*, 2009).

The objective of this study was to evaluate the utility of intra-operative touch imprints of surgically excised tissue for rapid decision making in surgical treatment. This technique will be assessed to determine the sensitivity, specificity, positive and negative predictive values which will be compared to histopathology as a gold standard technique.

1.3 Justification

Cancer which remains as a major world concern has not been considered as a major health issue in developing countries until recently according to a 2005 report by the World Health Organization (WHO) (Loncar *et al*, 2007). Oral cancer is a global health problem with increasing incidence and mortality rates with approximately 300,000 patients annually estimated to have oral cancer worldwide(Mehrotra, 2006). According to the world health report (2004) cancer accounted for 7.1 million deaths in 2003. Head and neck cancer is the 6th most common cancer in the world with Squamous cell carcinomas being the most prevalent form of oral malignancies according to Warnakulasuriya S (2009).

According to GLOBOCAN 2012, lip and oral cavity cancer is the 15th most common cancer in Africa and 7th most common cancer in Middle Africa, however there is limited data from few hospital based cancer registries available Warnakulasuriya S(2009). A study found head and neck cancer to be second commonest cancer in the urban and peri-urban regions in Nairobi, Kenya between years 2000-2002(Limo *et al*, 2007).

There is increase in the incidence of cancer in developing nations due to change of habits for example increase in smoking and increased pollution due to increasing industrialization. The prevalence of oral cancer is relatively higher in men, older people and among people of low education and low income.

Proper management of cancer treatment involves proper diagnosis. The ability of a surgeon to assess margins for presence of cancer cells and extent of cancer spread will aid in complete excision thus increase chances of survival rates and also aid in future management in case of extensive spread.

A study by Amhareen (2004) comparing touch imprint cytology versus frozen section in intra-operative consultation found that touch imprint provided better cellular

morphology and fewer artifacts in comparison with frozen section which provided better architectural clarity but was hampered by freezing artifacts. The study found touch imprint cytology to be cheap and fast way of assessing tumor and can be used during intra operative consultation. The need for safe, simple, cost effective method of sampling is therefore necessary to assist in intra operative consultation..

1.4 Research Questions

1. Can touch imprint cytology be used to make diagnosis of oral squamous cell lesions**?**
2. How reliable is touch imprint cytology in comparison to histopathology which is the gold standard technique?
3. What are the cyto-morphological patterns presented in touch imprint cytology?

1.5 Hypothesis

1.5.1 Alternative hypothesis

Touch imprint cytology is a reliable and effective technique to histopathology which is the gold standard technique.

1.5.2 Null hypothesis

Touch imprint cytology is not a reliable and effective to histopathology which is a gold standard technique.

1.6 Objectives

1.6.1 Broad Objective

To evaluate utility of touch imprint cytology in diagnosis of patients with suspected oral Squamous cell carcinoma.

1.6.2 Specific Objectives

1. To conduct cyto-diagnosis and compare it with histopathology diagnosis.
2. To determine the agreement between touch imprint cytology and Histopathology by comparing sensitivity, specificity and p-value.
3. To describe the cytomorphological patterns observed in touch imprint cytology.

1.7 Conceptual Framework

The independent variable influences the dependent variable as illustrated in fig 1.7

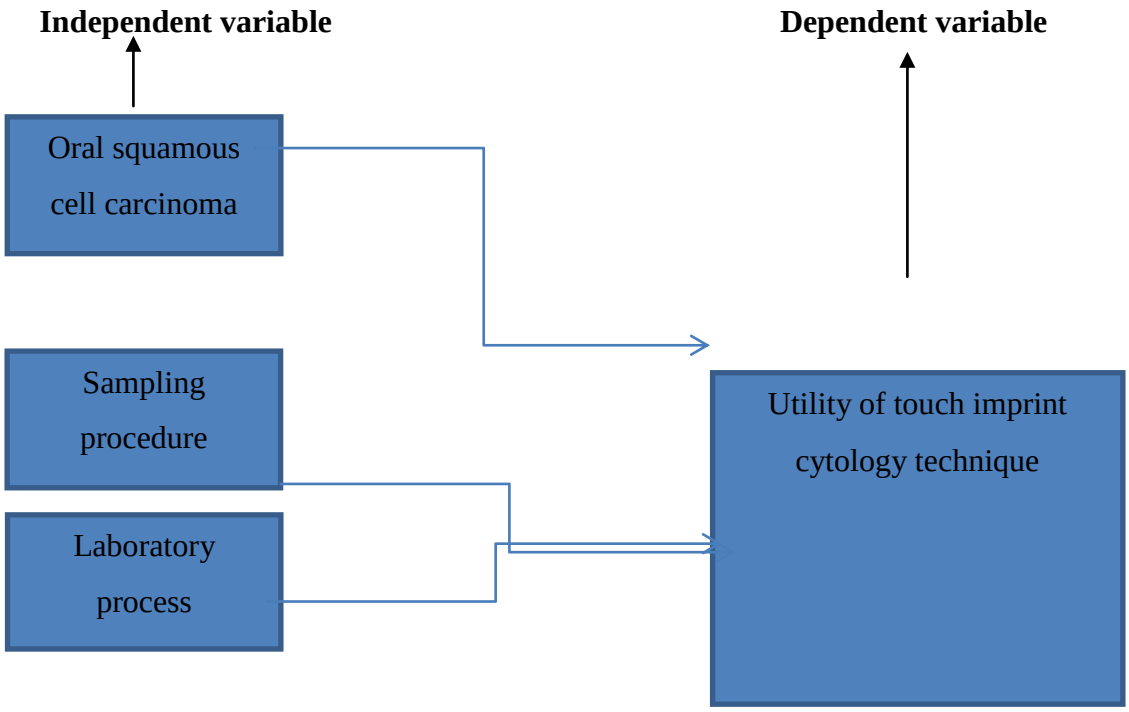

Figure 1.7: Conceptual framework. Independent and dependent variables

1.8 Operationalization

Presence of oral squamous cell in tissue biopsy should be identified by the touch imprint cytology. The process of sampling and staining and analyzing will have effect on touch imprint cytology. This study will be limited to study the following variable.

1.8.1. Dependable variable- The utility of touch imprint cytology

1.8.2. Independent variable-

a. Demographic characteristics- age, gender, site of lesion
b. oral squamous cell in biopsy samples.
c. Sampling procedure
d. Lab process

CHAPTER TWO

LITERATURE REVIEW

2.1 Global perspective of oral squamous cell carcinoma

Cancer remains a worldwide disease that is of great concern. According to world cancer report 2008 (Boyle, 2008), until recently, cancer was considered a disease of westernized, industrialized countries. Today the situation has changed dramatically, with the majority of the global cancer burden now found in low- and medium-resource countries. An increase in the incidence of cancer in developing nations means that the international implications of the disease now require greater attention.

According to (WHO) oral cancer is the eleventh most common cancer worldwide (Stewart et al, 2003). The incidence of oral cancer shows extensive variation with differences across countries particularly relating to distinct risk factors and availability and accessibility of health services. Oral cancer is a global health problem with increasing incidence and mortality rates. Around 300,000 patients are annually estimated to have oral cancer worldwide (Babshet *et al,* 2011).

A study done to assess the distribution and differentiation of squamous cell carcinoma from the head and neck region in Zimbabwe population between the periods of 1982-1991 found the most common site of SCC distribution to be the mandible, gingival, flour of the mouth and the tongue. The most common type being well differentiated squamous cell carcinoma occurring mostly in 40-60 age groups (Chidzonnga *et a*l, 2006).

In Kenya a study done between years 2000-2002 found head and neck cancer to be second most common cancer; overall oral squamous carcinoma accounts for 90% of head and neck malignancies (limo *et al,* 2007; Boyle *et al,* 2008).

2.2 Epidemiology of oral squamous cell carcinoma

The incidence of OSCC remains constant but appears increased in some parts of the world (Iamaroon *et al*, 2004; Peterson *et al*, 2005).Squamous cell carcinoma is the most frequently occurring malignant tumor of the oral structures it accounts for about 90% of the malignant oral lesions(Boyle *et al* 2008). Epidemiological studies have shown that the incidence of oral cancer varies significantly among the continents and within developed and developing countries (Javaoivc *et al*, 1993).

Mortality rates for carcinoma of the mouth and oropharynx in some areas in Brazil are among the highest in the world (Othon *et al*, 2001). According to Owings (Owings *et al*, 1984) the most important determinant of poor prognosis is advanced disease stage at diagnosis. The dental practitioner plays a particularly important role in the detection of oral cancer in its early stages when treatment is most effective and morbidity is minimal (Schnetler, 1992).

2.3 Pathology of Oral Squamous Cell Carcinoma

2.3.1 Etiology

The development of head and neck cancer is multi-factorial, either as a result of the interaction of environmental factors, behavioral factors or genetic inheritance according to Haddad et al 2008. However, alcohol and tobacco are two of the most important risk factors for development of oral squamous cell carcinoma. Past and present studies have identified that consumption of alcohol and tobacco as two main factors in the development of oral cancer (Bundgaard *et al*, 1994 & Krishna et al 2013). Many viruses have been associated with oral epithelial dysplasia and squamous carcinoma. Oral epithelial dysplasia, which is a precursor to squamous carcinoma, is infected with HPV. HPV is also found in 33–50% of oral squamous cell carcinomas and again most of these contain HPV-16 and HPV-18. These viruses contain gene products (E6 and E7) that bind wild-type p53 and Rb proteins and eliminate the ability of these proteins to

stimulate DNA repair or apoptosis.35 Another virus that has been implicated in malignant transformation is human herpes virus 6 (HHV-6). HHV-6 has been reported in oral squamous carcinomas but it is unclear whether this finding has any clinical relevance (Bundgaard *et al*, 1994; Williams, 2000).

2.3.2 Pathogenesis

The pathogenesis of squamous cell carcinoma is multi-factorial, like all epithelial neoplasms, the development of squamous cell carcinoma is thought to be a multi-step process involving the sequential activation of oncogenes and inactivation of tumor suppressor genes in a clonal population of cells. Defects in cell cycle checkpoint components are a major cause of genetic instability in cancer cells. This can result in increased production of growth factors or numbers of cell surface receptors, enhanced intracellular messenger signaling, and/or increased production of transcription factors. In combination with the loss of tumour suppressor activity, this leads to a cell phenotype capable of increased cell proliferation, with loss of cell cohesion, and the ability to infiltrate local tissue and spread to distant sites(Bundgaard *et al*, 1994; Williams,2000; Robins, 2012).

The genetic alterations observed in head and neck cancer are mainly due to oncogene activation and tumor suppressor gene inactivation, leading to de-regulation of cell proliferation and death. These genetic alterations include gene amplification and over expression of oncogenes such as myc, erbB-2, Epidermal Growth Factor Receptor (EGFR), cyclin D1 and mutations, deletions and hypermethylation leading to *p16* and *p53* tumor suppressor gene inactivation (Mehrotra *et al*, 2006).

There is a concept of two-step process of cancer development in the oral mucosa; the initial presence of a precursor (premalignant, precancerous) lesion which subsequently develops into cancer (Babshet *et al*, 2011).A potentially malignant lesion is a morphologically altered tissue that has a greater than normal risk of malignant

transformation. The presence of epithelial dysplasia is generally accepted as one of the most important predictors of malignant development in pre-malignant lesions. Several oral lesions like leukoplakia, erythroplakia, lichen planus and actinic keratosis are considered to be premalignant lesions for oral squamous-cell carcinoma, since an increased risk of malignant transformation is associated with them. These lesions are often subtle and asymptomatic, requiring a high index of suspicion on the part of clinician, especially if the risk factors such as tobacco use or alcohol abuse are present. In a recent study (Scheifele *et al*, 2003) based upon epidemiological data of European patients, it was concluded that the upper limit of the annual transformation rate of oral leukoplakia is unlikely to exceed 1%. On the basis of such data, it should be deduced that the first aim in the management of leukoplakia or any other potentially malignant lesion is the early diagnosis and prevention of malignant transformation.

2.3.4 Treatment of oral squamous cell carcinoma

Treatment of OSCC would include a single modality surgery, radiotherapy and various combinations of these modalities. Selection of treatment is based on different factors, including tumor accessibility, functional outcome, patient's health and preference, and the availability of treatment expertise. (Hui S *et al*, 2013; Pignon JP, *et al*, 2009).

2.3.5 Clinical presentation

In the early stages, cancers of the oral cavity appear either as raised, firm, pearly plaques or as irregular, roughened, or verrucous areas of mucosal thickening, possibly mistaken for leukoplakia. Symptoms are uncommon in earlier stages of the disease but become frequent with advanced local invasion. Overt squamous cell carcinoma typically presents as a persistent mass, nodule, or indurated ulcer with color changes that are common and consist of red or red and white hues (Robins *et al*, 2012).

2.3.6 Histopathology of OSCC

The histological presentation of OSCC under a microscope is demonstrated in a progression on the squamous epithelium from normal, to hyperkeratosis, to mild/ moderate dysplasia, to severe dysplasia and finally as cancer/malignant (Robins *et al*, 2012). These cancers begin as dysplastic lesions, which may or may not progress to full-thickness dysplasia (carcinoma in situ) before invading the underlying connective tissue stroma. Squamous cell carcinomas range from well-differentiated keratinizing neoplasms to anaplastic, sometimes sarcomatoid, tumors, and from slowly to rapidly growing lesions. However, the degree of histological differentiation, as determined by the relative degree of keratinization, is not correlated with behavior (Robins et al, 2012).

2.3.7 Cytopathology of OSCC

The squamous epithelium is essentially identical to that of the uterine cervix although squamous cells originating in the mouth often show mild cytologic atypia. Keratinizing squamous cell carcinoma is characterized by malignant cells with heavy keratinization. The heavier the keratinization, the more dissociation the single cells are seen. The malignant cells of non-keratinizing squamous cell carcinoma are more cohesive than those of keratinizing squamous cell carcinoma, typically forming irregular, disorderly sheets(Demay,2012).The tumor is composed of abnormal squamous cells with marked pleomorphism, including bizarre cell shapes (snakes, tadpoles, etc) .The cytological smears will be interpreted based on the following parameters: enlarged nuclei, variation in nuclear size and shape (pleomorphism), nuclear membrane irregularity, nucleo-cytoplasmic ratio, number of nuclei, binucleation, keratinization, tadpole forms, hyperchromatism, chromatin pattern and distribution as well as discrepancy in nucleocytoplasmic maturation(Babshet,2011)

2.3.8 Role of cytopathology in diagnosis of OSCC

The definitive diagnosis of cancer disease is mainly through tissue biopsy specimen or cytology processing of exfoliative specimen (Naveed *et al*, 2017). Cytology as a diagnostic technique has mainly been used in gynealogical specimen and fluids or aspirates from tumors or cystic lesion or body cavities. The utilization of cytology as a diagnostic tool alongside histopathology as a rapid technique has been attempted in various researches to evaluate its accuracy (Geetha *et al*, 2014; Naveed *et al*, 2017) .This is useful since cytology is less expensive due to its utilization of less costly equipment. It is also fast and can be useful in intra-operative consultation in theatres to enable proper management of cancer.

2.3.9 Grading of oral squamous cell carcinoma

The grading of squamous-cell carcinoma is based on the degree of differentiation and the amount of keratin present in the cytoplasm of the predominating tumor cells as indicated in studies. Broder's suggested a system of grading tumors in which a grade I lesion was highly differentiated (its cell were producing much keratin) while grade IV was poorly differentiated (the cells were highly anaplastic and showed practically no keratin formation) (Anneroth, 1984 Zuher, 1996).

Grade I**:** A well-differentiated squamous-cell carcinoma exfoliates mainly keratinized malignant cells that stain deep orange with a Papanicolaou stain.

Grade II**:** At this grade at least 50% of the malignant cells are large, moderately differentiated, non-keratinizing squamous cells.

Grade III**:** A poorly differentiated carcinoma exfoliates mainly non-keratinized cells which stain blue and resembling abnormal parabasal cells.

2.4 Cytology

2.4.1 Principle

Diagnostic cytology is the microscopic examination of cellular material for the diagnosis of disease particularly cancer. This is in comparison with histological diagnosis which relies on the architecture of the tissue to help determine the disease process, whereas cytological diagnosis relies on examination of individual cells and their morphology to give clues as to the cell's health and activity (Freida *et al*, 2009).

To prepare a good cytology preparation one requires an extensive understanding of fixation and staining. A poorly prepared, fixed and stained slide can alter the diagnosis, a false-positive or false-negative diagnosis can result from negligence in cytology preparation. After cytology preparation and staining, the specimens are examined by cytologist and pathologist. The cytology preparation is carefully screened to identify potential cell abnormalities. Any potentially abnormal cells are noted and marked. The cytologist or pathologist uses all clues found on the slide to make decision about disease process present (Ankur *et al*, 2010).

2.4.2 Cytological techniques

Cytology is a diagnostic technique that utilizes various sampling methods; touch imprint, smear, fine needle aspirate among others. Cytology procedures are simple and inexpensive method that provides excellent preservation of cellular details without freezing artifacts and with no loss of tissue as with the cryostat (Palouse *et al*, 2004; Veneti *et al*, 1996).Cytology sampling is relatively painless, non-invasive technique and requires minimal technical skills(Babshet,2011). It is also useful in providing diagnosis where specimen in limited and where patients refuse surgery or are medically compromised.

The shortcomings in cytology can be due to lack of sufficient clinical information that can assist in diagnosis, improper sampling and fixation of cytological samples; this can be caused by inexperienced and careless cytotechnologist (Babshet, 2011).Other limitations noted in studies include not being able to distinguish in-situ from infiltrating carcinoma and its inability to evaluate depth of invasion and margins of resection (Kolte, 2010) this according to studies done.

The ability to deliver an immediate diagnosis by cytology makes it an important part of treatment at places where frozen section facility is not available; owing that it needs an advanced setup. In utilization of cytology as an intra-operative procedure the most important indication is to establish or confirm diagnosis rapidly, this is an important aspect of surgical pathology that often guides the surgeon's hand. The rapidity of preparation should not be at the expense of results accuracy. Cytology can be applied as a learning tool by promoting interpretation of cytology and its histological correlation (Kolte, 2010). It has limitations as it can provide inadequate cellular sample especially when sampling hemorrhagic biopsies.

2.4.3 Cytology touch imprints

The origin of imprint cytology can be traced back to 1927 by Leonard S. Dudgeon and Vincent Patrick at the University of London. They introduced use of rapid cytology for diagnosis of freshly cut specimens with reliable accuracy rates (Dudgeon, 1927).Following this, several studies have been done to evaluate usefulness of cytology as a tool for intra operative diagnosis. The use of cytology preparations during intra-operative consultation is yet to be appreciated as a rapid technique in favor of traditional examination of frozen sections. This appears to be due to the surgical pathologist's relatively higher level of confidence in frozen sections, though many studies have demonstrated that the diagnostic efficacy of intra-operative cytology is comparable to that of frozen section (Shidham, 2000).

Touch imprints prepared from fresh surgical specimen give excellent cytological clarity and when used intra operatively it can provide valuable information where frozen section interpretation is equivocal (Suen, 1978). The study also noted the importance of cytology when specimen is limited in quantity and large enough only to provide sufficient cells for cytological analysis (Babshet, 2011). The study pointed out limitation of imprint in diagnosing well differentiated tumors and tumors with dense fibrous stroma.

A retrospective study (Esteban, 1987) done to review lumpectomy specimens and to evaluate the intra-operative imprint cytology margins found it to be accurate, simple, rapid, and cost-effective method for determining the margin status of breast conservation therapy specimens intra-operatively.

Imprint cytology diagnoses from patients with laryngeal and pharyngeal tumors were correlated with the histological result of a corresponding biopsy. The imprint cytology proved to be a useful, quick and reliable method with complete diagnostic accuracy, sensitivity, specificity, positive predictive value and negative predictive(Lončar *et al*, 2007; Savargaonkar *et al*,2001; Clarke *et al*,2010).

In a study of correlation between the results of the biopsy specimens and touch imprint preparations in patients with 30 head and neck mass lesions (nasal, pharyngeal, laryngeal and oral lesions (Hussein *et al*, 2005). The concordance between touch imprint and paraffin sections was 90%. The sensitivity and specificity of TIC in detecting malignancy were 88% and 92%, respectively. In frozen sections, many factors contribute to the false-negative rate, including the suboptimal preparation of the specimen and sampling errors.

Touch imprint cytology has proved its accuracy in diagnosing surgical specimens belonging to thyroid, parathyroid, breast cancer margins, sentinel lymph nodes and

prostate but its role in the evaluation of resection margins of squamous cell carcinoma remains unclear (Meyskens, 1991).

Several studies have been conducted on different cytological techniques to assess its utility in diagnosis, a study done on touch preparation found the technique to have less cellularity but more cellular clarity (Suen *et al*, 1978; Khinamompona *et al*, 2003; Kolte *et al*, 2010; Esteban *et al*, 1987).

2.4.4 Papanicolaou technique

Cytology preparations are routinely stained by Papanicolaou technique (Papanicolaou 1954). This stain provides optimal microscopic visualization of the nuclear and cytoplasmic morphology. It is not specific for any compound related to malignancy but provides good visualization of cellular morphology so that cellular health and disease processes can be evaluated.

Five dyes in three different solutions make up the papanicolou staining protocol. Hematoxylin is used as the nuclear stain. The first counterstain, OG-6, consists of orange G, which stains keratinized cells. The second counterstain, EA, contains light green SF yellowish, eosin Y, and Bismark brown Y. EA differentially stains the cytoplasm of cells allowing the cytologist to distinguish between different cell types. Both stains are used with phosphotungstic acid as a mordant. Well stained cells are transparent, allowing the observer to interpret any underlying cells. Differentially stained cytoplasm, exhibiting a spectrum of colors, demonstrates the health status and activity level of the cell (Freida, 2009).

2.4.5 Hematoxylin and eosin technique

This is a proven technique that utilizes Hematoxylin and Eosin stain (H&E).This is a popular staining method in histology it involves the application of hemalum, which is a complex formed from aluminum ions and hematein , which is an oxidation product of

haematoxylin. Hemalum stains nuclei of cells (and a few other objects, such as keratohyalin) blue. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colors other, eosinophilic structures in various shades of red, pink and orange. (Freida, 2009).

While the use of conventional H&E stained paraffin sections is considered the gold standard, the preparation takes time thus making it unsuitable as an intraoperative guide where rapid results are required. Frozen sections, on the other hand, can also be used intra-operatively with fairly good results and however their use requires specific & expensive instruments and additional personnel (Veneti *et al*, 1996).

CHAPTER THREE

MATERIALS AND METHODS

0.7 **Study Site**

School of Dental sciences in University of Nairobi is a teaching and referral hospital diagnosing and treating head and neck patients. Touch imprint cytology and biopsy samples suspected of OSCC were received and processed in Oral pathology laboratory in the department of oral and maxillofacial surgery in the school of dental sciences.

0.7 **Study design**

This was a descriptive cross-sectional study.

0.7 **Study population**

The target population comprised of patients presenting with clinical signs and symptoms suggestive of OSCC attending university dental clinics.

0.7.7 **Inclusion criteria**

All patients who had lesions suspicious of OSCC with no previous confirmatory tests done and who consented to participate were included in this study.

0.7.7 **Exclusion criteria**

All Patients not presenting with clinical signs and symptoms suggestive of OSCC were excluded from the study.

0.7 **Sample size determination**

Fisher's formula (1998) will be used to calculate the population size of the study.

$$(n) = \frac{Z^2 pq}{d^2}$$

Where :

(n)=the desired sample size

Z=the standard normal deviate that provides 95% confidence interval of (1.96)

(p)=prevalence of suggestive of OSCC (90%=0.9)

(q)=1-p

(d)=absolute precision (error bound) (0.05)

Hence:

$$n = \frac{1.96^2 \times 0.9 \times 0.1}{0.05^2} = 138$$

Since the population size is less than 10,000 the final sample estimate (n*f*) will be calculated using the following formula:

$$nf = \frac{n}{1 + (\frac{n}{N})}$$

Where:

(n*f*) = the desired sample size (when population is less than 10,000)

(n) = the desired sample size (when population is more than 10,000)

N = the population of patients diagnosed with OSCC within 6 months is 126

Hence:

$$nf = \frac{138}{1 + (\frac{138}{126})} = 65.87 \approx 66$$

NB: a 10% attrition rate was included hence 66+7= 73 respondents.

0.7 **Sampling Technique**

The patients with lesions suspicious of squamous cell carcinoma were selected irrespective of their age and gender, they underwent thorough clinical examination by dentist and those who consented to participate in the study were selected and convenience sampling was applied consecutively until the desired sample size was achieved.

0.7 **Recruitment**

The 69 patients with suspected OSCC who consented and scheduled to undergo incision surgical procedures were recruited in the study. The cytologist conducted all the sampling procedures. The patients used in the study were undergoing routine checkup in the clinics. The patients suspected samples were obtained after thorough clinical examination by an oral dental surgeon. All specimen which were queried for squamous cell carcinoma were selected irrespective of patients age their age and gender. All patients who underwent surgical procedure and gave consent to participate in the study were selected consecutively until the sample size was achieved.

0.7 **Data and specimen collection**

Data collection was obtained in the following manner; socio demographic data was obtained from patient data form filled by investigator after the patient gave consent.

0.7 **Procedures in Laboratory**.

The touch imprint cytology and histopathology biopsy samples from the same patient had the same serial number and was attached with a request form with all information

0.7.7 **Cytology touch imprints slide preparation**

Study samples were obtained from excised biopsy by directly sampling the site of lesion to produce maximum number of diagnostic cells. This was obtained by holding fresh biopsy sample using forceps blotting firmly on tissue paper to remove excess blood and then touching gently the tissue site on a pre-labeled frosted microscope slide while avoiding any gliding movement. The two touch imprint slides per patient were prepared and immediately immersed in 95% ethyl alcohol to avoid drying then stained using papanicolaou stain using (hematoxyline papanicolaou 1, JT BAKER, Tuegseweg Netherlands). (Appendix II). The stained cytological slides were examined by principal investigator together with the supervising pathologists.

0.7.7 **Histopathology slide preparation**

The biopsy sample of the suspected OSCC was then well preserved in 10% buffered formalin for histopathology processing. The preserved tissue was the dehydrated in different changes of ascending alcohols, 80%, 95% and three changes of Absolute alcohol. The samples were cleared in three changes of Toluene and finally infiltrated by two changes of molted wax. These samples were then blocked in embedding rings before sectioning using microtome equipment. Sections of 5microns were obtained and stained using H &E technique (Hematoxylin and Eosin stain, OXFORD, Navghar, India) (appendix I)

(appendix I).The stained sections were then reported by the hospital pathologists.

0.7.7 **Reporting**

The screening of cytology preparation was done by the principal investigator and the slides of both touch imprint cytology and histopathology examined by three consultant pathologists. The grading of SCC in TIC slides was based on the degree of differentiation and the amount of keratin present in the cytoplasm of the predominating tumor cells as indicated in a text Zuher, 1996. ·

Cytology results were evaluated as follows malignant, suspicious for malignancy and negative for malignancy. Non-diagnostic specimens were excluded (no cellularity, air drying or distortion artifact, obscuring blood).

i. .**Malignan**t (SCC): isolated cells or clusters of malignant cells showing keratinization. The cells have distinct cell borders, vesicular nuclei and prominent nucleoli. Adenocarcinoma: cells are usually arranged in cohesive groups of various sizes in the form of loose clusters or acini with central lumina. The individual cells may show eccentric nuclei, mostly with prominent nucleoli and evidence of mucin production in the form of cytoplasmic vacuolation.)

ii. **Suspicious for malignancy** (suggestive of malignancy but uncertain due to limited number of cells or degree of atypia)

iii. **iii. Negative for malignancy** (no evidence of malignancy like high N/C ratio, pleomorphism, hyperchromasia, coarse chromatin, irregular nuclear outlines).

iv. iv. **Non-diagnostic** (no cellularity, air drying or distortion artifact, obscuring blood).

v. There was consensus review in discordant reports between the two pathologists, to prevent analytical errors such as missing abnormal cells or misinterpretation of abnormal cell with normal cells.

3.10.4 Quality Control

3.10.4.1 Pre analytical

Sample collection and processing

All procedures pertaining to labeling of slides, specimen collection, transportation, analyzing data and posting of results were adhered to. The sampling slide was properly labeled with a serial number with its accompanying request form which had same serial number.

0.7.7.3. **Analytical**

The correct samples were obtained from patients after assessment by qualified oral maxillofacial surgeon to ensure that only suspicious cases of oral squamous cell carcinoma were selected. Oral touch imprint samples and biopsy were collected as per stipulated procedure, preserved in 95% alcohol for 10minutes and biopsies in 10% buffered formalin to prevent cellular deterioration. Staining of touch imprint with papanicolaou stain and was done by the Principal Investigator while observing the right SOPs. Stains were changed regularly and covered to ensure good staining quality. Blocks for histology were also processed by the Principal investigator under the guidance of a qualified laboratory technologist. All slides were reviewed by consultant pathologists supervising the study.

0.7.7.3 **Post-analytical:**

All slides were verified before the release of reports to ensure accurate interpretation of results. A tie breaker helped in cases whereby the supervisors disagreed.

0.7.7 **Ethical Consideration**

Approval to carry out the research was obtained from the UON/KNH Ethics and research committee (appendix VI)

0.7.7 **Data Management and Analysis**

Data was collected and stored in spreadsheet both in hard and soft copies. Data in hard copies were stored in a lockable cabinet while the ones in soft copies were protected by passwords and only authorized persons were allowed to access the information. Data was captured and cleaned for errors in Statistical Package for the Social Sciences (SPSS) version 17. Descriptive statistics for continuous outcomes including means or medians (including the corresponding standard deviation or inter-quartile range as appropriate) were calculated. Categorical outcomes such as gender were presented using counts and respective proportions (percentages). Sensitivity, specificity, negative predictive value and positive predictive value were calculated using a 2 x 2 table. Cohen's Kappa and overall agreement were done to assess the agreement between the two tests using histology as the gold standard. Results have been presented in tables and charts as appropriate.

0.7.7 **Study limitations**

Blood obscuring artifacts and insufficient sample was still a challenge but as the investigator I acquired the expertise afterwards and was required to add more samples and discard initial samples.

CHAPTER FOUR

RESULTS

A total of 73 samples were collected but only 69 were diagnostic which were analyzed and statistical analyses conducted. These samples included biopsies for histopathology and touch imprint cytology slides; these were analyzed in oral pathology laboratory in the department of oral and maxillofacial surgery, University of Nairobi.

4.1 Socio-demographic factor

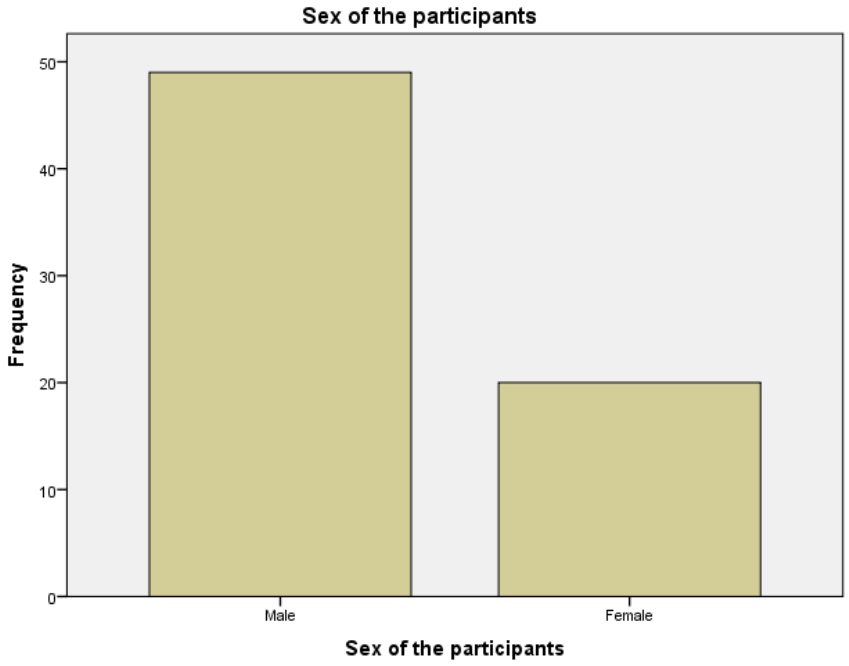

Figure 4.1: Sex Distribution of Patients (n=69)

Males formed the majority of the cases at 49 out of 69 while females were 20 out of 69 and these represented 71% and 29% respectively with a ratio of 2.45:1 for M: F.

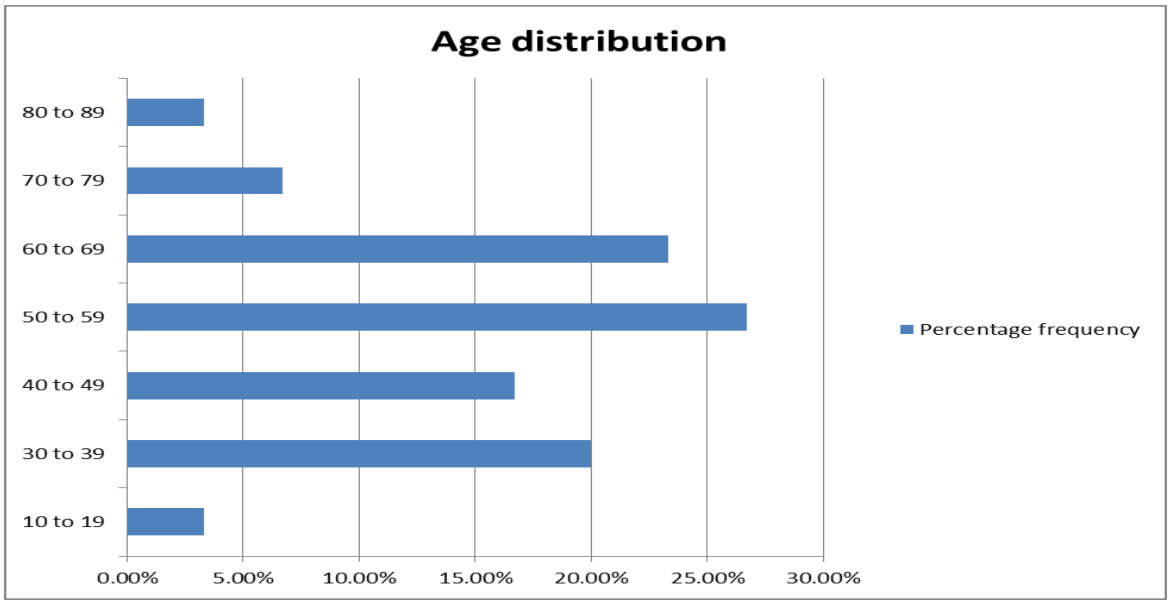

Figure 4.2: Age distribution of Study Patients (n=69)

The youngest diagnosed with OSCC was 19 years old while oldest was 86 years old. Majority of the participants were 50-59 years old making a (17)24.6% proportion of the entire study population while the least being (1)1.4% : others were 20.3% for 60-69 years, 15.9% for 70-79 years, 14.5% for 30-39years, 11.6% for 40-49 years , 8.7% for 80-89 years and 2.9% for 20-29 years age group.

4.2 Site distribution of lesion

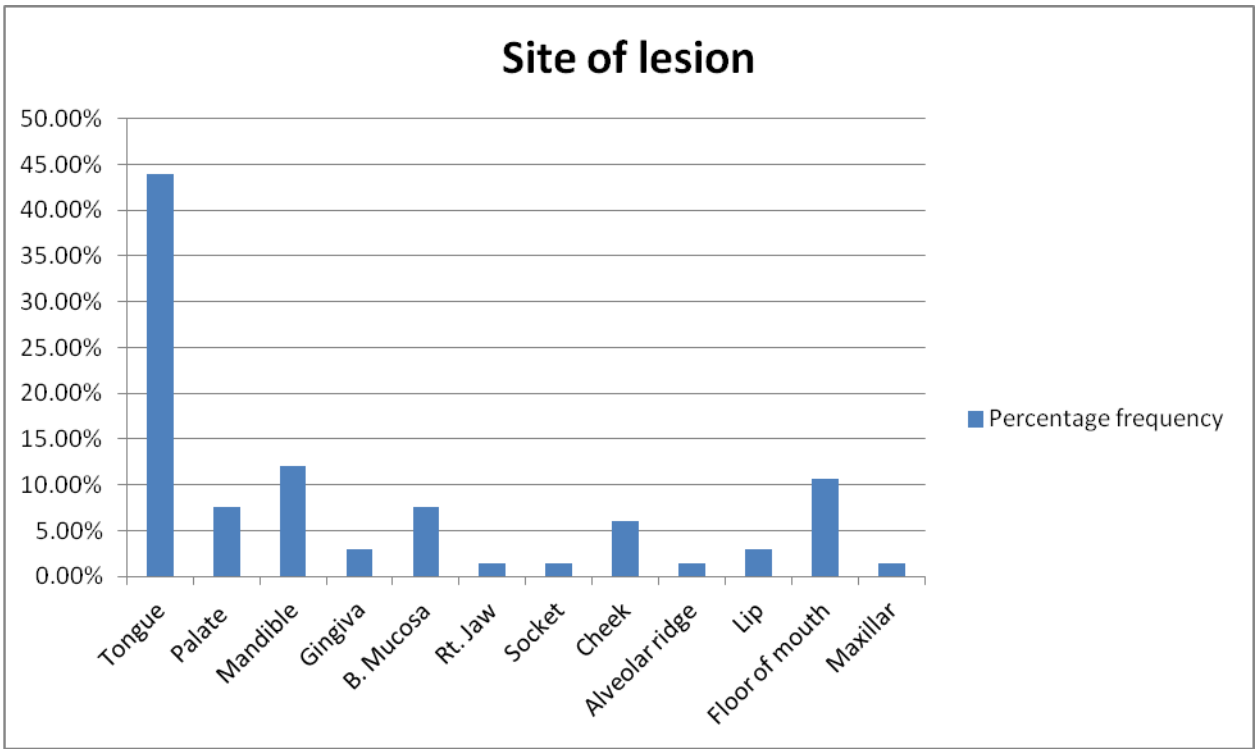

Figure 4.3: Site distribution

The majority of samples were obtained from tongue 30(43.5%) and the least from the alveolar ridge and socket each 1 (1.4%). Others were Mandible 10 at 14.5%, floor of the mouth 7 at 10.1%, both palate and buccal mucosa each 5 (7.6%), cheek 4(5.8%), Lip, Gingiva and maxilla each 2 (2.9%).

4.3 TIC cytology patterns

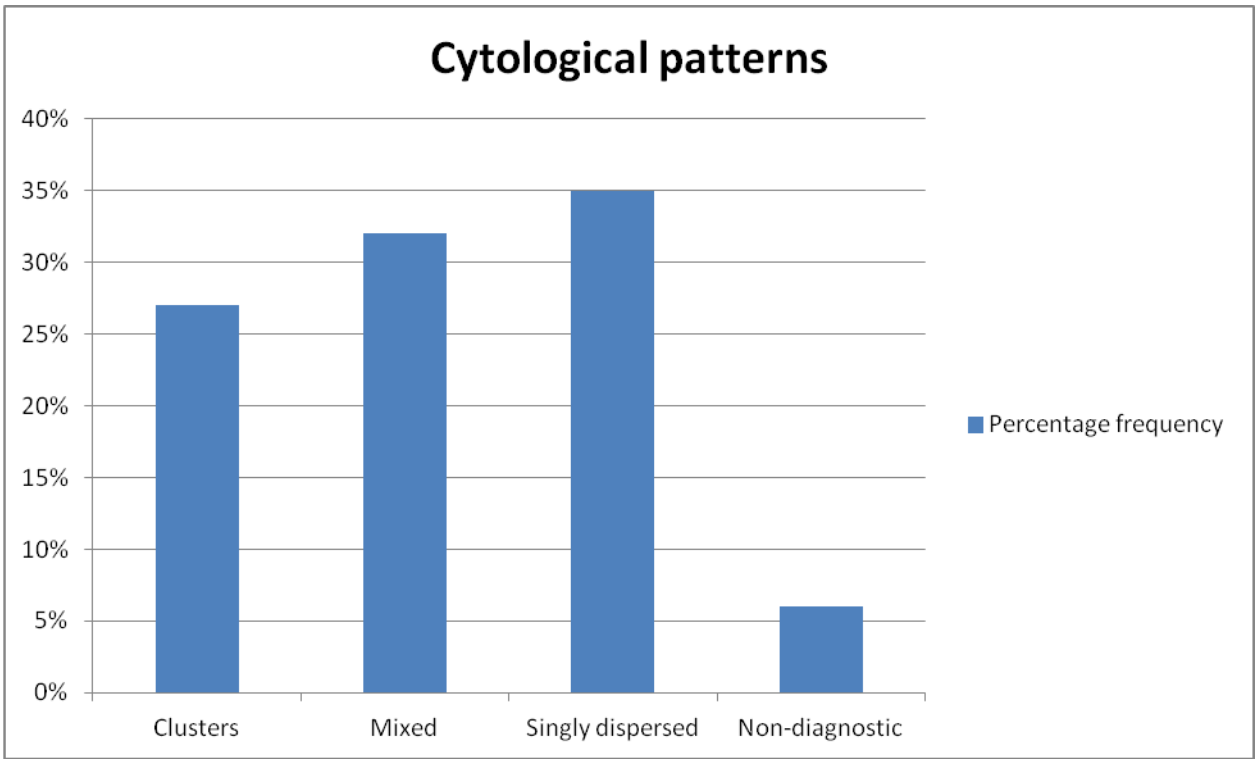

Figure 4 4: Cytological Patterns

TIC cytological patterns appeared as clusters, mixed (clusters and singly dispersed) and singly dispersed. Singly dispersed were 24(34.7%), Mixed pattern (singly and clusters) was 21(30.4 %) while clusters were 18 (26.0%) and samples 6(8.7%) pattern could not be established.

4.4 Touch imprint cytology results

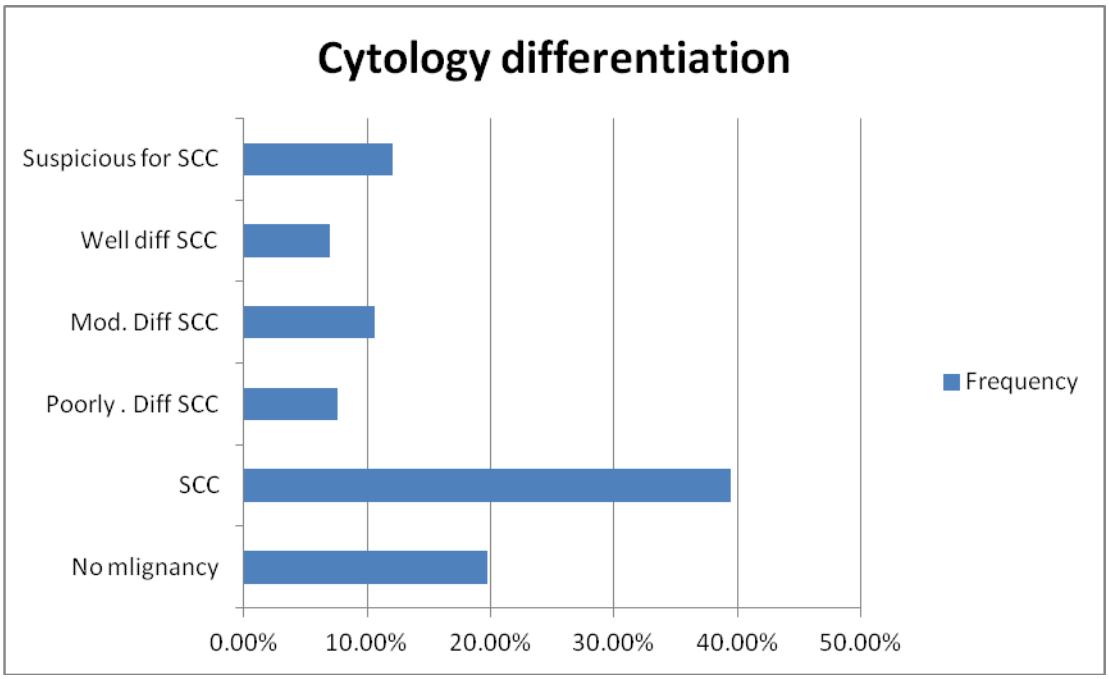

Figure 4.5: Touch Imprint Cytology Differentiation Results

Out of 69 samples examined 45 (65.2%) were diagnosed as malignant, 8 (11.6%) as suspicious and 16(23.2%) diagnosed negative or with no malignancy. Out of the 45(65.2%) malignant cases 26 (37.7%) were diagnosed as SCC, 7(10.1%) as MDSCC and WDSCC each, and 5(7.2%) as PSCC.

4.5 Histolopathology results

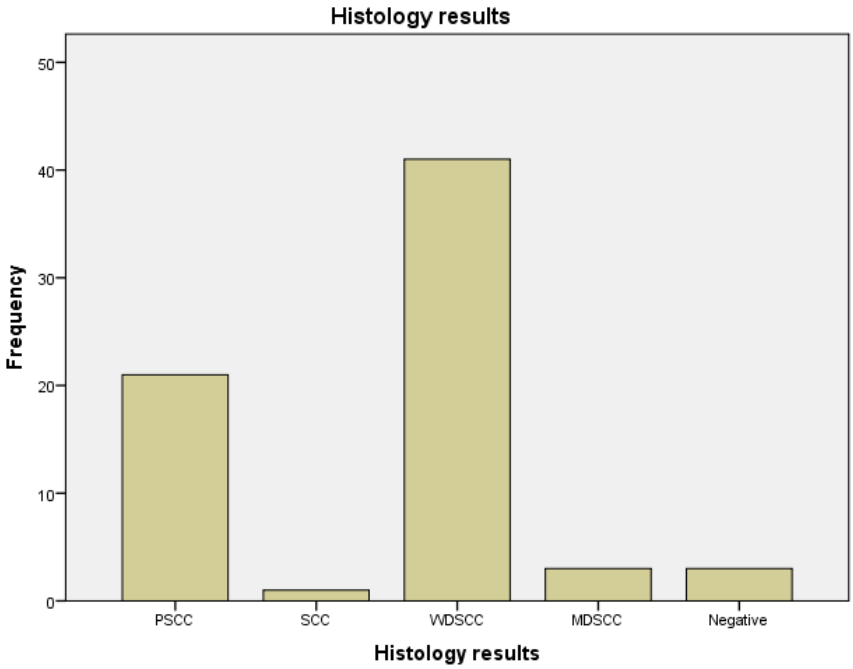

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Figure 4.5: Histopathology results

Out of 69 histopathology diagnosis 40 (57.9%) were diagnosed as WDSCC, 21(30.4%) diagnosed as PDSCC, 3(4.3%) MDSCC while 4(5.7%) had no malignancy.

4.7 Histopathology versus Cytopathology

Out of 69 cases, there were 53 true positives, 12 false negatives, 4 true negatives and 0 false positive results when imprint cytology was compared with histology in a 2 X 2 table.

Table 4.1: Cytopathology versus Histopathology results

		Cytology		Total
		Positive	**Negative**	
Histology	Positive	53	12	65
	Negative	0	4	4
Total		**53**	**16**	**69**

Table 4.2: Sensitivity, Specificity, Ppv, Npv And P Value

Sensitivity	**81.5%**
Specificity	100%
Positive predictive value	100%
Negative predictive value	25%
p-value	0.033
Kappa level of agreement	53%
Standard error	0.130
Kappa value	0.339

The sensitivity, specificity, PPV and NPV were 81.5%, 100%, 100% and 25% respectively and a p-value of 0.033 with a kappa level of agreement of 53%..

4.8 TIC Photomicrographs demonstrating Cytomorphological patterns and cytological diagnosis

TIC slides showed variety of cells some singly dispersed, in clusters. Others obscured by blood artifact, while others with normal cells (figure 4.7.1, figure4.7.2, figure 4.7.3 and figure4.7.3)

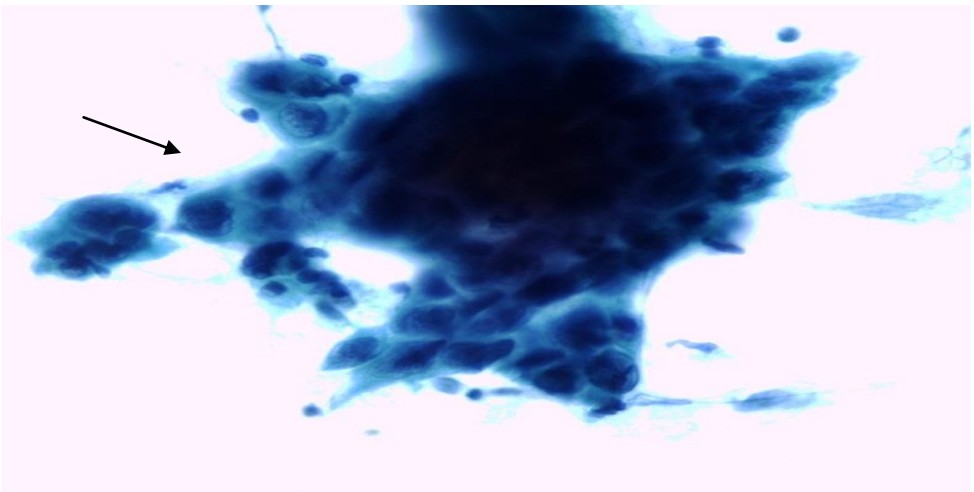

Figure 4.7: Photomicrograph of clustered pattern of poorly differentiated SCC(x400)

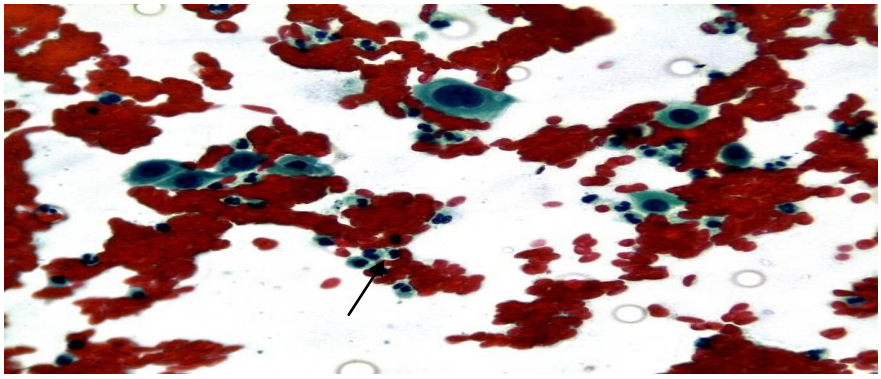

Figure 4.8: photomicrograph of TIC Show singly dispersed cells suspicious of SCC with overlying blood artifact.(x400)

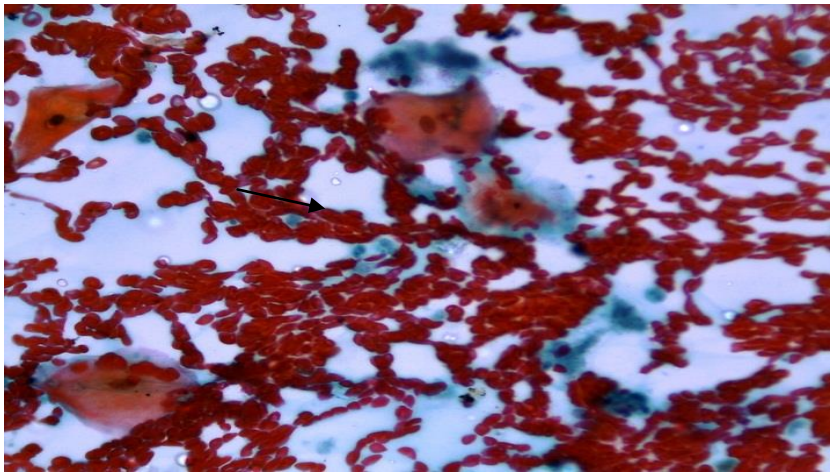

Figure 4.9: TIC showing hemorrhagic imprint with underlying cells.

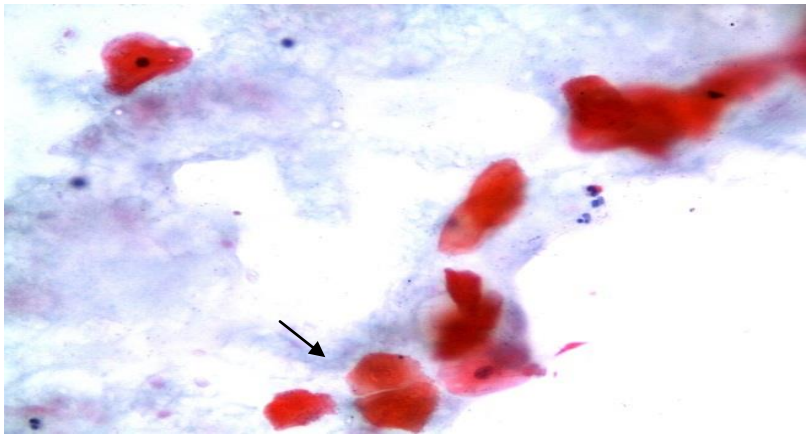

Figure 4.10: TIC showing normal looking squamous cells

CHAPTER FIVE

DISCUSSION

Oral squamous cell carcinoma is an aggressive malignant neoplasm with high morbidity and mortality rates whose therapy, when advanced, requires disfiguring surgical procedures that should accomplish complete excision of the malignant lesion (Suttichai *et al*, 2012). Intra-operative consultation is useful in providing information that can assist in immediate therapeutic decision during surgical procedure; it can also assist to assess tumour margins and adequacy of specimen and also the extent of spread of a tumor during surgical procedure. Touch imprint cytology is a simple, cheap and quick way of assessing presence of malignancy during intra operative analysis. For decades touch imprint cytology has been used as a diagnostic tool for breast cancer(Helpap, *et al* 1978) .Touch imprint cytology have also been used as an adjunct to assess adequacy of samples that were obtained by CT guided or ultrasound, this reduced the time a patient was exposed to radiology(Fotou *et al*, 2007; Mangia *et al*, 2008) . The only challenge encountered in touch imprint is its ability to differentiating invasive from insitu samples (Valdes *et al*, 2007). In this study we were not able to clearly differentiate and grade malignancy using TIC.

Frozen section which is currently being used for intra operative consultation in resourceful countries has its limitation due to interpretational difficulties caused by freezing artifacts; it is expensive and needs specialized personnel. This is not suitable for resource limited countries contrary to touch imprint which is cheap, quick and excellent cellular clarity and does not require expertise in machinery to use (Ahmareenm & Anwar, 2007) .

A total of 73 were collected to evaluate the diagnostic accuracy of touch imprint cytology in comparison to histopathology which is the gold standard technique. Only 69 were assessed in this study since others were non diagnostic. The majority of the

participants were male comprising of 46(71%) and female comprised 20(29%) which is in ratio of 2:45: 1 for M: F. In other studies showed male predominance to female to be 2:1 (Naveed *et al*, 2017).The high number of males could be due to predisposing factors such as smoking tobacco and drinking of alcohol; males have been found to engage in higher numbers(16.8%) compared to women at (2.1%) according to Kenyan National authority for campaign against alcohol and drug abuse (NACADA) data in the year 2017. Smoking being a significant etiological factor of OSCC and combined with use of alcohol greatly increases the risk of acquiring SCC (Zygogiann *et al*, 2011)

The majority of patients were in the age range 50-59years comprising of 17 patients and the least was age range 10-19 years comprising of 1 patient. This is comparable to a study done by Mehorta who found the majority of patients being in age range 50-59years (Mehrotra *et al*, 2006).

The majority of samples were obtained from tongue 30(43.5%) while there is a study that found buccal mucosa to be most common followed by tongue then hard palate(Naveed *et al*, 2017).

The cellular pattern that was most observed was singly dispersed cell pattern. According to (Demay, 2012) the heavier the keratinization, the more dissociation the single cells are seen. Non –keratinizing squamous cell are more cohesive than keratinzing cell. In this study histological results showed high level of well differentiated squamous cell carcinoma. The most common type of oral squamous cell was well differentiated squamous cell this is comparable to study by Chidzoga et al, 2006 which found well differentiated squamous cell carcinoma (Chidzonnga *et al*, 2006).

The sensitivity and specificity of touch imprint cytology for squamous cell carcinoma in our study was 81.5% and 100% respectively. A study on malignancy of upper aero digestive tract found sensitivity and specificity of touch imprint cytology to be 96% and 100% respectively (Naveed *et al*, 2017) while another study found sensitivity and

specificity to be 92.3% and 94.4% respectively (Geetha, *et al* 2015). These findings indicate that TIC is a good screening tool that should be useful intra operative procedure where rapid results are required for effective surgical management. Histopathology is still important as a confirmation report.

Positive Predictive Value and Negative Predictive Value were 100%, 25% in this study, while another study found PPV and NPV of 96% and 89.5% respectively (Geetha *et a*l, 2015). There was low percentage of NPV (25%) this is due to high number of false negatives; this could be attributed to negative results which could be due to sampling from sites with no tumor cells causing high level of false negatives, this was also a recommendation by Naveed *et al*, 2017 who recommend that multiple imprints to be made.. This can be improved if more than one sampling site is applied. A study by Chao *et al*, 1996 recommends careful screening be done to detect scanty malignant cells.
The overall accuracy in this study was 90.75% while a study done had overall accuracy of 96.7%. We found that TIC of OSCC showed accuracy due to highest number of 53 true positive cases to 12 cases of false negatives and four true negatives results which agreed with gold standard technique which is histopathology.

There is statistical significance in the results with a p-value of 0.033 which is significant at 5% level of significance being $P<0.05$. There is a statistical significance of touch imprint cytology as a screening and diagnostic tool.

CHAPTER SIX

CONCLUSION AND RECCOMENDATION

6.1 Conclusion

The objective of this study was to determine the agreement between touch imprint cytology and histopathology by comparing sensitivity, specificity and p-value. Touch imprint was found to be specific and sensitive technique with specificity of 100% and

sensitivity of 81.5% and an overall accuracy of 90.75% with 53 true positive cases in agreement with the 65 positive histopathology results and 4 true negative in agreement with histopathology.

Touch imprint cytology has a overall accuracy of 90.75% and with a kappa level agreement of 53% and a p-value of 0.033 which is statistically significant. Touch imprint cytology is therefore a sensitive technique which can be useful in screening and reliable where rapid preliminary diagnosis is needed in the surgical management; it is also an affordable technique for developing countries with limited resource.

6.2 Recommendation

We recommend sampling from multiple sites should be applied while preparing touch imprints to increase harvesting of malignant cells from biopsies.

Touch imprint cytology requires practice and experience and should be adopted in pathology routine work to enable technical staff develop and acquire expertise.

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APPENDICES

Appendix I: Client consent information forms

Title: **The utility of touch imprint cytology in histopathology diagnosis of oral squamous cell carcinoma**

My name is Alice Kangogo Limo a Masters student at the Jomo Kenyatta University of Agriculture and Technology in the department of Pathology and Laboratory Medicine. I would like to conduct a study on tissue sample that will be excised from you by the surgeon for diagnosis. Kindly read the information below and feel free to ask any questions or you can request me to read and explain to you if you are unable to read. After understanding the information I will then request you to participate in this study if you are willing.

Introduction: There is limited study conducted on the use of cytology alongside histopathology preparation in diagnostic process where rapid preliminary diagnosis may help in surgical management.

Purpose: The purpose of this study is to evaluate the use of cytology in diagnosis alongside histopathology diagnosis of surgically excised specimens. This would asses if cytology can assist in rapid diagnosis and thus facilitate individualized treatment. The sensitivity and specificity of Cytology diagnosis in comparison with Histopathology diagnosis will provide information on the accuracy of cytology application in Head and neck lesions. It can promote interpretation of cytology smears and its histological correlation and provide more information on the role of cytology in diagnosing oral squamous cell carcinoma.

Procedure: The investigator will acquire cytology imprint by touching tissue excised from you on a slide; the slide will then be preserved and processed using cytology techniques. It will then be examined by investigators and two pathologists alongside the tissue sample excised.

Benefits: This research project will hopefully assist in improving diagnosis and management patients undergoing oral surgery by evaluating the usefulness of cytology in diagnosing oral lesions.

Risks and inconveniences: There are no risks or inconveniences to you or on processing of your sample. No extra payment will be required for cytology analysis.

Voluntarism: Your participation in this study is voluntary. Declining to participate will by no means affect the services you are seeking for. Therefore, upon reading the above information feel free to participate or not to without any consequences.

Do you have any questions? 1Yes_____ No _____

If yes I will clarify them to you.

Do you agree to participate in the study? Yes _____ No _____

CONSENT

Declaration by patient

I……..have read/have been read for and understood the purpose of the study, procedure, the benefits of the study and I have agreed to participate without force or coercion of any kind.

Thumb print/signature: ……... Date: ……..

Witness name: ……Signature: ……. Date: …….

Study, contact:

Research (principal investigator): Alice Kangogo Limo

Jomo Kenyatta University of Agriculture and Technology

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If you have questions about your rights as part of this research project, contact:

KNH/UON Ethical Research Committee chairperson

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Appendix II: Papanicolou Staining Technique

Principle: Papanicolaou stain contains both acidic and basic dyes. Acidic dye stains the basic components of the cell and basic dye stain the acidic components of the cell. Hematoxylin stains the nuclei blue by dye lake formation. The eosin azure solution being acidic stains the cytoplasm which is basic so that the eosin has affinity for the mature cells while light green has affinity for the young cells. Orange G also being an acidic dye has an affinity for the cytoplasm and stains keratin.

Materials:

- Coplin jars
- Staining dishes
- Slide staining racks
- Mounting media
- Coverslips
- Microscopic slides
- Staining solutions

Procedure

1. Dip in 95% ethyl alcohol for ten minutes.
2. Running water for 10 seconds.
3. Stain in Harris hematoxylin for 1-6 minutes.
4. Differentiate in Scotts tap water substitute for 30 seconds.
5. Rinse in running water for 10 seconds.
6. Dip in 70% ethyl alcohol for 10 seconds/ 10 dips.
7. Dip in 95% ethyl alcohol for ten seconds.
8. Stain with OG-6 for 1 minute.
9. Rinse in tow changes of 95% ethyl ten dips/ seconds each.
10. Stain in Modified EA, EA-50 or EA-65 for three minutes.
11. Rinse in three changes of 95% ethyl alcohol for ten seconds each.

12. Rise in three changes of Absolute alcohol for three changes each.
13. Clear with three changes of xylene one minute in each change.
14. Mount in DPX.

Results Interpretation

Nuclei -------Blue

Acidophilic cells ------Red

Basophilic cells ------Blue Green

Erythrocytes --------Orange-red

Keratin -----Orange-red

Superficial cells -------Pink

Intermediate and Parabasal Cells -----Blue Green

Eosinophil ------Orange Red

Appendix III: Tissue Processing

As soon as tissues are biopsied they are preserved in a life-like manner in 10% buffered formalin for 24 hours in a ratio of 1:10 of tissue to fixative. The tissue is then described macroscopically and trimmed by pathologist. The trimmed tissue is then placed in labeled embedding ring for tissue processing explained below.

Principle: Transfer of tissues in container within a basket through a series of stationary reagents arranged in-line or in a circular carousel to allow the diffusion of various substances into and out of porous tissues. Diffusion results from the tendency of processing reagents to equalize concentrations both inside and outside blocks of tissue. The reagent molecules diffuse down a concentration gradient and move from where they are at a high concentration to where they are at a lower concentration.

Materials:

- Tissue processor
- Microtome
- Knife
- Ruler
- HB pencil

Procedure:

1. There are 10-12 reagent stations with temperatures adjustable between 30-45°C, 3-4 paraffin wax stations with variable temperature settings between 48-68°C, and vacuum-pressure options for each station and agitation is achieved by tidal action.
2. The first stage in tissue processing is dehydration (the removal of water). In tissues, water is present in both free and bound forms and needs to be removed

before processing can continue. Dehydration is usually carried though ascending grades of alcohol, 70%. 80%. 95% and three changes of absolute alcohol.

3. Clearing is the next step where tissues are processed through three changes of chloroform which is a clearing agent this is necessary since dehydrants (alcohols) are immiscible with paraffin wax. Clearing agent has a high refractive index thus rendering tissue transparent.
4. Infiltration is the next step, tissue are saturated with molten paraffin wax in a vacuum reduced pressure. This is the saturation of tissue cavities and cells by a supporting substance which is generally the medium in which they are finally embedded.
5. Paraffin embedding is done next it involves surrounding the tissues by a medium such as paraffin wax which when cooled and solidified will provide sufficient support for section cutting or microtomy.
6. Sections of 4-5um are made and placed on labeled slides by fishing method.
7. Once dry the sections are dewaxed to remove excess wax in section in a oven of 60^0c for two hours.
8. The sections are left to cool before staining in hematoxylin and eosin technique.

Appendix IV: Manual hematoxylin and eosin staining procedure for formalin fixed paraffin embedded tissues

Principle: Alum acts as mordant and hematoxylin containing alum stains the nucleus light blue. This turns red in presence of acid, as differentiation is achieved by treating the tissue with acid solution. Bluing step converts the initial soluble red color within the nucleus to an insoluble blue color. The counterstaining is done by using eosin which imparts pink color to the cytoplasm.

Materials:

- Staining jars
- Staining rack
- Microscopic slides
- Microscopic coverslips
- Staining reagents

Procedure

1. Dip slides in one change in of xylene each for 5 minutes.
2. Two changes in xylene for 2 seconds each.
3. Dip in two changes of absolute alcohol for 10 seconds each.
4. Dip in one change of 95% Ethanol for 10 seconds.
5. Dip in one change of 75% ethanol for 10 seconds.
6. Rinse in water for two minutes.
7. Stain in Harris Hematoxylin for 10minutes.
8. Rinse in tap water.
9. Differentiate in 0.5% acid/alcohol two dips.
10. Rinse in blue in Scott's tap water for 5 minutes till slides turn blue.
11. Counter stain with 0.5% alcoholic Eosin for 2 minutes.

12. Dehydrate in ascending grades of alcohol, 70%, 95% and two changes of absolute alcohol for ten seconds each.
13. Clear in three changes of xylene ten seconds each
14. Mount and cover slip using DPX.

Interpretation

Nuclei ---- blue, black

Cytoplasm ---Pink

Muscle fibres ---deep red

RBCs ---orange red

Fibrin ----deep pink

Appendix V: Cytology Report Form

Social Demographic Data	
Patient hospital number	
Study number	
Age	
Sex	
Site	
Surgeon	
Date	

Cytology Specimen assessment

Adequacy Satisfactory □

Unsatisfactory □ Reasons:

……

Negative for malignancy □

Suspicious□

Inflammatory □

ASC-US □ LSIL □

ASC – H □ HSIL □

SCC □

□ Others, Specify…….

Principal investigator's name……

Sign……..

Pathologist's name……

Sign……. Date**……**

Appendix VI: Histopathology Report Form

Social Demographic Data	
Patient hospital number	
Study number	
Age	
Sex	
Site	
Surgeon	
Date	

History**:**

Histopathology Report:

Principal investigator's name……

Sign……..

Pathologist's name……

Sign……Date**……**

Appendix VII: Ethical Approval

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Ref: KNH-ERC/A/324

11th October 2013

Alice Kangogo Limo
TM 300-1180/2012
Medical Laboratory Sciences Dept.
JKUAT

Dear Alice

RESEARCH PROPOSAL: THE UTILITY OF TOUCH IMPRINT CYTOLOGY IN HISTOPATHOLOGY DIAGNOSIS OF ORAL SQUAMOUS CELL CARCINOMA (P399/07/2013)

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 11th October 2013 to 10th October 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.**

"Protect to Discover"

Appendix VIII: Publication

AJOHS 2019V (6) 1 African Journal of Oral Health Sciences

THE UTILITY OF TOUCH IMPRINT CYTOLOGY IN HISTOPATHOLOGY DIAGNOSIS OF ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is an aggressive neoplasm with limited therapeutic options and poor survival rates. Assessment of surgical margins in OSCC during intra-operative procedure is important and influences its local recurrence, patient's survival rates, management and overall prognosis. Currently histopathology technique is the gold standard diagnostic technique; it is reliable but time consuming while touch imprint cytology is cheap and quick technique that can be useful where rapid results are required

Aim: The objective of this study was to evaluate the utility of touch imprint cytology in assessing OSCC.

Methods: A descriptive cross-sectional study of 73 participants with suspected oral squamous cell carcinoma and on analysis, 66 of the samples were diagnostic. Touch imprints were done and results were compared with histopathology. Data was analyzed using SPSS version 17.0 and descriptive summary statistics done. The level of agreement between the diagnostic techniques was calculated using the chi-square test and kappa statistics. A p value less than 0.05 was considered statistically significant.

Results: The most participants were male (69.7%) and the highest age being 50-59 years. The most common site of lesion was tongue (43.9%) and cytological pattern most demonstrated was a singly dispersed cell pattern. There were 53 true positives, 12 false negatives and 1 true negative and no false positive. The sensitivity, specificity, positive predictive value, negative predictive value and the overall accuracy were 100%, 7.7%, 81.0%, 100% respectively with an overall accuracy of 53.85% and p-value of 0.033 which is statistically significant.

Conclusion: Touch imprint cytology is a sensitive technique that can be useful in screening and reliable where rapid preliminary diagnosis is needed; it is also affordable, hence useful in resources challenged settings.

Key words: Touch imprint cytology, Histopathology

Background

Oral Squamous Cell Carcinoma (OSCC) is one of the major leading and the most frequently occurring malignant tumor of the oral structures, accounting for about 90% of the malignant oral lesions. Epidemiological studies have shown that the incidence of oral cancer varies significantly among the continents and within developed and developing countries [2,3] .The dental practitioner plays a particularly important role in the detection of oral cancer in its early stages when treatment is most effective and morbidity minimal.[10] The ability to deliver an immediate diagnosis by cytology makes it an important part of management especially in places where frozen section facility is not available. In utilization of cytology as an intra-operative procedure the most important indication is to establish or confirm diagnosis rapidly, this is an important aspect of surgical pathology, though; rapidity of preparation should not be at the expense of accuracy[11]. Currently diagnosis is done using histopathology technique which is considered definitive; however, histo-

pathology is time consuming and may not be useful in intra-operative consultation where quick decisions are required. The frozen section for intra operative consultation has been widely used however it employs expensive equipment's which is not affordable especially in developing countries like Kenya. There is need for cheaper and less time consuming technique which can aid in intra operative consultation. Touch imprint cytology has an advantage due to the relative ease of performing the procedure, it is non-invasive, and is a rapid procedure that does not alter tissue and according to a study done[4] provides excellent cytological clarity[4]. Cytology as a technique is applicable since it is possible to assess tumor cells that are generally characterized by reduced cohesiveness which increase their presence in tissue fluid thus selectively enriching the surface with detached tumor cells giving a unique source for cytological analysis [9].

This study aims to evaluate the utility of touch imprint cytology of surgically excised tissue for rapid diagnosis and to determine the sensitivity, specificity, positive and negative predictive values as well as correlate cytological and histological patterns of these lesions. The study will provide additional data on intra operative techniques and hopefully provide affordable alternative that can be applied for intra operative assessment.

Methodology:

A descriptive cross-sectional study of 73 participants with suspected oral squamous cell carcinoma was conducted, whereby on analysis 66 of the samples were diagnostic. All patients who underwent surgical procedure and gave consent to participate in the study were selected consecutively until the sample size was achieved. Demographic characteristics were captured using a predesigned questionnaire. Study samples from consenting patients, were obtained from excised biopsy tissue by oral surgeons and imprint made directly carefully sampling the site of lesion to produce maximum number of diagnostic cells.

The imprint cytology sample was obtained by holding fresh biopsy sample using forceps blotting firmly on tissue paper to remove excess blood and then gently touching the tissue site on a pre-labeled microscope slide while avoiding any gliding movement. The two touch imprint slides per patient were prepared and immediately immerse in 95% ethyl alcohol to avoid drying which can alter cellular morphology. The stained cytological slides were examined by principal investigator together with the supervising pathologists and compared with histopathology diagnosis which is the gold standard technique.

Ethical consideration

Approval to carry out the research was obtained from the University of Nairobi/Kenyatta national Hospital (UON/KNH) Ethics and research committee before commencement of the study. The participants were carefully taken through the consent process and a signed consent was filled before they participated in the study. The information obtained from participants was kept strictly confidential.

Study Site

The study was conducted at the University of Nairobi, department of oral and maxillofacial surgery in the oral pathology laboratory. This is a diagnostic laboratory for head and neck samples and receives samples from all over the country.

Study population

Oral tissue samples from 66 participants with suspected oral squamous cell carcinoma who gave consent to participate in the study were used.

Inclusion criteria

All fresh unfixed samples of suspected Oral squamous cell carcinoma.

Exclusion criteria

Samples from normal, inflammatory lesions, benign lesions and those with confirmed diagnosis of oral squamous cell carcinoma was not included in the study.

Laboratory procedure

Standard operating procedures of KNH/UON were adhered to those pertaining to labeling of slides, specimen collection, transportation, analyzing data and posting of results. Cytology touch imprint were obtained by pressing two pre-labeled glass slide gently on the freshly cut surgical tissue specimen as already been described. The biopsied tissue sample of the suspected OSCC was fixed in 10% buffered formalin and then subjected to histopathology processing during which 4µm thick tissue sections were stained using Hematoxylin and Eosin technique. The touch imprint cytology was first read followed by histopathology report by both the principle investigator and by three blinded pathologists differently; the results were compared with histopathology diagnosis as a control. Cytology results were evaluated

as; Malignant (SCC), suspicious for malignancy, negative for malignancy and Non-diagnostic were excluded from the study; this included those with scant cellularity, air drying or distortion artifact, obscuring blood.

Data analysis

The cytological findings were compared to histological findings and data was analyzed using SPSS version 17.0. Descriptive summary statistics was done and presented as proportions and percentages in the form of tables and charts where relevant. The level of agreement between the diagnostic techniques, namely the Touch Imprint Cytology and Hematoxylin & Eosin was calculated using the chi-square test and kappa statistics. A p value less than0.05 was considered statistically significant.

Results

Demographic characteristics

A total of 73 were samples were taken but only 66 were satisfactory for this study. The majority of the participants were males comprising of 46(69.7%) and female comprised 20(30.3%) which is in ratio of 2:1: The highest age range was 50-59 years consisting of 17 (25.5%) and the highest site of occurrence was tongue with 29 samples (43.9%). The cytological pattern most demonstrated by imprint cytology was singly dispersed pattern comprising 23 (34.8%).

Histopathology and cytopathology results

Out of 66 cytology samples examined 45 (68.2%) were diagnosed as malignant, 8 (12.1%) as suspicious and 13(19.7%) were negative. Out of the 45(68.2%) malignant cases; 26 (39.4 %) were diagnosed as SCC, 7(10.6%) as MDSCC and WDSCC each, and 5 (7.6%) as PSCC. While out of the 66 histopathology samples 41 (62.1%) were WDSCC, 21 (31.8%) were PDSCC, 3(4.5%) were MDSCC and 1(15%) negative. Therefore in comparison with histopathology, there were 53 true positives, 12 false positive, 1 true negative and 0 false negative results when imprint cytology was compared with histology in a 2 X 2 table.

TIC Photomicrographs demonstrating cytomorphological patterns and cytological diagnosis

TIC slides showed variety of cells some singly dispersed, in clusters. Others obscured by blood artifact, while others with normal cells. (figure 4.7.1, figure4.7.2, figure 4.7.3 and figure4.7.3.

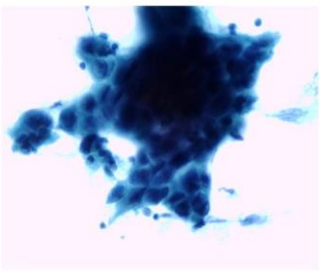

Figure .1: Photomicrograph of clustered pattern of poorly differentiated SCC(x400)

Table 1: Cytopathology versus Histopathology results

			Total
	Positive for SCC	Negative for SCC	
Positive for SCC	53	12	65
Histology results	0	1	1
Negative for SCC	53	13	66
Total			

Table 2: Sensitivity, specificity, PPV, NPV and p value

Sensitivity	100%
Specificity	7.7%
Positive predictive value	81%
Negative predictive value	100%
p-value	0.033

The sensitivity, specificity, PPV and NPV were 100%, 7.7%, 81% and 100% respectively and a p-value of 0.033.

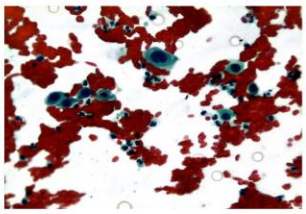

Figure 2: photomicrograph of TIC Show singly dispersed cells suspicious of SCC with overlying blood artifact. (x400)

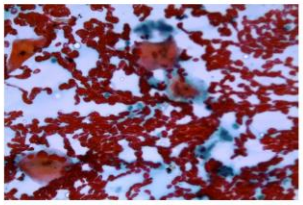

Figure 3: TIC showing hemorrhagic imprint with underlying cells.

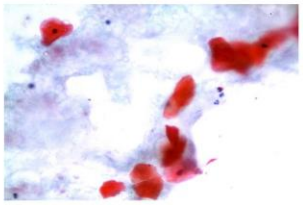

Figure 4: TIC showing normal looking squamous cells.

DISCUSSION

Oral squamous cell carcinoma is an aggressive malignant neoplasm with high morbidity and mortality rates whose therapy, when advanced, requires disfiguring surgical procedures that should accomplish complete excision of the malignant lesion[1]. Intra-operative consultation is useful in providing information that can assist in immediate therapeutic decision during surgical procedure; it can also assist to assess tumour margins and adequacy of specimen and also the extent of spread of a tumor during surgical procedure. This can assist surgeons ascertain of clear margins during surgery of malignant cases and thus prevent recurrence or spread of tumor.

Touch imprint cytology is a simple, cheap and quick way of assessing presence of malignancy during intra operative analysis. For decades touch imprint cytology has been used as a diagnostic tool for breast cancer[24] .Touch imprint cytology have also been used as an adjunct to assess adequacy of samples that were obtained by CT guided or ultrasound, this reduced the time a patient was exposed to radiology [17,18]. The only challenge encountered in touch imprint is its ability to differentiating invasive from insitu samples[16].

Frozen section which is currently being used for intra operative consultation in resourceful countries has its limitation due to interpretational difficulties caused by freezing artifacts; it is expensive and needs specialized personnel. This is not suitable for resource limited countries contrary to touch imprint which is cheap, quick and excellent cellular clarity and does not require expertise in machinery to use [23].

A total of 73 were collected to evaluate the diagnostic accuracy of touch imprint cytology in comparison to histopathology which is the gold standard technique. Only 66 were assessed in this study since others were non diagnostic. The majority of the participants were male 46 (69.7%) translating to 2.1: 1 for M: F ratio. Other studies show similar male predominance [11]. Smoking is the most significant implicated etiological factor of OSCC and combined with use of alcohol greatly increases the risk of acquiring OSCC[20]. Kenyan National authority for campaign against alcohol and drug abuse (NACADA) data in the year 2017 indicates that 16.8% male are users of tobacco in comparison to 2.1% females.

The majority of patients were in the age range 50-59years comprising of 17 patients and the least was age range 10-19 years and 20-29 years comprising of 1 patient each. This is comparable to a study done by Mehorta who found the majority of patients being in age range 50-59years[15].

The majority of samples were obtained from tongue 29(43.9%), while there is a study that found buccal mucosa to be most common followed by tongue then hard palate[11].

The sensitivity and specificity of touch imprint cytology for squamous cell carcinoma in our study was 100% and 7.7% respectively. A study on malignancy of upper aero digestive tract found sensitivity and specificity of touch imprint cytology to be 96% and 100% respectively[11] while another study found sensitivity and specificity to be 92.3% and 94.4% respectively[14]. These findings indicate that TIC is a good screening tool that should be useful intra operative procedure where rapid results are required for effective surgical management. Histopathology is still important as a confirmation report.

The Positive Predictive Value and Negative Predictive Value were 81.0%, 100% in this study,

while another study found PPV and NPV of 96% and 89.5% respectively[14].

The overall accuracy in this study was 53.85% while a study done elsewhere had an overall accuracy of 83%[15]. We found that TIC of OSCC showed accuracy due to highest number of 53 true positive cases to 12 cases of false positive that agreed with gold standard technique which is histopathology.

There is statistical significance in the results with a p-value of 0,033 which is significant at 5% level of significance being $P<0.05$. There is an agreement between histology and TIC as a diagnostic tool which can be useful in intra operative consultation.

CONCLUSION

Touch imprint cytology is therefore a sensitive technique which can be useful in screening and reliable where rapid preliminary diagnosis is needed in the surgical management; it is also an affordable technique for developing countries with limited resources.

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