CHAPTER ONE

1-1Introduction

Nasopharyngeal carcinoma (NPC) is a cancer originating in the nasopharynx, the uppermost region of the pharynx or "throat", where the nasal passages and auditory tubes join the remainder of the upper respiratory tract. NPC differs significantly from other cancers of the head and neck in its occurrence, causes, clinical behavior, and treatment. It is vastly more common in certain regions of East Asia and Africa than elsewhere, with viral, dietary and genetic factors implicated in its causation (Pingpin Wei,2014).

Nasopharyngeal cancer, is classified as Nasopharyngeal carcinoma a malignant neoplasm, or cancer, arising from the mucosalepithelium of the nasopharynx, most often within the lateral nasopharyngeal recess. There are three types of NPC moderately-differentiated non keratinizing type, and an undifferentiated type, which typically contains large numbers of non-cancerous lymphocytes, thus giving rise to the name lymphoepithelioma. The undifferentiated form is most common, and is most strongly associated with Epstein-Barr virus infection of the cancerous cells (Hildesheim and Wang, 2012; IARC, 2014).

NPC is uncommon in the United States and most other nations, but is extremely common in southern regions of China (Serhat,.2010). Particularly in Guangdong accounting for 18% of all cancers in China (Srinivasan,2013). It is sometimes referred to as Cantonese cancer because it occurs in about 25 cases per 100,000 people in this region, 25 times higher than the rest of the world (Hu, 2006). It is also quite common in Taiwan. This could be due to the South East Asian diet which typically includes consumption of salted vegetables, fish and meat (Kietly,2002). While NPC is seen primarily in middle-aged persons in Asia,

a high proportion of African cases appear in children. The cause of increased risk for NPC in these endemic regions is not entirely clears (Ushiki, 2002). With the proximity of Sudan to the North African country of Morocco whereby the study revealed that EBV is commonly associated with NPC in Moroccan patients and that NPC tumors from Moroccan patients harbor high-risk HPV genotypes. Thus, NPC in Sudan may not been a unique case within the Africa (Laantri*et al.*, 2011).

In Sudan, there had been cases or reports describing the pathology of NPC in Sudan (El Hassan ,*et al.* 2011) they observed that the EBV is strongly associated with nasopharyngeal cancinoma at a frequency comparable to that in countries with intermediate degree of endemicity for the tumour.

During the past 30 years it has become exceedingly apparent that several viruses play significant roles in the multistage development of human neoplasms, in fact approximately 15% to 20% of cancers are associated with viral infections (ZurHausen, 2001; Parkin, 2006). Oncogenic viruses can contribute to different steps of the carcinogenic process, and the association of a virus with a given cancer can be anywhere from 15% to 100% (Parkin, 2006). In addition to elucidating the etiology of several human cancers, the study of oncogenic viruses has been invaluable to the discovery and analysis of key cellular pathways that are commonly rendered dysfunctional during carcinogenesis in general(McLaughlin-Drubin and Munger, 2008).

In Sudan Cancer Registry (SCR) Three hundred and seventy-four cases of nasopharyngeal carcinoma (NPC) and 512 cases seen at the Radiation and Isotope Centre, Khartoum (RICK) were analysed. NPC formed 5.8% of all cancer cases in the SCR and 7.2% at the RICK; this is the highest frequency so far reported outside the Chinese. The male/female ratio was 3:1; NPC was the commonest tumour in males at the RICK (12.1%) and second commonest in the SCR (9.2%). It tended to occur in younger patients (youngest, 3 *yr*), with 14 and 12.1% of cases in

children 14 *yr* or under in the SCR and RICK respectively; it is the commonest childhood malignancy in the Sudan (hedayatallah ,2012)

There are many techniques help in diagnosis of nasopharyngeal carcinomaincluding histopathology, immunofluorescent, immunohistochemistry, instuhybridization and DNA micro array (Hoffrand, 2001).

Diagnosing nasopharyngeal carcinoma usually begins with a general examination; include observation of early signs and symptoms of nasopharyngeal carcinoma. It may take months of investigating other avenues before a definitive diagnosis is made. Also the use the endoscope or another instrument to take a biopsy to be tested for cancer. Beyond diagnosing nasopharyngeal cancer, a biopsy also describes the type of nasopharyngeal carcinoma (Hoffrand, 2001).

Detection of nasopharyngeal carcinoma can be easly by the technics of thePolymerase Chain Reaction (PCR)& Immunohistochemistry and detict the relationship between the cancer and viruses(Bartlett, 2003).

Polymerase chain reaction (PCR) : Is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations (Bartlett and Stirling, 2003).

Immunohistochemistry:

Is the technique for identifying cellular or tissue constituents (antigen) by means of antigen- antibody interactions. The site of antibody being identified either by direct labeling of the antibody, or by use of secondary labeling method (Ramos-Vara, 2005).

The technique can be helpful in tumor diagnosis, predicting disease prognosis, and directing its plan of therapy (Helal, 2005).

Avidin biotin technique which is applied in this study is one of indirect methods depend on the affinity of avidin to biotin. Avidin is a glycoprotein extracted from streptoavidini, which have a four identical subunit binds one molecules of biotin.

Epstein Barr Virus (EBV) is tightly associated with different human cancer, and several malignancies as Burkett's lymphoma, Hodkgins lymphoma and nasopharyngeal carcinoma (IARC, 1997).

Cytomegalovirus (CMV) is a common infection that is usually harmless. Once CMV is in a person's body, it stays there for life. Among every 100 adults in the United States, 50–80 are infected with CMV by the time they are 40 years old (Hu, et al. 2006).

Herpes simplex virus (HSV) also known as human herpesvirus 1 and 2 are two members of the herpesvirus family, Herpesviridae, that infect humans. Both HSV-1 (which produces most cold sores) and HSV-2 (which produces most genital herpes) are ubiquitous and contagious. They can be spread when an infected person is producing and shedding the virus. Herpes simplex can be spread through contact with saliva, such as sharing drinks(Ryan and Ray 2004).

1-2 Objectives

General Objective

To detect Immunohistocchemiccal and Molecular Detiction of Keratin and Epithelial Membrane Antigen, Cytomegalovirus, Epstein Barr Virus and Herpes Simplex Virus among Sudanese Patients with Nasopharyngeal Carcinoma.

Specific objectives:

- Todetect Keratin and Epithelial Membrane Antigenusing immunohistochemistry technique.
- To corelate between EBV , CMVand HSV infection and nasopharyngeal carcinoma types among Sudanese patients using PCR techniques.
- To find out (if any) association between EBV, CMV and HSV with other demographic factors.
- To correlate betweenKeratinand Epithelial Membrane Antigen and and nasopharyngeal carcinoma types among Sudanese patients using immunohistochemistry.

1-3 Rationale

The numbers of new cases with (NPC) is increasing each year in the Sudan and globally. Although, the etiology of NPC is well established in many countries but still obscure in Sudan. Therefore, this study was done to detect validity of (Keratin and EMA) tumor markers in differential diagnosis of NPC . Furthermore screening Sudanese patients with NPC for possible EBV , CMV and HSV association, since these viruses have potential risk in NPC. There is a need for focused researches to unravel the causes of nasopharyngeal carcinoma.

Chapter Two

Review of Literature

2-1 Scientific background:

NPC is fairly rare in most parts of the world. The number of nasopharyngeal carcinoma in Sudan increased due to the increased exposure to carcinogenic factors. Smoking and other tobacco use are associated with about 75 % of (NPC) cases; tobacco causes irritation of the mucous membranes of the mouth& nasal cavity (Abuidris, *et al*. 2008).

This cancer is, however, much more common in certain parts of Asia and North Africa, particularly in Southeast China. It is also more common among Inuits of Alaska and Canada, and among some immigrant groups in the United States, such as recent Chinese and Hmong immigrants (Elbeshir, et al. 2001; and Idris, et al. 1995).

Abuidris, *et al*. (2008) stated that infection by high-risk human papillomaviruses and Epstein-Barr virus are very frequent in the adult human population, and have been associated with several human carcinomas, especially NPC.

In Morocco an investigation of the presence of EBV and HPV in Moroccan patients with nasopharyngeal carcinoma, was conducted using the polymerase chain reaction (PCR). Biopsies of nasopharyngeal carcinoma were obtained from 70 patients, Sixty two cases (88.9%) were classified as type 3 (undifferentiated carcinoma), 6 (8.6%) as type 2 (non keratinizing NPC) and only 2 (2.9%) cases were classified as type 1 (keratinizing NPC). EBV was detected in all NPC tumors, whereas HPV DNA was revealed in 34% of cases (24/70). Molecular analysis showed that 20.8 % (5/24) were infected with HPV31, and the remaining were

infected with other Oncogenic types (i.e., HPV59, 16, 18, 33, 35 and 45). In addition, statistical analysis showed that there is no association between sex or age and HPV infection. Where as their data indicated that EBV is commonly associated with NPC in Moroccan patients and showed for the first time that NPC tumors from Moroccan patients harbor high risk HPV genotypes (Duensing et al, 2001).

Lutzky et al. (2008) urges that NPC is a "human malignancy derived from the epithelium of the nasopharyngeal cavity." He further notes that the carcinoma is considered to be one of the most "striking examples of a human malignancy" which is connected with the virus. Nasopharyngeal carcinoma cells contain the EBV genome and they encode the viral proteins which contribute to the development of a malignant phenotype. The disease is much remarkable and noted in some parts of the world with certain geographical factors and some demographic issues of distribution.

According to the *American Cancer Society* (2013), NPCis a type of cancer which "starts in the nasopharynx, the upper part of the throat behind the nose and near the base of the skull."

NPC is an endemic in southern Asia, southern China, and North Africa, although it is a rare epidemic in most parts of the world (Parkin et al. 2005). One of the highest incidence that witnessed NPC greatly was in the central region of the Guangdong Province in southern China as well as in Hong Kong, which recorded about 25-30 per 100,000 individuals per year (Blot et al. 1988). The disease is relatively not frequent in some western parts (Lutzky*et al*., 2008).

Zeka, Gore, and Kriebel (2003) point out that, even though patients within stages I and II diagnosed, they are having a high chance of the disease being cured with the assist of radiotheraphy (RT) alone, whereas those diagnosed with stages

III and IV, of whose they are the most locally advanced diagnosis are much more distanced and its prevention and treatment remains not clearly understood and poor. This poor result and outcomes were mostly being held accountable as a result of increased toxicity of the concurrent chemoradiation of the ordinary tissue, thus making most of the patients to become unmanageable only through the chemotherapy process which will take a duration of time (Sugano et al., 1978). Thus, the need for an urgent need to look for an innovative biological agent which will potentiate the efficiency and lastly overcome chemotherapy resistance while not exacerbating the toxicity. Approximately 30% to 40% of the patients with advanced stage NPC develop local recurrences and metastatic disease. The prognosis for patients with metastatic disease is poor, with a median survival of less than 12 months (Lutzky*et al*., 2008).

2.2. Histology of Nasopharynx :

2.1 Anterior wall

Extends from the choanae or posterior nares to include the incomplete floor which is formed by the postero-superior surface of the soft palate. (Wood Jones ,2003).

2.2Posterior wall (including roof).

A continuous arched wall with the roof extending from the superior margin of the choanae caudally to the level of the free border of the soft palate (Frazer, 2003).

2.3 Lateral walls

There, the nasopharynx communicates with the right and the left tympanic cavities by the Eustachian tubes. The pharyngeal ostium of the auditory tube shows prominence of its posterior lip and adjacent to that is the pharyngeal recess or fossa

of Rosenmiiller. Passing directly downwards from the posterior lip is the salpingopharvingeal fold (Frazer, 2003).

2.3.1Intermediate or transitional epithelium

This epithelium has similar morphological features to the transitional epithelium of the urinary tract; it has five or six cell layers and no cilia. The deepest layer of (UICC, 2001). Cells has a cuboidal or at times a columnar shape, followed by polyhedral cells and a superficial row of rounded cells. There are no intercellular bridges and no flattening of the superficial layers (Bryant, 2003).

The transitional or intermediate epithelium had some features similar to those of the transitional epithelium of the urinary system. The stratified nature of this epithelium was shown clearly in contrast to that of the pseudo-stratified ciliated variety (Friedmann, 2001).

However, its nuclei showed vertical alignment similar to that of the ciliated epithelium but were easily differentiated, even under the low power, from the flattened cells of the squamous epithelium. There were also no cilia and nointercellular bridges. Although histologically separable from the squamousepithelium, the cells of the intermediate epithelium contained keratohyalin and occasionally keratin. An aggregate of intermediate epithelium formed a wavy ring separating the nasophargyx from the oropharynx. The lateral walls also contained numerous islets of the intermediate epithelium. Similar distribution of the intermediate epithelium was seen in the mucous lining of the pharyngeal tonsil in the posterior wall (Adams, 1998).

2.3.2Pigmentation in the mucosa

The basal layer of the epithelium showed dark brown pigment. The pigment was found in patches of the squamous, ciliated and intermediate epithelia. The pigmented areas of the epithelium were confined to the posterior and lateral walls. Although most of the pigment granules were condensed in the basal layer, the ciliated and intermediate epithelia showed some pigment even amongst the upper layers of cells.

From individuals of Chinese origin showed pigmentation while fourtes and Malays (that is 41 % of the specimens from Indians and Malays). None of the Europeans showed pigmented epithelium (Johnson, 1999).

The tunica propria of the nasopharynx had a framework of collagenous and elasticfibres and numerous blood and lymphatic vessels. By the use of a modified Dominici stain (Ali, 1994). large numbers of mast cells were found throughout the tunica propria. Mixed mucous and serous secreting glands were found in the submucosal space and occasionally deeper to the muscle bundles.

Three pharyngeal tonsils were formed by the accumulation of lymphoid tissue in the median dorsal wall and within, or close to, the orifices of the Eustachian tubes. **(**Hoffrand, 2001).

2.4 Definition of NPC :

Nasopharyngeal carcinoma (NPC) is a malignant tumor of nasopharyngeal epithelium. It is the maintype in nasopharyngeal malignant tumors in both endemic areas and regions with low incidence. Epidemiological studies in NPC with the focus on etiology and biological behavior of the disease werestrongly encouraged as a result of the International Union against Cancer (UICC) Symposium on Cancer of Nasopharynx held in Singapore in 1964 (Muir et al. 1997), and

investigations in the past four decadeshave produced many important fi ndings in those aspects. NPC has unique epidemiological features,including obvious regional, racial, and familial aggregation. The aim of this chapter is to detail the incidenceand distribution of NPC, as well as risk factorsof the development of the disease.

2.5 Epidemiology of NPC:

Nasopharyngeal cancer is a type of tumor with extremely unbalanced endemic distribution. It can be seen in many countries and areas of the fi ve continents.

However, the incidence of NPC is lower than 1/105 in most areas. High-incidence areas are centralized in the southern part of China (including Hongkong). The highest incidence is found in Guangdong province, and the incidence in male canreach 20–50/100000. According to the data of International Agency for Research on Cancer (IARC),approximately 80,000 cases of NPC were newly diagnosed worldwide in 2002, and about 50,000 cases deceased, with Chinese accounting for 40%. Intermediaterates were seen in local inhabitants of Southeast Asia, Eskimos from the Arctic area, and inhabitants from North Africa and the Middle East (Parkin et al. 1992, 2002; Waterhouse et al. 1982; Muir et al. 1987).There is prominent difference in the incidence of NPC between the Northern and Southern parts of China. High-incidence areas are centralized in fi ve Southern provinces (Guangdong, Guangxi, Hunan, Fujian, Jiangxi). The incidence is highest in Guangdong province, so NPC is also called “Canton tumor.” Within Guangdong province, the Pearl River delta and Xijiang River basin, especially Zhaoqing, Foshan, and Guangzhou, form a high-incidence core region (Jia et al. 2005).

2.6 Etiology of NPC :

In endemic regions, NPC presents as a complex diseasecaused by an interaction of the oncogenic gammaherpesvirusEBV chronic infection, environmental,and genetic factors, in a multistep carcinogenic process.The highest

incidence of NPC in SouthernChinese strongly indicates that both genetic susceptibility andenvironmental factors contribute to thetumorigenesis of NPC in its development and progression.In addition, a small population of cellssharing properties of normal stem cells (NSC) withintumor has been suggested to be involved in the etiologyof NPC. This chapter will focus mainly on threemajor etiological factors including genetic, environmental,and viral factors. (Jia et al. 2004; Zeng et al. 2002).

2.7 Histopathological Typesof Nasopharyngeal Carcinoma NPC

Most cases of NPC can broadly be classified as nonkeratinizing and keratinizing. The nonkeratinizing group can further be separated into undifferentiatedcarcinoma and differentiated carcinoma group.

2.7.1 Undifferentiated Carcinoma

Undifferentiated carcinoma is the major histopathological type of NPC, although the exact percentage in different populations varies. In endemic populations, undifferentiated carcinoma takes up between 47% and 92% of all cases of NPC (Tan and Putti 2005). In a major series on a western (non-endemic) population, this subtype of NPC constituted only 44% of all NPCs (Al-Sarraf et al.1998). Undifferentiated carcinoma is characterized microscopically by tumor cells with spindle-to-oval vesicular or hyperchromatic nuclei bearing prominent nucleoli and which also feature scattered mitotic activity Variable numbers of intermixed lymphocytes and plasma cells are seen. There are two classically described patterns of growth: the "*Regaud*" pattern denotes a solid sheet-like tumor cell growth pattern, while the "*Schmincke*" pattern is typified by the presence of apparently separated or loosely attached tumor cells (sometimes described as a reticulated pattern) with prominent intermixed lymphocytes (Glanzmann et al. 2001). This latter pattern exemplify es the previous terms for NPC: "*lymphoepithelial carcinomaor lymphoepithelioma*" (*vide supra*). These histological patterns have no prognostic significance.

2.7.2 Differentiated Nonkeratinizing Carcinoma

Differentiated nonkeratinizing NPC is very similar histopathologically to undifferentiated carcinoma, except that the tumor cells have a stratified or pavemented arrangement with cell borders being readily discernable (Chan et al. 2005) .The tumor cells may have a plexiform arrangement, a growth pattern akin to that of transitional cell carcinoma of the urinary tract. In series from Singapore, this tumor subtype constitutes between 7% and 49% of cases of NPC (Shanmugaratnam et al. 1979; Tan and Putti 2005). Nonkeratinizing NPC can rarely have a papillary architecture with tumor epithelium covering fi brovascular tissue cores. Apart from histological similarities, differentiated nonkeratinizingNPC and undifferentiated NPC have comparable prognosis and their distinction is not thought to have clinical significance (Chan et al. 2005).

2.7.3 Keratinizing Squamous Cell Carcinoma

Keratinizing squamous cell carcinoma (SCC) is uncommon in NPC-endemic regions. In endemic areas such as Singapore, it constituted between 1% and 20% of all cases of NPC (Tan and Putti 2005). In contrast, the proportion of this subtype in non-endemic western populations has been reported in up to 67% of casesof NPC (Shedd et al. 1999). Histologically, the tumor shows prominent features of keratinization, including presence of squamous pearl formation and evidentintercellular bridges. The tumor islands infiltrate within a desmoplastic stroma and the tumor can be graded as well, moderately and poorly differentiated, as in SCCs of other body sites.This subtype of NPC has been described to have the most guarded prognosis of all NPC subtypes, probably contributed by its relative radioresistance(Weiland 1985; Wenig 2007). The 5-year survival is reportedly 20%–40% as compared with about 65% for nonkeratinizing NPC subtypes (Wenig 2007). *Basaloid SCC* is the most unusual variant of NPC with only a handful of cases reported in the literature (Wan et al. 1995; Müller et al. 2000). It is histologically very similar to other basaloid SCCs that occur in the rest of the

upper aerodigestive tract and displays tumor cells with hyperchromatic nuclei, increased nuclear–cytoplasmic ratio with peripheralpalisading of tumor nuclei.

The World Health Organization (WHO) classifies NPC into three histopathological types based on the degree of differentiation. Type 1, SCC, is seen in 5%–10% of cases of NPC and is characterized by well-differentiated cells that produce keratin and demonstrated the presence of intracellular bridges when observed under the electron microscope. Type 2, nonkeratinizing squamous carcinoma, varies in cell differentiation (from mature to anaplastic cells) but does not produce keratin. Type 3 or undifferentiated NPC constitutes the bulk of the tumors seen in patients with NPC, is also nonkeratinizing, but is less differentiated, with highly variable cell types (clear cell, spindle cell, anaplastic) (Shanmugaratnam 1978).

Types 2 and 3 NPC are Epstein–Barr virus (EBV) associated and have better prognoses than type 1; EBV infection is generally absent in type 1, especially in nonendemic areas (Marks et al. 1998). However, more recent data suggest that almost all NPC tumors in the endemic areas, regardless of histologic subtype, have comorbid EBV infections, which is a strong evidence for EBV as the etiology of NPC (Vasef et al. 1997). Undifferentiated NPC or type 3 was frequently characterized as lymphoepithelioma owing to the heavy infiltration of the primary tumor with lymphocytes. In endemic areas such as Southern China, WHO Type 3 accounts for more than 97%, while keratinizing SCC is more common in the Western countries (up to 75%) (Marks et al. 1998). There is no uniform morphological characteristic of NPC-affected tissues; thus, diagnosis of undifferentiated NPC is usually based on the location of the tumor in the nasopharynx and the presence of EBV transcripts in the tumor cells (Gullo et al. 2008). Clonal EBV genome is present in the early preinvasive dysplastic lesion or carcinoma in situ, illuminating that the development of malignant invasive tumor drop behind the infection of EBV (Pathmanathan et al. 1995). This close

association with EBV is what makes NPC unique from other head and neck cancers.

2.8 Risk factors of nasopharyngeal carcinoma

2.8.1 Diet

A poor diet may increase your risk of nasopharyngeal cancer. This may be because of a lack of some vitamins and minerals. People who eat more fresh vegetables, fresh fruit and other sources of vitamin C may have a lower risk of nasopharyngeal cancer. This is important throughout life, including in childhood (Greenland, 2000).

Nasopharyngeal cancer is more common in parts of Asia, Northern Africa, and the Arctic than it is in Europe. Diets very high in salt cured meats and fish or pickled foods are more common in some of these places. These foods can be very high in nitrates and nitrites, which react with protein to form nitrosamines. These chemicals can damage DNA.(Ahlbom, 2000).

Studies in China and Hong Kong have shown that babies and young children who eat Chinese cured and salted fish are at an especially high risk of nasopharyngeal cancer later in life. People from China, or with Chinese ancestry living in the UK, have higher rates of nasopharyngeal cancer than other ethnic groups, and it may be that this is due to their diet. Some studies have shown an increased risk of nasopharyngeal cancer among people who drink tea made from Chinese medicinal herbs (American cancer society, 2002).

2.8.2Viruses

Viruses can help cause some cancers. But this does not mean that you can ‘catch’ these cancers like an infection. The virus can cause genetic changes in cells that make them more likely to become cancerous in the future. Many people are affected with a cancer causing virus but never get cancer. The virus only causes cancer in certain situations.

Nasopharyngeal cancers have been linked to the Epstein Barr virus (EBV). Most people carry EBV and it does them no harm. This virus is linked to a number of cancers, including Hodgkin’s disease and Burkitt’s lymphoma (a type of non Hodgkin's lymphoma) as well as nasopharyngeal cancer (Kenneth, 2004).

2.8.3 Inherited risk

The risk of nasopharyngeal cancer is higher in people who have a relative who had had it. The risk to other family members appears higher if the relative was diagnosed before the age of 40 and is a first degree relative. A first degree relative is a parent, brother, sister, son or daughter (Decker and Goldstein ,1982)

2.8.4 Chemicals

People exposed to wood dust through their work have an increased risk of nasopharyngeal cancer. Treated wood contains several chemicals, and we don’t know which of these causes the increased risk.

Exposure to wood fires for cooking in the home over many years and using solvents in the workplace have been linked to nasopharyngeal cancer in one study. But other studies do not show a link with solvents in the workplace, or fumes from wood fires. A study in Africa showed that exposure to compact charcoal oven fumes in childhood could increase nasopharyngeal cancer risk but there didn't seem to be an increased risk from exposure to the fumes in adults.

One study has shown an increased risk of nasopharyngeal cancer for people exposed to chlorophenol, which is used in pesticides and as a wood preservative (Kenneth, 2004).

2.8.5 Ear, nose and throat diseases

People who have had chronic ear nose and throat diseases at some point in the past are at an increased risk of nasopharyngeal cancer. This includes chronic blocked and runny nose (rhinitis), middle ear infections (otitis media) and polyps (Christian and Moormann, 2008).

2.8.6 Smoking

Research has shown that smoking causes an increase in the risk of nasopharyngeal cancer, which can be up to 3 times higher in long term smokers (25 years or longer) (De la Chica, 2005).

2.8.7 Salt-cured foods

Chemicals released in steam when cooking salt-cured foods, such as fish and preserved vegetables, may enter the nasal cavity, increasing the risk of nasopharyngeal carcinoma. Being exposed to these chemicals at an early age may increase the risk even more (Peters, 1994).

2.8.8 Alcohol

Unlike most other types of head and neck cancer, alcohol is not a known risk factor for nasopharyngeal cancer (Little, 1999).

2.8.9 Viruses and cancers

For 40 years following the discovery of the first tumor virus by Peyton Rous in 1911, viruses were viewed as peculiar infectious agents capable of inducing

cancer in animals but having no relevance to humans. However, by the end of the 20th century, complete evidence had accumulated that six different human viruses, including EBV, HBV, HPV, HTLV-1, HCV, and KSHV, were bona fide etiologic agents of human cancer and caused 15% to 20% of all human tumors worldwide. Most knowncellular oncogenes have been identified through studies of RNA tumor viruses, whereas identification of the p53 tumor suppressor and many functions of the Rb tumor suppressor were gleaned through studies of DNA tumor viruses (Ronald, 2008).

2.9 Epstein - Barr virus

Epstein–Barr virus (EBV) is a ubiquitous human γ-herpes virus infecting more than 90% of the adult population worldwide. EBV was first identified in 1964 when Anthony Epstein's group discerned virus-like particles by electron microscopy in a cell line that had been established from a Burkitt's lymphoma biopsy (Epstein, et al. 1964). EBV is an enveloped virus that contains a DNA core surrounded by an icosahedral nucleocapsid and a tegument (Matthew, 2004).

2.9.1 The natural history of EBV infection

EBV infects approximately 95% of the world's adult population and, after primary infection; the individual remains a lifelong carrier. The oropharynx is the primary site of infection and is believed to be the site for virus replication persistent active lytic infection in this region ensures the production of new virions in the oropharyngeal secretions for transfer in saliva to susceptible hosts. In underdeveloped countries, primary infection with EBV usually occurs during the 1st few years of life and is often asymptomatic. Primary infection can often be delayed until adolescence or adulthood, in many cases producing the characteristic clinical features of infectious mononucleosis including sore throat, fever, malaise, lymphadenopathy, and mild hepatitis (Baumforth, 1999).

2.9.2Pathogenesis of EBV

EBV initially infects epithelial cells; the hallmark of EBV disease is subsequent infection of B lymphocytes and polyclonal B lymphocyte activation with benign proliferation. The virus enters B lymphocytes by means of envelope glycoprotein binding to a surface receptor CD21, which is the receptor for the C36 component of complement; 18 to 24 hours later, EBV nuclear antigens are detectable within the nucleus of infected cells. Expression of the viral genome, which encodes at least two viral proteins, is associated with immortalization and proliferation of the cell. The EBV-infected B lymphocytes are polyclonally activated to produce immunoglobulin and express a lymphocyte-determined membrane antigen that is the target of host cellular immune responses to EBV-infected B lymphocytes. During the acute phase of infectious mononucleosis, up to 20% of circulating B lymphocytes demonstrate EBV antigens. After infection subsides, EBV can be isolated from only about 1% of such cells (Kenneth, 2004).

2.9.3 Immunity of EBV

EBV expresses more than 80 lytic antigens, latently infected cells express up to 8 proteins and several non-translated RNAs (Young and Rickinson, 2004). The six latent EBV proteins, six are nuclear antigens (EBNA1, 2, 3A, 3B, 3C, LP) and two are membrane proteins (LMP1 and 2). This limited antigen expression is probably one of the essential immune escape mechanisms of latent EBV infection, while EBV expresses more than 80 antigens during lytic replication. In addition to reduced viral protein expression, the virus performs some latency functions with non-translated RNAs which cannot be detected by T cells looking for small peptides presented on MHC class I and II molecules. EBV reduces antigenic protein expression further, dependent on the differentiation stage of the latently infected B cell. While all eight latent EBV antigens can be found in naïve B cells

in tonsils, the primary site of EBV infection after transmission in saliva, germinal center B cells express only EBNA1, LMP1 and LMP2. Furthermore, infected peripheral blood memory B cells express no EBV antigens or EBNA1 during homeostatic proliferation. Therefore, memory B cells, harboring the EBV genome without any EBV protein expression, are probably the site of long-term EBV persistence, and invisible to the immune system (Christian and Moormann, 2008).

2.9.4 Oncogenic features of EBV

To be oncogenic, EBV must maintain its viral genome in the cell, avoid killing the cell and prevent the cell from becoming a target for destruction by the immune system; finally the virus must activate cellular growth control pathways. To maintain viral DNA in the cell, EBV establishes latent infection in B-lymphocytes. The EBV genome is maintained in these cells, either as a multicopy circular episome in the host cell or by integrating the viral DNA into the host genome, the virus thus ensures transmission to cell progeny when B-lymphocytes replicate. EBV latent genes induce an activated phenotype in the infected B cell which is not transformed, but they can proceed unchecked or acquire oncogenic mutations, and become neplastic. In normal individuals cytotoxic T-cells responses against latent viral proteins prevent the expansion of these activated B cells. Through normal differentiation of these cells, EBV eventually enters the resting B-cell memory compartment .Only EBNA-1 is expressed in these cells. The EBV growth –promoting latent genes products are not expressed, and so the cells are not pathogenic. The limited responses of gene products also prevent frequent viral replication because cytotoxic responses to EBNA-1 are rare, EBNA-1 expressing lymphocytes escape immune surveillance. This then constitutes the viral reservoir. Intermittently, these cells may enter lytic cycle where it lysis/death releasing virions to infect more cells. With immune suppression latently infected cell in the peripheral blood or on the oropharynx in the case of NPC increase in number. The

final mandate of the virus in achieving oncogenecity must activate intracellular signaling involved in control of proliferation. years after primary EBV infection, malignancies such as Burkett's lymphoma, nasopharyngeal carcinoma, and Hodgkin`s disease can emerge which can initiate from a clone of EBV –infection cells. Role of EBV in these late-onset malignancies is complicated. Because EBV is clonal, it clearly sets the stage for progression to frank tumor. However, other factors may be important: specific failure of immune recognition, stimulation of B-cell proliferation by other infections and /or appearance of secondary genetic aberrations or mutation (Matthew and Razelle, 2004).

2.9.5 EBV and Cancers

Epstein-Barr virus (EBV) is tightly associated with diver's human cancers (Sylvie and Tadamasa, 2008). EBV is associated with several malignancies (e.g. Burkitt`s lymphoma, Hodgkin`s lymphoma and nasopharyngeal carcinoma) and thus has been classified as a group 1 carcinogen (International Agency for Research on Cancer, 1997). Several studies have recently hinted at a possible role for EBV in the pathogenesis of breast carcinoma, which represents the most common carcinoma of females in the Western world (Labrecque, 1995; Bonnet, 1999). If substantiated this would have potential implications for the prevention and treatment of breast cancer Immunotherapeutic strategies based on EBV specific cytotoxic T cells are currently being developed for the treatment of EBV positive Hodgkin's lymphoma and nasopharyngeal carcinoma, and they could potentially also be applied to EBV associated breast carcinoma (Ambinder 1996; Roskrow 1998). EBV has been linked occasionally with other types of malignancies, including a pseudolymphomatous lung carcinoma (Begin, 1987) and gastric adenocarcinoma (Shibata and Weiss, 1992; Pittaluga, 1992).

2.10 Cytomegalovirus (CMV):

Is a member of the herpesvirus family. Infection is worldwide and usually asymptomatic. CMV may cause a mononucleosis infection in healthy individuals but can cause severe illness in congenital infection and in an immunocompromised host.

The most common disease manifestation is gastrointestinal disease. CMV pneumonia is the most serious complication, but has become less common with prevention strategies for at-risk patients. Rare manifestations include retinitis and encephalitis. CMV also has an immunosuppressive effect, which can lead to an increased susceptibility to invasive bacterial and fungal disease as well as graft-versus-host disease (GVHD) (Boeckh M,2011).

- After initial infection, human CMV remains in a persistent state within the host. Immunity against the virus controls replication, although intermittent viral shedding can still take place in the immunocompetent person.
- Complications are therefore mainly seen if the immune system is immature, or is suppressed by drug treatment or co-infection with other pathogens.
- Clinically significant CMV disease frequently develops in patients immunocompromised as a result of HIV, solid organ transplantation and bone-marrow transplantation (Akhter K,2012).
- Primary CMV infection of the immunocompromised host may cause disease in almost every organ of the body - eg, pneumonia, hepatitis, encephalitis, colitis, uveitis, retinitis and neuropathy.

2.10.1 Epidemiology

- 50-80% of all adults are infected with CMV.
- Infection may be passed via body fluids - eg, kissing, sexual intercourse, blood transfusion, or by tissue donation.

- Most HIV-infected individuals are seropositive for CMV. HIV infection accelerates the development of CMV-dependent immunological abnormalities (Barrett L, Fowke KR, Grant MD, 2012)export a summary to use in yourpraisal

2.10.2 Infection in immunocompetent hosts

CMV is usually an asymptomatic infection. In immunocompetent individuals, symptomatic disease usually manifests as a mononucleosis syndrome (Herpes simplex - oral;1012)

- Infection is usually asymptomatic in infants and children.
- In adolescence and early adulthood, primary infection is also usually asymptomatic but may cause a mild flu-like illness.
- CMV may also produce a mononucleosis syndrome similar to Epstein-Barr virus (causing a febrile illness with splenomegaly, impaired liver function, and abnormal lymphocytes in the blood), but without characteristic pharyngitis and lymphadenopathy.

2.11 Herpes simplex virus type 1 (HSV-1) :

Is usually the cause of oral infection. After primary infection, HSV-1 becomes latent, usually in the dorsal root ganglia of the trigeminal nerve. Rarely, herpes simplex virus type 2 (HSV-2) may cause primary infection of the oral cavity, typically in association with orogenital sex, but recurrent oral HSV-2 disease is rare. (Karjala Z, Neal D, Rohrer J;2011)

2.11.1 Epidemiology

About 1% of primary care consultations are for cold sores.

- 56-85% of people have serological evidence of HSV-1 infection by early adulthood. Prevalence depends on their resident country.
- 20-40% of young adults who are seropositive for HSV-1 have recurrent cold sores.
- Recurrences occur typically between two and six times a year.

- A study from Amsterdam found that HSV-1 seroprevalence varied according to ethnicity and was more prevlalent in older age groups and those of low economic status (Kramer M et al, 2008).
- Transmission is due to viral shedding into saliva and can occur by direct contact with saliva (eg, kissing). Viral shedding into saliva may occur during asymptomatic infection but it is thought that the risk of infection is much smaller than during symptomatic infection.
- Viral shedding can occur up to 60 hours after the onset of symptoms.[1]
- Factors that may trigger a recurrence of oral herpes simplex include immunosuppression (eg, corticosteroids), upper respiratory tract infections, fatigue, emotional stress, physical trauma, exposure to sun (ultraviolet light), trauma and menstruation.
- Obesity may increase susceptibility to HSV-1 infection(Karjala Z, Neal D, Rohrer J;2011)

2.11.2Presentation

- Infection with HSV can cause pain and blistering within the mouth (gingivostomatitis or recurrent oral ulceration) or on or around the lips (cold sores or herpes labialis).

2.11.3Prognosis

Oral herpes simplex is usually a self-limiting disease.Lesions (whether due to primary infection or recurrent disease) usually heal within 7-10 days, without scarringPrevention:

- Sunscreen may be useful for people who have recurrences triggered by sunlight but the evidence is equivocal. (Opstelten W,2008)
- There is some evidence that application of a topical antiviral before exposure to a triggering factor (eg, sunlight) offers some protection. (Opstelten W,2008)

- Prophylactic oral antivirals are not generally recommended for immunocompetent individuals. There is support for the use of systemic aciclovir and valaciclovir for the prevention of recurrent attacks and this is an appropriate prophylaxis for people with frequent or severe episodes, or for immunocompromised individuals (specialist advice should be sought)(Rahimi H,2012).
- Laser phototherapy may help to reduce the frequency of attacks in recurrent herpes labialis(Munoz Sanchez PJ,2011)

2.12 Diagnosis of Nasopharyngeal carcinoma: -

2.12.1 Histopathology:

Diagnosis of the cancer must be proven by the presence of cancer cells as seen under microscope. Only a biopsy can definitely confirm nasopharyngeal carcinoma, although negative biopsy doesn't exclude the presence of cancer, cancer may still be present (Mustofi, 1992).

2.12.2 Immunohistochemistry (IHC):

Refer to the process of localizing antigens in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. 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 cell death. IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore(Ramos, 2005).

2.12.3Molecular biology:

EBV is a herpes virus with a 184-kbp long, double-stranded DNA genome that encodes _85 genes (Kieff and Rickinson, 2001). The viral genome consists of a series of 0.5 kb terminal direct repeats at either end or internal repeat sequences that serve to divide the genome into short and long unique sequence domains that have most of the coding capacity (Cheung and Kieff, 1982). EBV, as with other herpesviruses, has a toroid-shaped protein core wrapped with double-stranded DNA, a nucleocapsid with 162 capsomeres, a protein tegument between the nucleocapsid and envelope, and an outer envelope with external glycoprotein spikes (Rickinson and Kieff, 2001).When EBV infects a cell, the DNA becomes a circular episome with a characteristic number of terminal repeats, depending on the number of terminal repeats in the parental genome, with variation introduced during viral replication (Kieff and Rickinson , 2001; Baumforth, 1999).

2.12.4Routine blood tests:

Perform routine blood work, including a CBC count and chemistry profile. Liver function test results may be abnormal in those rare cases with hepatic metastases. Uric acid levels may be elevated in patients with rapidly growing tumors(Henle, 1976).

Epstein-Barr virus (EBV) titers, including immunoglobulin A (IgA) and immunoglobulin G (IgG) antibodies to the viral capsid antigen, early antigen, and nuclear antigen should be performed. These titers may correlate with tumor burden and decrease with treatment (Gastpar 1981).New data have emerged that plasma EBV-DNA levels may be a helpful marker for pretreatment risk categorization, for initial treatment response, and at the time of relapse (Leung, 2006).

2.12.5Other tests:

A baseline audiogram is helpful prior to radiotherapy, especially in children who receive cisplatin.

Creatinine clearance rates (24-hour collection or nuclear GFR testing) should be obtained at baseline and during treatment for those patients being treated with platinum-based chemotherapy because decreases in renal function, requiring dose modifications, have been reported. (American Cancer Society, 2002).

2.13 Management of Nasopharyngeal carcinoma:

2.13.1Surgery:

Due to the anatomical position of NPC and its tendency to present with cervical lymph node metastases, it is not amenable to surgery for local control. Biopsy of the involved lymph node is the usual surgical procedure. The nasopharyngeal primary tumor is rarely biopsies (Kietly,2002).

2.13.2Chemotherapy:

Several factors are taken into account in deciding the chemotherapy regimen. Firstly, efficacy: the figures for event-free survival are similar for most small chemotherapy series but therapy usually involves fairly high-dose radiotherapy to the nasopharynx – 60 to 65 Gy. However, the most promising results with a recent update, are those obtained using the Martens protocol NPC-91-GPOH (Society of Pediatric Oncology and Hematology). This protocol should therefore be considered as the best current treatment. Uniquely, the NPC-91-GPOH protocol includes immunotherapy with interferon-beta after chemotherapy and radiotherapy, which may explain its superior results compared to regimens

without interferon treatment. Secondly, late effects: in terms of chemotherapy, the Manchester regimen – doxorubicin, methotrexate and cyclophosphamide – would produce infertility in boys (total dose of cyclophosphamide 12 gm/m2) and possible anthracycline toxicity (total dose of doxorubicin 360 mg/m2) (Cheung and Kieff, 1982).

2.13.3Radiotherapy:

Although treatment with radiotherapy controls the primary tumor , it does not prevent the appearance of distant metastases . Radiotherapy is given with megavoltage equipment after initial chemotherapy. A maximum dose of 45 Gy is given to the clinical target volume, which is a 1 cm margin around the MRI-detected primary site, and inferiorly down to the clavicles to include the lymph nodes(Udhiki,2003).

Chapter Three

Materials and Methods

3.1 Study design:-

This is retrospective perspective study, aimed to detectof Keratin and Epithelial Membrane Antigen, Cytomegalovirus, Epstein Barr Virus and Herpes Simplex Virus among Sudanese Patients with Nasopharyngeal Carcinoma using Immunohistochemistry and PCR.

3.2 Study area:-

The study was conducted in Radiation and Isotope center, Khartoumduring the period from May 2012to December 2014.

3.3 Study population:-

One hundred and fifty NCP paraffine blocks were obtained from patients diagnosed with NPC at Radioisotope centre in Khartoum State during the period from May 2012to Janaury 2015. Patient identification data and other information were obtained from patient's file.

3.4 Sample size:-

One hundred fifty blocks previously diagnosed as nasopharyngeal tumors Sample processing and staining:-

From each block Six sections were cut:

One section used for morphological changes after stained by hematoxylin and eosin, two section stained with Keratin and Epithelial Membrane Antigen tumor markers.three section putting in epindorf tube for molecular technique.

3.5 Histopathology tissue processing:

One section of 5 µm in thickness were obtained from formalin – fixed. Paraffin wax embedded tissues using rotary microtome. Sections required for histopathology were stained using Hematoxylin and eosin (Mayer's technique)

Sections were de-waxed in hot plate oven and cleared in 2 changes of xylene for 2 minutes, then hydrate through ethanol (100%, 90%, 70%, 50%) and water 2 minutes for each, then stained in Mayer's Hematoxylin for 7 minutes, then washed and blued in running tap water for 10 minutes, then stained in eosin for 3 minutes, then washed in distilled water, then dehydrated through ascending ethanol concentrations, then cleared in xylene and mounted in DPX mounting.

3.5.1 Immunohistochemical tissue processing:

Sections were dewaxed in hot plate oven and cleared in two changes of xylene for two minutes, then hydrated through descending concentrations of ethanol (100%, 90%, 70%, 50%) and water two minutes for each, then retrieved by water bath retrieval technique for one hour, then treated with hydrogen peroxide solution for fifteen minutes, then washed in phosphate buffer saline (PH 7.4) for five minutes, then treated with protein blocker for fifteen minutes, then treated with primary antibody (The kit from Sacace technologies - Casera–Italy was able to detect cytokeratin and EMA) for thirty minutes, then rinsed in phosphate buffer saline (PH 7.4), then treated with avidin biotin complex for thirty minutes, then rinsed in phosphate buffer saline (PH 7.4), then treated with DAB for ten minutes, then washed in phosphate buffer saline (PH 7.4) for five minutes, then counterstained in Mayer's haematoxylin for one minutes, then washed and blued in running tap water for ten minutes, then dehydrated through ascending concentrations of ethanol (50%, 70%, 90%, 100%), then cleared in xylene and mounted in DPX mountant (Bancroft and Marilyn, 2002).

3.5.2 Tissue sections preparation for polymerase chain reaction (PCR)

From each paraffin block, sections of 30-50 microns were collected into a screw capped Eppendorf tube. To avoid cross contamination, each block was cut with new gloves and new disposable microtome blade.

- De-paraffinization and re-hydration of sections

One ml of xylene was added two times to a screw capped Eppendorf tube containing sections of oral embedded in paraffin. Then sections were incubated at 37°C for 30 minutes, vortexed and centrifuged at 800g per 5 minutes. The pellet was re-hydrated with serial dilutions of absolute ethanol, 75% ethanol, 50% ethanol, 25% ethanol and sterile water, vortexed and centrifuged for 3 minutes at maximum speed after each washing and the supernatant was discarded.

- **DNA Extraction:**

DNA was extracted according to the steps described in DNA extraction kit purchased from Sacace biotechnologies-Casera –Italy. The pellet obtained from previous steps was treated with 1500 µl of Reagent 2 (lysis buffer), vortexed, incubated at 65°C for 5 min and centrifuged at 3000g for 15 min. The supernatant was discarded. 150 µl of Reagent 3, 25 µl of Reagent 4 (proteinase K) and 10 µl of Reagent 5 were added to the pellet and incubated at 50°C overnight. 90 µl of Reagent 6 were added to each tube, vortexed vigorously and centrifuged at 800g for 15 min. The supernatant was transferred to a new sterile 1.5 ml Eppendorf tube. 750 µl of absolute ethanol were added to each tube and mixed by inverting. Then centrifuged at 7000 g for 5 minutes and the supernatant was discarded. This step was repeated by using 1ml 70% cold ethanol and tubes were incubated with open caps for 10 minutes. The pellet (containing the DNA) was re-suspended in 50 µl of

Reagent 7 (DNA eluent), shacked for 2 hours by use of a shaker and stored at -20°C until used.

Polymerase chain reaction (PCR):

Amplification of EBV,CMV and HSV:

Type specific primers were used to detect EBV,CMV and HSV in NPC . Amplification was performed according to kit from Sacace technologies- Casera – Italy. The final reaction volume of 40 µl containing 20µl mix-1(contained in PCR tubes), 10 µl of mix-2 and 10 µl of extracted DNA (sample). Samples and controls were amplified using Gene Amp PCR system 9700. The PCR program was described in Table 1.

Table 1 Show PCR Program used for amplification of HPV genes

Steps	Temperature	time	Cycles
0	95°C	Pause	
1	95°C	5min	1
2	95°C	15sec	42
	65°C	25sec	
	72°C	25sec	
3	72°C	1min	1
4	4°C	Storage	

Gel-electrophoresis

The PCR products were visualized in 2% Agarose gel with 0.5 µg/ml Ethidium bromide. Ten micro liters of 100bp DNA ladder and PCR product was loaded on the gel. Gel-electrophoresis was performed at 120V and 36 mA for 60

minutes. Pictures were taken by Gel documentation system (Gel mega, digital camera and software in a computer).

Interpretation of PCR results

All samples were examined after adopting all quality control protocols during preparing, staining, and reading.

According to manufacture kit (from Sacace technologies –Italy*)* manual, the PCR product length for EBV should be 290bp ,500bp forCMVand 430bp for HSV.

3.5.3 Data analysis:-

Data was analysis using SPSS computer programming to calculate mean, frequencies and chi squar were calculated.

3.5.4 Ethical cosideration

All samples were taken ethically after informing the doctor (who is resbonsableaboute the histopathology lab.)and take his permission,all of them were agreed to cooperate in this study. In addition the proposal was approved by the Faculty Research Board, College of Medical Laboratory Science, Sudan University of Science and Technology.

Chapter Four

Results

A total of 150 patients diagnosed with nasopharyngeal carcinoma (NPC) were investigated by conventional histopathology, immunohistochemistry and special histochemicalmethods.Their ages ranged between 17 to 88 years old with a mean age of 50 years. Most patients were older than the age of 50 years representing 77 (51.3%) and the remaining 73(48.7%) were younger than50 years as indicated in Table 1.

Table 2: frequaency of gender, 97(64.7%) of study subjects were males. While, 53 (35.3%) of study subjects were females.

Table3: Shows the location of the respondents. The respondents were selected according to their population size in the area of interest. Many of the patients were from West at 36.7%, they were followed by those from the South at 19.3%. 18% were from Khartoum, 14% from East, and 12% from North location. All these patients were either in Khartoum Radiation and Isotope Center who had been diagnosed with NPC or those who were under the treatment in RICK.

Table 4 :indicates the positivity and negativity of keratin among the sampled patients . As displayed by the result, 149 (99.3%) of the respondents had a positive keratin while only a minimal number at 0.7% had negative keratin.

Table 5: shows the description of epithelial membrane antigen (EMA) at its distribution among the 150 patients of different ages. The results show that 82% of the interviewees had a positive s of epithelial membrane antigen while 18% had negative EMA .

Table 6: displays the result of the EBV and its detection of its positivity among patients with nasopharyngeal carcinoma when using PCR techniques.58% of the respondents indicated that positive serum of Epstein-Barr virus, while the rest at 42% had a negative EBV.

Table7: shows that cytomegalovirus (CMV) distribution among the sampled respondents. Majority of these interviewees at 64% were negative towards CMV while the rest at 36% shown positivity about CMV.

Table 8: shows the negativity and positivity of the herpes simplex virus (HSV) on the interviewed respondents and its association with NPC. Majority of the respondents at 89.3% were negative about HSV association while the rest 10.7% positive.

Table9and figer 1 and 2 :shows thatRelation between results of CK and Herpex Viruses(EBV, CMV and HSV)**.**CK expression was demonstrated by immunohistochemistry using pan CK antibodies. EBV, CMV and HSV were identified by polymerase chain Reaction (PCR). Of the 150 samples, 144/150(96%) were CK positive and the remaining 6/150(4%) were CK negative (internal control). 144 tissue samples from patients with NPC were studied for immune-expression of CK and molecular identification of EBV, CMV and HSV correlations. All of the 144 samples were Pan CK positive. Six additional samples (CK negative) were added as internal control. EBV was detected in 92/144 (64%) of CK positive samples, consequently, the 95% confidence level and Odd Ratio (OR) is 22.9 (1.2649 to 414.7633), P< 0.003). No EBV was found in the samples

negative by CK. CMV was identified in 53/144(37%) of CK positive samples consequently, the 95% confidence level and Odd Ratio (OR) is 7.6 (1.2649 to 414.7633), P< 0.003). No CMV was found in the samples negative by CK. HSV was detected in 18/144 (12.5%) of CK positive samples (P< 0.4). No EBV was found in the samples negative by CK, as indicated in Table3, Fig1 and 2.

Table.10and figure 3: Shows the description of immune-expression of EMA and molecular identification of herpes viruses (EBV=92, CMV=53 and HSV=18) correlations. Of the 92 specimens with EBV infection, loss of EMA (negative) was identified in 10/92 (10.9%) and the remaining 82/92(89.1%) were positive. Furthermore, 20 EMA negative were foundEBVnegative,accordingly, the 95% confidence level and Odd Ratio (OR) is 2.2439(0.9929 to 5.0713), P< 0.05.Of the 53 positive CMV loss of EMA (negative) was identified in 10/53 (18.9%) and the remaining 43/53(81.1%) were positive.Furthermore, 20 EMA negative were found CMV negative, consequently, the 95% confidence level and Odd Ratio (OR) is 2.4651(1.0441 to 5.8203), P< 0.04).

Of the 18 positive HSV loss of EMA (negative) was identified in 4/18 (22.2%) and the remaining 14/18(77.8%) were positive. Furthermore, 26 EMA negative were found HSV negative, consequently, the 95% confidence level and Odd Ratio (OR) is 8.3571(2.3627 to 29.5604), P< 0.001), as indicated in Table3, Fig3.

Table.11and figure 4&5 : Shows the description of Immunohistochemical Expression of Cytokeratins (CK) and Epithelial Membraneantigen (EMA) in nasopharyngeal carcinoma were 150 tissue samples obtained from Sudanese patients with NPC were investigated their ages ranging from 17 to 88 with a mean age of 50 years. Immunohistochemical expression of Cytokeratin was identified in 144/150(96%) and could not be identified in 6/150(6%). Epithelial Membrane

antigen was identified in 120/150(8%) and could not be identified in 30/150(20%). Of the 144 samples positive for CK, 120/144(%) were also found to be positive for EMP2 and the remaining 24/144(%). However, the 6/150 (%) found negative in Ck, have also revealed loss of expression in EMA, as indicated in Photomicrographs 1, 2 and 3.

According to World Health Organization (WHO) classification of NPC, Type I, Type II, Type III, Type II&III, were identified in 0%, 56/150(37.3%), 85/150(56.7%) and 9/150(6%), respectively, as indicated in Fig4 &5.

Table 12 : show the relationship between NPC types and age.28(18.7 %)were diagnosedby histopathology as type2in less than 50 years old and the same percentage in less than 50 years old.26(40 %)were diagnosed as type3in more thane 50 years old and 45(30%)in less than 50 y/o.5(3.3%)were diagnosed as type 2&3in less than 50 y/o and 4(2.7%)when patients more than 50 y/o . the correlation between histopathological diagnosis and age was found to be statistically insignificant (P>0.75).

Table 12: shows the relationship between the histopathological diagnosis and epithelial membrane antigen (EMA).in type2,48(32%)were detected positive with EMA and negative with 8(5.3%). In type3, 70(46.7%)were detected positive and15(10%)were negative. In type 2&3 were detect 5(3.3%) were positive and4(2.7%)were negative. The correlation between thehistopathological diagnosis and epithelial membrane antigen (EMA) was found to be statistically insignificant (P>0.09).

Table 13: show the relationship between age group and Epstein Barr Virus .In less than5 0 years old 44(29.3%)were diagnosed as positive and 29(19.4%)were negative.in more than 50 years old 43(28.7%)were positive and34(22.7%)were negative . . The correlation between the age group and EBVwas found to be statistically insignificant (P>0.86).

Table 14: show the relationship between age group and Cytomegalovirus .In less than 50 years old 26(17.3%)were diagnosed as positive and 47(31.3%)were negative.in more than 50 years old 28(18.7%)were positive and49(32.7%)were negative . . The correlation between the age group and CMV was found to be statistically insignificant (P>0.59).

Table 15 : show the relationship between age group and Herpes Simples Virus .In less than 50 years old 6(4%)were diagnosed as positive and 67(42.7%)were negative.in more than 50 years old 10(6.7%)were positive and67(44.7%)were negative . . The correlation between the age group and HSV was found to be statistically insignificant (P>0.42).

Table 1: Frequency of age group among study population

Age	Frequency	Percent %
Less than 50	73	48.7
More than 50	77	51.3
Total	150	100

Table 2:Frequency of SEX group among study population

SEX	Frequency	Percent %
Male	97	64.7
Female	53	35.3
Total	150	100

Table 3: Frequency of Location group among study population

Location	Frequency	Percent %
Khartoum	27	18
West	55	36.7
North	18	12
East	21	14
South	29	19.3
Total	150	100

Table 4: Frequency of Keratin results

Keratin	Frequency	Percent %
+ve	149	99.3
- ve	1	0.7
Total	150	100

Table 5:Frequency of EMA results

EMA	Frequency	Percent %
+ve	123	82
- ve	27	18
Total	150	100

Table6:Frequency of EBV results

EBV	Frequency	Percent %
+ve	87	58
-ve	63	42
Total	150	100

Table 7: Frequency of CMV results

CMV	Frequency	Percent %
+ve	54	36
-ve	96	64
Total	150	100

Table 8: Frequency of HSV results

HSV	Frequency	Percent %
+ve	16	10.7
-ve	134	89.3
Total	150	100

Table9: Relationship between CK and Herpes Viruses (EBV, CMV and HSV).

Virus	CK		Total
	Positive	Negative	
EBV	**p=0.003**		
Positive	**92**	**0**	**92**
Negative	**52**	**6**	**58**
CMV	**p=0.003**		
Positive	**53**	**0**	**53**
Negative	**91**	**6**	**97**
HSV	**p=0.4**		
Positive	**18**	**0**	**18**
Negative	**126**	**6**	**132**

Table 10. Relationship betweenEMA and Herpes Viruses (EBV, CMV and HSV).

Virus	EMA		Total
	Positive	Negative	
EBV		p=0.05	
Positive	82	10	Positive
Negative	0	20	Negative
CMV		p=0.04	
Positive	43	10	Positive
Negative	0	20	Negative
HSV		p=0.001	
Positive	14	4	Positive
Negative	0	26	Negative

Table 11. Relationship betweenNPC types and Immunoexpression of tumor markers (CK& EMA).

NPC type	CK P=0.06		EMA P=0.09	
	Positive	Negative	Positive	Negative
I	0	0	0	0
II	56	0	48	8
III	81	4	67	18
II&III	7	2	5	4
Total	144	6	120	30

Table 12: Relationship between NPC typesand Age group

Age	Types		
	Type 2	Type 3	Type 2 &3
Less than 50	28 18.7%	40 26.7%	5 3.3%
More than 50	28 18.7%	45 30%	4 2.7%
Total	56 37.3%	85 56.7%	9 6%

P=0.75

Table 13:Relationshipbetween age group and EBV results

Age	EBV		Total
	+ve	-ve	
Less than 50	44 29.3%	29 19.4%	73 48.7%
More than 50	43 28.7%	34 22.7%	77 51.4%
Total	87 58%	63 42%	150 100%

P= 0.09

Table 14 :Relationshipbetween CMVand age group of study population

Age	CMV		Total
	+ve	-ve	
Less than 50	26 17.3%	47 31.3%	73 48.6%
More than 50	28 18.7%	49 32.7%	77 51.4%
Total	54 36%	96 64%	150 100%

P=0.86

Table 15: Relationshipbetween HSV resultsand age group of study population

Age	HSV		Total
	+ve	-ve	
Less than 50	6 4%	67 44.7%	73 48.7%
More than 50	10 6.7%	67 44.7%	77 51.4%
Total	16 10.7%	134 89.3%	150 100%

P=0.59

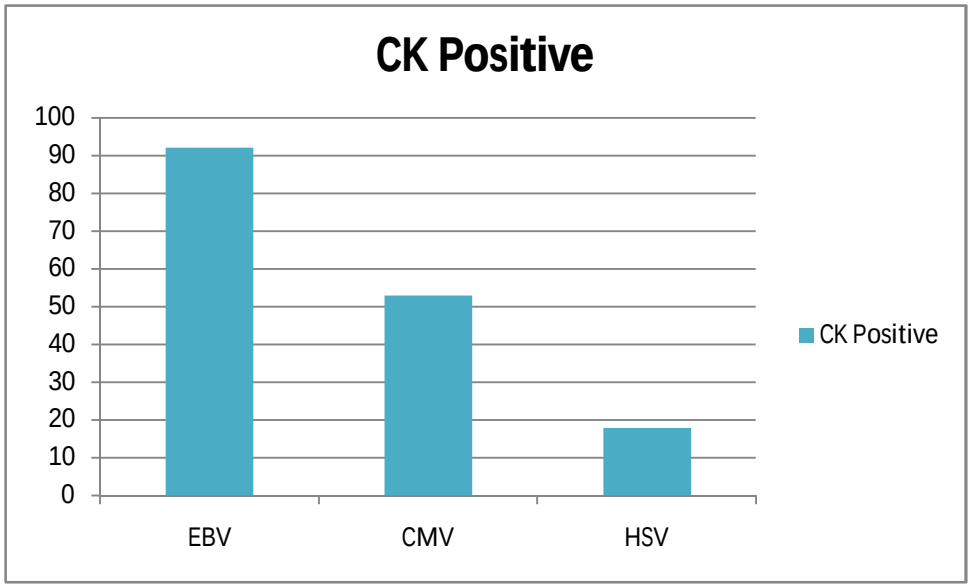


Figure. 1. Description of positive immune-expression of CK by presence of EBV, CMV and HSV.

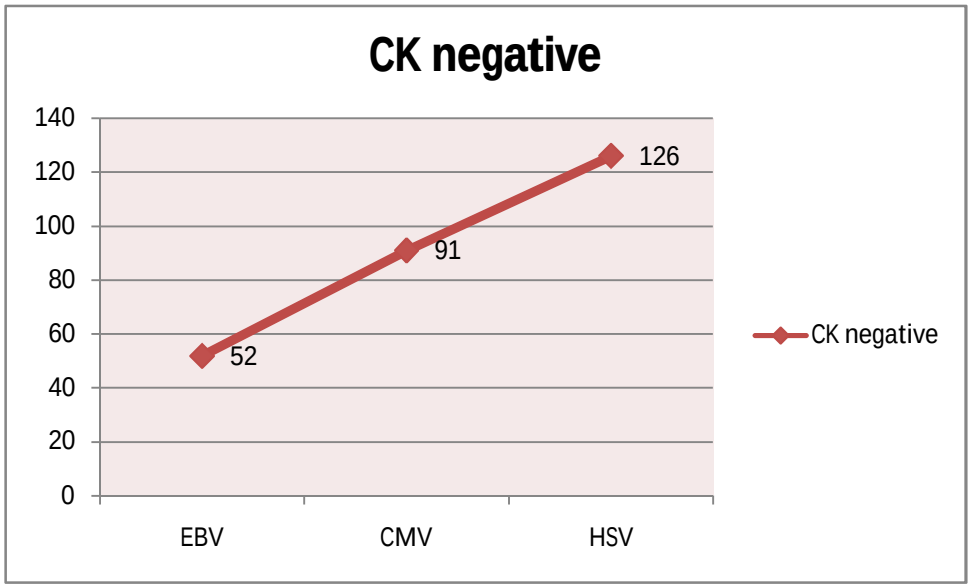


Figure. 2. Description of negative immune-expression of CK by presence of EBV, CMV and HSV.

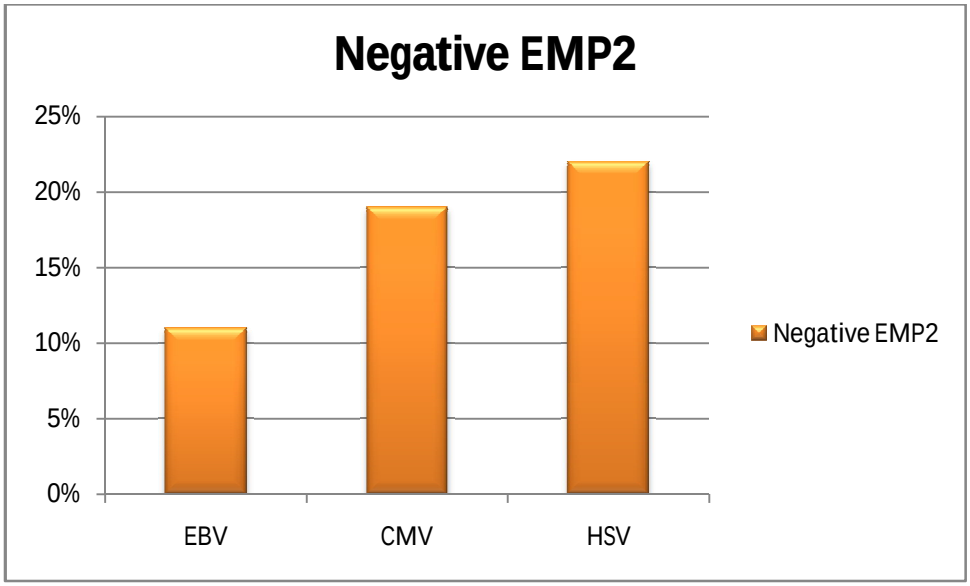


Figure.3. Description of loss of EMA by human herpes viruses

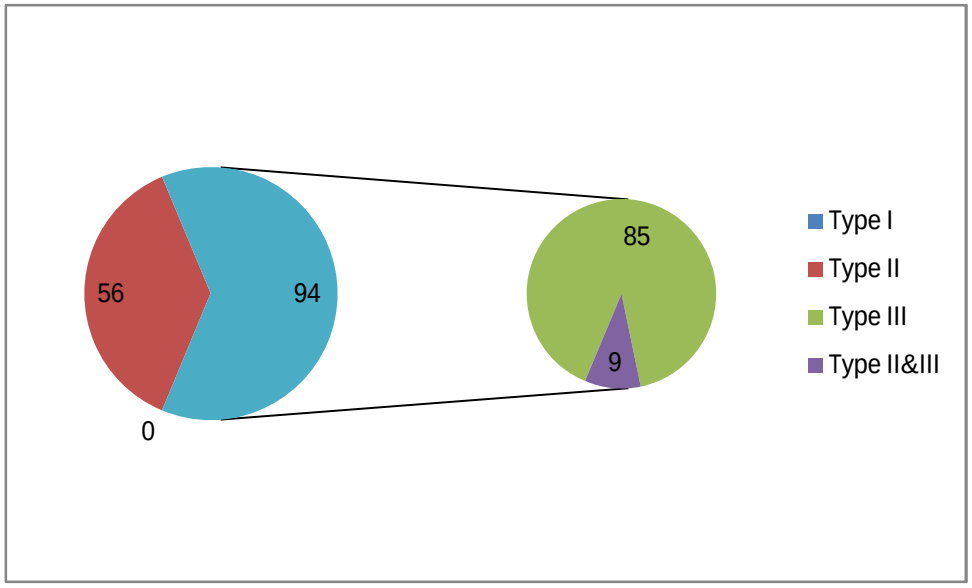


Figure 4. Description of the study population by NPC types (WHO classification)

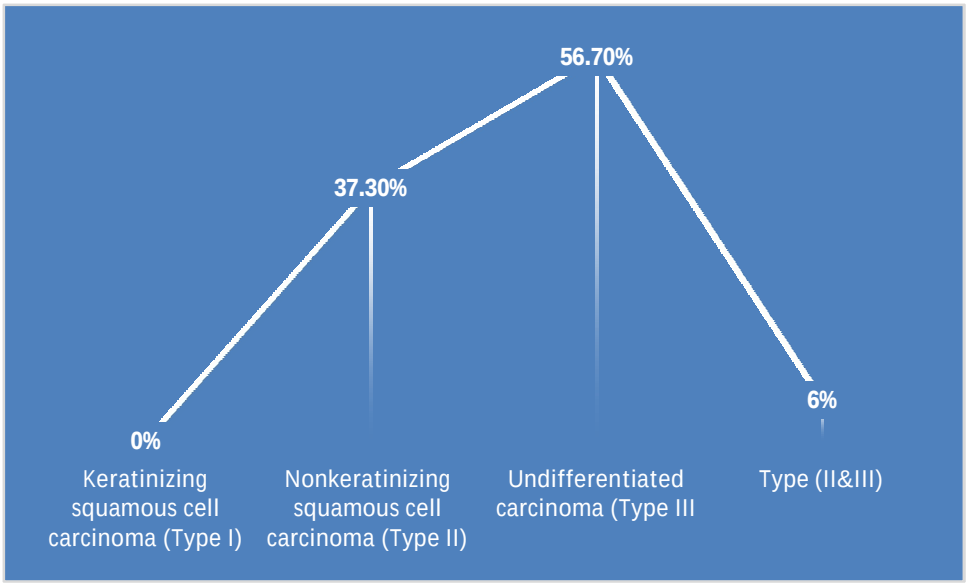


Figure 5. Description of the study population by NPC histological types

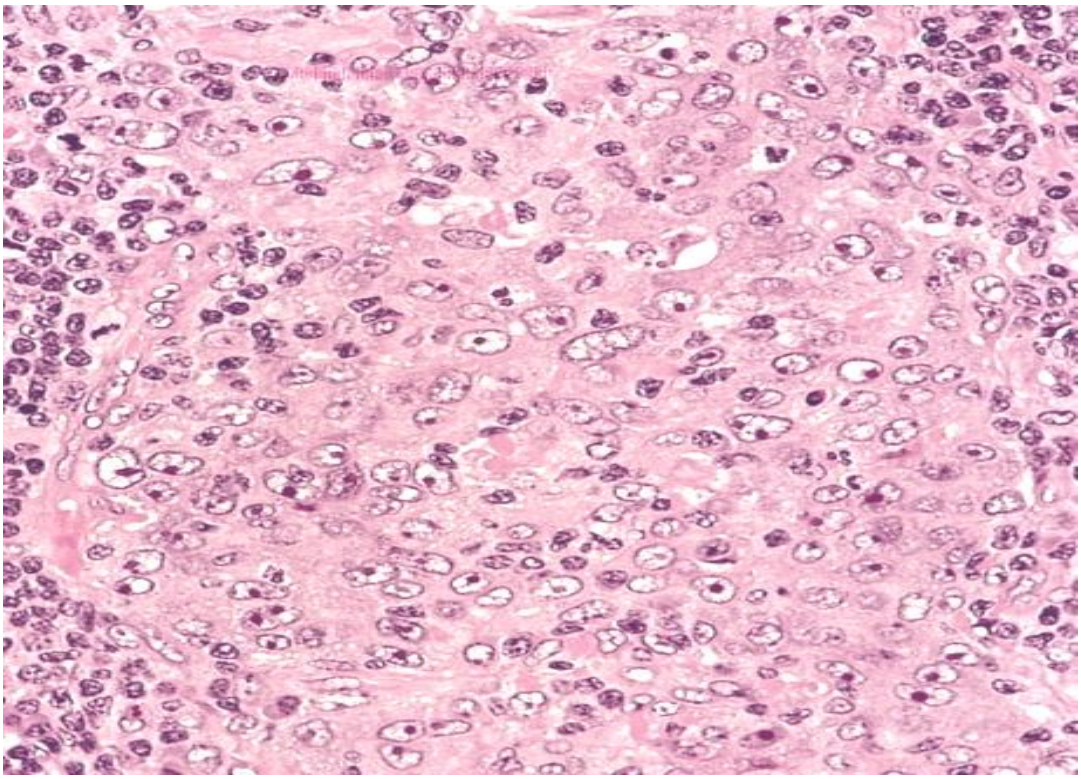


photo .1. H& E stained section of Nasopharyngeal carcinoma , obtained from 54 years old patient(x 40).

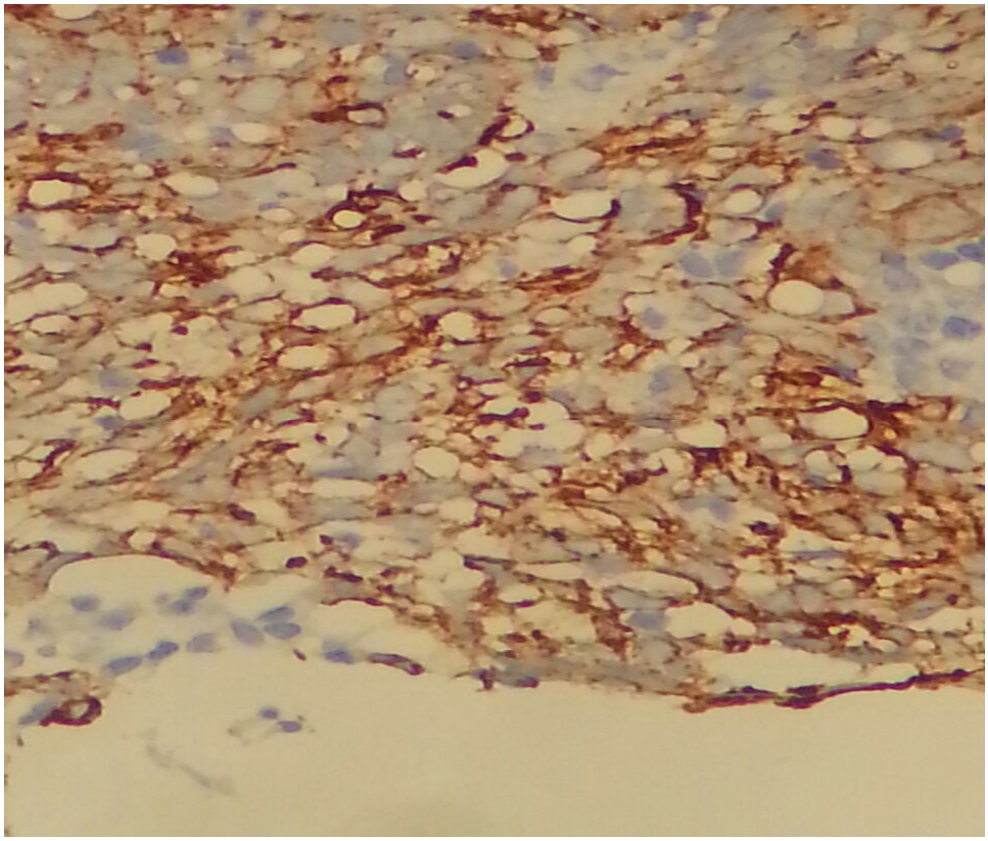


Photo .2.

Formalin fixed paraffin wax embedded section of Nasopharyngeal carcinoma immunostained for cytokeratin tumor marker using water bath heating and avidin biotin peroxidase diaminobenzidine labeling system, show cytoplasmic positivity (x 40).

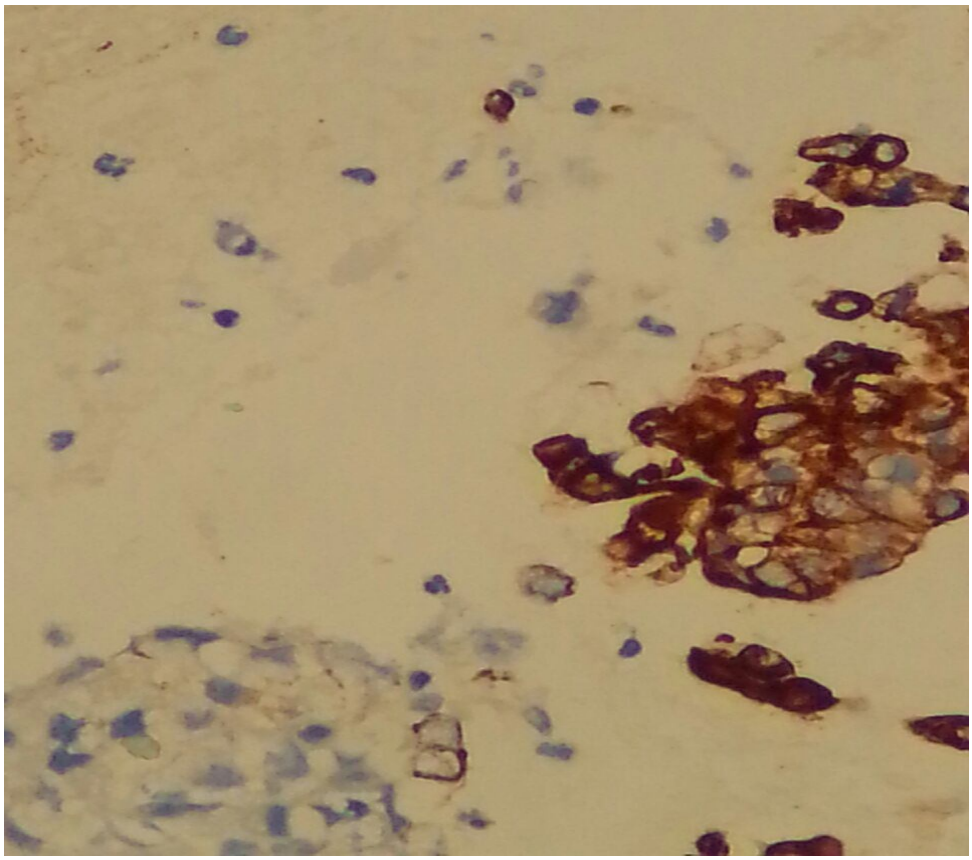
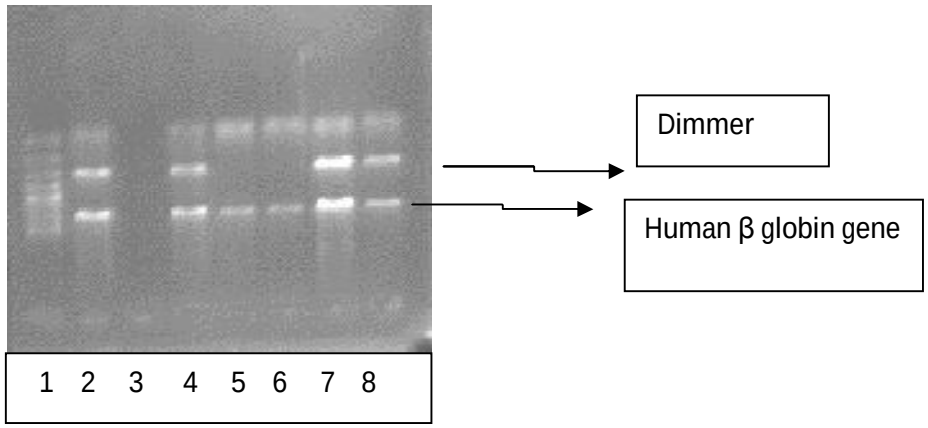


Photo .3

Formalin fixed paraffin wax embedded section of Nasopharyngeal carcinoma immunostained for EMA tumor marker using water bath heating and avidin biotin peroxidase diaminobenzidine labeling system, show cytoplasmic positivity (x 40).



5

Photo 4: Lane 1 modular weight marker (100bp) 1, 2 positive control, 3negative control for amplification, 4, 7 and 8 positive for EBV (290bp), 5 and 6 negative for EBV .

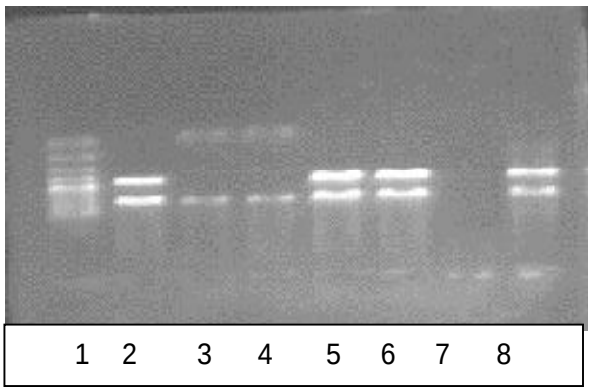


Photo 5: Lane 1 modular weight marker (100bp) 1, 2 positive control, 7negative control for amplification, 3, and 4negative for HSV (430 bp), 5 and 6 and 8 positive for HSV

CHAPTERFive

Discussion

Cancer continues to be the most serious disease thatafficts our life and become one of the most serious problem world wide, which gives a reason to be a targeted from researchers to find new ways of early diagnosis, treatment and follow up. Nasopharynx carcinoma (NPC) is the most common cancer originating in mucosal lining epithelium of the nasopharynx. World Health Organization classifies nasopharyngeal carcinoma in three types. Type I (squamous cell carcinoma), Type II (keratinizing undifferentiated carcinoma) and Type III (nonkeratinizing undifferentiated carcinoma)(Paul, et al. 2010).

Tumor markers are substances in the body that usually indicate the presence of cancer. These markers are usually specific to certain types of cancer and are usually found in the blood or/and tissue samples. Therefore, in this study we assessed the presence and validity of certain tumor markers that might be produced by nasopharynx tissues or by the body in response to the occurrence of NPC. These markers include pan cytokeratin and EMA. The sensitivity, specificity, positive predictive value and negative predictive value determine whether a marker will be useful in the screening and diagnosis of cancer. In this study the sensitivity, specificity, positive predictive value and negative predictive value of these markers were measured depending upon the results obtained by histopathology. While discussing this topic, it is important to bear in mind that; not all cancers produce tumor markers; having a high level of a marker does not always mean that a cancer is present. Because of this, tumor markers are not usually used to diagnose cancer. Instead, tumor markers are often used to monitor cancer after it has been diagnosed. It is also used to monitor the treatment of certain types of cancer. What is more, these facts do not rule out the role of some tumor markers in diagnosis of some cancers.

Cytokeratins, belonging to the intermediate filament protein family. This biomarkers are useful in oncology diagnostics. At present, more than 20 different cytokeratins have been identified, of which cytokeratins 8, 18, and 19 are the most abundant in simple epithelial cells. Upon release from proliferating or apoptotic cells, cytokeratins provide useful markers for epithelial malignancies, distinctly reflecting ongoing cell activity (Baraket al., 2004.It has been suggested that cytokeratin 18 is a potential biomarker for the differentiation and prognosis of NPC, and its dysregulation might play an important role in the pathogenesis of NPC (Li et al., 2009).

Cytokeratins are proteins of keratin-containing intermediate filaments found in the intracytoplasmiccytoskeleton of epithelial tissue. Cytokeratins are usually found in pairs: Basic or neutral cytokeratins include; CK1, CK2, CK3, CK4, CK5, CK6, CK7, and CK8. Acidic cytokeratins are; CK9, CK10, CK12, CK13, CK14, CK16, CK17, CK18, CK19 and CK20 (Schweizer, et al. 2006). Several studies have indicated the role of different types of CK in the subsequent management of NPC (Wei, et al. 2014; Li, et al. 2009). High levels of CK19-2G2 fragment expressed in tissue and serum are present in patients with nasopharyngeal carcinoma. The serum level of CK19-2G2 is helpful in the diagnosis of nasopharyngeal carcinoma and, the combination of serum CK19-2G2 and EB-VCA IgA improves the detection sensitivity (Lei, et al. 2012). Therefore, the aim of this study was to assess the role of CK expression and presence of these herpes viruses as possible etiological agents in NPC.

EMA is a member of the four trans-membrane superfamily and is believed to mediate transferring of miscellaneous proteins such as alpha6beta1 integrin and MHC class I to lipid raft microdomains. EMA has been recognized as a tumor suppressor gene in certain model systems. Normally, EMA is expressed at discrete locations in the body including high levels in the eye, lung, heart, thyroid, and uterus (Wadehra et al.,2003). Loss of EMA expression is common in NPC and it is

associated with adverse prognosticators and might confer tumor aggressiveness (Yi-Hsien et al., 2012).

Human epithelial membrane Antigene (*EMA*), mapped to chromosome 16, is well-maintained across vertebrates (Wang,et al,2001). EMP2 was detected as a novel member within four-transmembrane (tetraspan) superfamily (Berditchevski F,1999).Practically, the best known tetraspan proteins are connexins, which form the major structural element of gap junctions. Connexins play vitalparts in the regulation of cell growth and differentiation. Cancer cells generally have down regulated levels of gap junctions. Moreover,numerous lines of evidence suggest that loss of gap junctional intercellular communication is an important step in carcinogenesis. Re-expression of connexins in cancer cells causes normalization of cell growth control and reduced tumorgrowth(Kandouz,2010).

In the present study we evaluated the relationship between cytokeratins as factors that are involved in the management of PNC and Herpes viruses as a major causes involved in the development of PNC. Several studies have shown that many cytokeratins are potential biomarker for the differentiation and prognosis of NPC, and its dysregulation might play an important role in the pathogenesis of NPC (Li, et al. 2009; Lei, et al. 2012; Wei, et al. 2014). Since, we have applied a pan cytokeratin in this study, spectrum of cytokeratins have been expected to be expressed.

However, the relationship between Cytokeratin expression and Human herpes viruses (EBV or CMV) was found to be statistically significant. To the best of our knowledge there is no study correlated the relationship between these viruses and cytokeratins in NPC.

Cytokeratins, are useful protein markers which are related to epithelium tissues and their related tumors. There are more than 20 different cytokeratins, of which cytokeratins 8, 18, and 19 are the most frequently identified in simple epithelial cells. Upon release from proliferating or apoptotic cells, cytokeratins

provide useful markers for epithelial malignancies, specifically reflecting current cell activity (Vivian, et al. 2004). The expression of cytokeratins varies with epithelial cell type, degree of differentiation, and development of the tissue. Through the transformation of normal cells into malignant cells, the cytokeratin patterns are typically maintained, and this property has enabled cytokeratins to be applied as tumor markers (Chu, 2002). The processes that cause the release of soluble cytokeratin fragments have not yet been completely elucidated but appear to involve multiple pathways including proteolytic degradation of cytokeratins in dying cells, abnormal mitosis, spillover of monomeric cytokeratin polypeptides from proliferating cells, apoptosis, and/or neovascularization (Vivian, et al. 2004).

EBV is found to be associated with 100% of poorly or undifferentiated NPC, a tumor of epithelial origin. The latent membrane protein-1 (LMP1) of EBV may play a causal role in the development of this disease (Curran, et al. 2001). Expression of LMP1 in the epidermis of transgenic PyLMP1 mice induces hyperplasia, an early step in the carcinogenic process (Wilson et al. 1990). Furthermore, in cultured carcinoma cell lines, heterologous expression of LMP1 leads to low serum requirements, loss of anchorage dependence, increased invasive capacity, and, in some cases, inhibition of terminal differentiation (Nicholson et al. 1997). Furthermore, growth characteristics of NPC tumors have been correlated with LMP1 expression levels. Detectable LMP1 protein is linked with the expression of EGFR and Ki67 in NPC biopsies (Zheng, et al. 1994) and LMP1-positive NPC tumors appear to grow faster and more expansively than LMP1-negative NPC tumors (Hu, et al. 1995, Temple, et al. 2014)). These complex processes in the EBV carcinogenesity and cytokeratins development may express the correlation between these factors. However, one of limitations in this study is the use of pan cytokeratins which prevent the chance of identification of different cytokeratin types.

Human cytomegalovirus (HCMV), a widely-spread β-herpesvirus, is a major cause of birth defects. CMV a large DNA virus, blocks host DNA synthesis and deregulates cell cycle progression (Qian, et al. 2010). A major strategy employed by many DNA viruses to replicate their genomes is to promote host cell entry into S-phase in order to utilize the cellular resources needed for viral DNA synthesis. The CMV UL97 protein has cyclin-dependent kinase (CDK) activity, allowing the virus to inactivate Rb-family proteins and activate transcription of S-phase genes (Kamil, et al. 2009). How far these interactions between viral genome and epithelial host cells can indicate intracellular process that involved in the production of cytokeratins require further investigations.

Although, HSV showed no correlation with cytokeratins, but still there are some positive samples in the present study. The HSV virion carries its own specific transcription initiation factor (alpha-TIF), which functions together with other components of the cellular transcriptase complex to mediate virus-specific immediate early (IE) transcription. The virus-coded IE proteins are the transactivator and regulatory elements modulating early transcription and subsequent translation of nonstructural virus-coded proteins needed mainly for viral DNA synthesis and for the supply of corresponding nucleoside components (Rajcáni andDurmanová, 2000). Skin keratinocytes represent a primary entry site for herpes simplex virus type 1 (HSV-1) in vivo. The cellular proteins nectin-1 and HVEM act as efficient receptors for both serotypes of HSV and are sufficient for disease development mediated by HSV-2 in mice. How HSV-1 enters skin, and whether both nectin-1 and HVEM are involved, is not known (Petermann, et al. 2014).

Future prospect: Further studies on the exact relationship between Human Herpes viruses and cytokeratin expression is needed, which may help in patients management. Study of the correlations between herpes viruses and different cytokeratins types is required.

In conclusion: There is strong correlation between Herpes viruses EBV and CMV) and cytokeratin expression in NPC. Knowledge of the exact interaction between cytokeratins and these viruses may stimulate new ideas that help in prognosis, treatment and overall management of patients with NPC.

In the current study all three herpes viruses were significantly correlated with loss of EMA. With the beginning of molecular targeted therapy and personalized medicine, novel therapies based on molecular targets of NPC have become the focus of research and progress over the last decade. What's more, as NPC is closely associated with the EBV infection, the role of tumor-associated viral antigens in NPC renders it an interesting nominee for cellular immunotherapy (Tsang ,2014).The close association of EBV infection with NPC suggests that EBV infection is a crucial in the occurrence of this cancer. The difficulties encountered in transforming primary epithelial cells in experimental systems suggest that the role of EBV in epithelial malignancies is complex and multi-factorial in nature. Genetic alterations in the premalignant epithelium may support the establishment of latent EBV infection, which is believed to be an initiation event. Oncogenic properties have been reported in multiple EBV latent genes. However, improved proliferation may not be the crucial role of EBV infection in epithelial malignancies. Genetic alterations in host cells as well as inflammatory stroma could modulate expression of EBV gene expression and alter the growth properties of infected premalignant epithelial cells encouraging their selection during carcinogenesis(Tsao,2014).

For the other herpes viruses (CMV and HSV), there is a lack in the literature, regarding their relation to NPC. Human cytomegalovirus (HCMV), a widely-spread β-herpesvirus, is a major cause of birth defects and opportunistic infections in HIV-1/AIDS patients. HCMV displays an intricate system-wide modulation of the human cell proteome. An impressive array of virus-host protein interactions occurs throughout the infection (Jean Beltran ,2014). There are two

types of herpes simplex virus, type 1 and type 2 (HSV-1 and HSV-2). HSV-2 is a common human pathogenic virus and is associated with sexually transmitted diseases. Acute HSV-1 infection generally involves gingiva stomatiti(Shen,2006) . Several independent studies suggest that HSV-2 infections correlate with a higher than normal incidence of cervical cancer (Jones.,1995).

Human epithelial membrane Antegen (*EMA*), mapped to chromosome 16, is highly conserved across vertebrates (Liehr T. et al,1999)High EMA expression was detected in ovarian cancer through triggering of caveolins/glycosylphosphatidyl inositol-linked proteins (Wadehra,2004).and was recognized as an early predictor of endometrial cancers with unfavorableconsequence(Wadehra,et al,2006).Loss of EMA expression confers an independent prognosticator in nasopharyngeal carcinoma, which might confer tumor aggressiveness through hindering its interaction with specific membrane protein(s) and hence the downstream signal transduction pathway(s) (Chen ,et al,2012).EBV oncoproteins may contribute to the highly metastatic phenotype of NPC (Raab-Traub,2002). so clarifying their underlying mechanisms should shed light on therapeutic strategies to block malignant progression of this cancer.Latent membrane protein 2A (LMP2A) is an EBV oncoprotein generally expressed in NPC and other EBV-associated cancers (Knipe,2007).LMP2A is detected in around half of NPC tumor specimens at the protein level and in more than 95% of the tumors at the mRNA level (Heussinger. et al.,2004).

However, and to the best of our knowledge no study have investigated the exact interaction (s) between EMA and Herpes viruses. So this study is a stimulation for further research in this area.

Although, there is are numerous malignant tumors that may arise in the nasopharynx, NPC represent the most predominant type. NPC was subdivided according to WHO histopathological grading system into: Keratinizing squamous cell carcinoma (Type I), Nonkeratinizing squamous cellcarcinoma (Type II)

andUndifferentiated carcinoma (Type III, most common subtype) (Shanmugaratnamet al., 1991;Bernadette Brennan, 2006).

In the present study the most common subtype is Type III followed by Type II, which is similar to the findings of several studies (Richard et al., 2002).

The clinical importance of measuring soluble cytokeratin in body fluids lies in the early detection of recurrence and the fast assessment of the effectiveness of therapy response in epithelial cell carcinomas. The three most applied cytokeratin markers used in the clinic are tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS), and CYFRA 21-1. TPA is a broad spectrum test that measures Cytokeratins 8, 18, and 19. TPS and CYFRA 21-1 assays are more specific and measure cytokeratin 18 and cytokeratin 19, respectively. Although the main use of these markers is to monitor treatment and evaluate response to therapy, early prognostic information particularly on tumor progression and metastasis formation is also provided for several types of cancers. Cytokeratin tumor markers can accurately predict disease status before conventional methods and offer a simple, noninvasive, cheap, and reliable tool for more efficient management (Barak, 2004). Therefore, in the present study we found very high immunoexpression of CKs in NPC, which may indicate the release of some CK protein fragment in the blood. Consequently, such body fluids measures may be very important in early prediction of NPC cancerous change. This because NPC is difficult to detect early. That's probably because the nasopharnyx isn't easy to examine and symptoms of nasopharyngeal carcinoma mimic those of other more common conditions (Keiji et al., 2011).

The positivity of tumor cells for pan-cytokeratin established the final diagnosis of non-keratinizing undifferentiated carcinoma (Boia et al., 2013). High levels of CK19-2G2 fragment expressed in tissue and serum are present in patients with NPC. The serum level of CK19-2G2 is supportive in the diagnosis of NPC. Furthermore, the combination of serum CK19-2G2 and EB-VCA IgA improves the

detection sensitivity (Lei et al., 2012). Furthermore, it was suggested that cytokeratin 18 is a potential biomarker for the differentiation and prognosis of NPC, and its dis-regulation might play an important part in the pathogenesis of NPC.

EMA plays a tumor suppressor role through interacting with specific integrin(s) in epithelial cells and, thereafter, manages regular signaling transduction in benign conditions.Loss of EMA expression (49.2%) was correlated with advanced primary tumor (p=0.044), nodal status (p=0.045) and the 7th American Joint Committee on Cancer stage (p=0.027). In multivariate analyses, loss of EMP2 expression emerged as an independent prognosticator for worse disease-specific survival (DSS; p=0.015) and local recurrence-free survival (LRFS; p=0.030), along with the American Joint Committee on Cancer stages III–IV (p=0.034, DSS; p=0.023, LRFS) (Yi-Hsien et al., 2012).Although the exact features of the EMA protein in NPC progression continue to be clarified, the potential value of EMA as a prognostic biomarker in NPCs is guaranteed (Xu et al., 2011).

Nevertheless, and to the best of authors' knowledge, no previous study showed a correlation between CK and EMA immuno-expression in NPC or other cancer. However, from the major limitations in this study was the use of small number of CK negative samples, which didn't give us a reliable assessment of the exact relation between EMA and Pan CK.

Regarding the relationship between expression of CK and/or EMA and NPC subtypes, the loss of expression of both markers significantly seen in type III. Though Subtype III represent the highest frequency in this study, there is still literature gap in this context.

ChapterSix

Conclusion and recommendations

6.1. Conclusion:

On the basis of this study we conclude that:

- The most frequent type of NPC among Sudanese patient is Type III followed by Type II .
- The frequency of NPC among the gender of Sudanese patient is 2 :1 males to females patents with NPC .
- Most patient with NPC in Sudan were older than 50 years.
- There is strong correlation between Herpes viruses EBV and CMV and cytokeratin expression in NPC. Knowledge of the exact interaction between cytokeratins and these viruses may stimulate new ideas that help in prognosis, treatment and overall management of patients with NPC.
- There is a high loss of EMA in NPC, which is corresponding to high NPC types. Loss of CK expression is relatively linked to high NPC types. Further studies to highlight the biological interrelation between theses markers and NPC is deemed necessary.

6.2 Recommendations:

On the basis of this study we recommend that:

- Studies should be continued to determine other markersspecialy for type one NPC .
- Molecular study should be done to detect more viruses that may cause NPC.
- Further studies should be done including the demographical factors of patients such as residence, occupation, family history.

References

1. Abuidris(2008). Histopathological patterns of nasopharyngeal carcinoma in Sudan. *Saudi Medical Journal*; 29:7.
2. Adamas, NV. S. (1968). The transverse dimensions of the nasopharynx in child and adult with and potential implications. *J ClinPathol* 58:535–538
3. Ahlbom, A., Day, N.2000. pooled analysis of magnetic fields and childhood leukaemia. *British Journal of Cancer*.**83:** 692- 698.
4. Akhter K;, Aug 2011 Cytomegalovirus, Medscape.
5. Al-Sarraf M, LeBlanc M, Giri PG, (1998) Chemotherapy versus radiotherapy in patients with advanced nasopharyngeal
6. Ambinder, R., Robertson K., Moore, S. and Yang J. Epstein–Barr virus as a therapeutic target in Hodgkin's disease and nasopharyngeal carcinoma. *Seminars in Cancer Biology*.7: 217- 226. 1996.
7. American Cancer Society, (2013). *Nasopharyngeal cancer. Retrieved 25th January.*
8. American Cancer Society:(2002) Cancer Facts and Figures.
9. Bancroft JD, Gamble M. Theory and practice of histological techniques5th ed. Churchil Livingstone: London .2002.877-897.
10. Barak V, Goike H, Panaretakis KW, Einarsson R.(2004) Clinical utility of cytokeratins as tumor markers. *ClinBiochem.* Jul;37(7):529-40.
11. Barrett L, Fowke KR, Grant MD(2012)Jul. Cytomegalovirus, aging, and HIV: a perfect storm. *AIDS* Rev.;14(3):159-67
12. Baumforth, K., Young, L., Flavell, K., Constandinou, C. and Murray P.G. (1999).The Epstein-Barr virus and its association with human cancers. *Molecular Pathology*.**52:** 307–322.

13. Begin, R., Eskandari, J., Joncas, J. and Panasci, L. Epstein-Barr virus Berditchevski F, Odintsova E.(1999) Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J Cell* Biol;146:477–92.
14. Blot, W.J. (1988). Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res*., 48:3282–7.
15. Boeckh M; (2011)Complications, diagnosis, management, and prevention of CMV infections: current and future. Hematology Am SocHematolEduc Program.:305-9.
16. Boia ER, Boia M, Balica NC, Rusu LC, Mazilu O, Solovan C, Baderca F.(2013) Non-keratinizing undifferentiated carcinoma of the nasopharynx. *Rom J MorpholEmbryol*.;54(3 Suppl):839-43.
17. Bryant, V. S. (2006). The transition of the ciliated epithelium of the nose into the squamouscancer: phase III randomized intergroup study *J ClinOncol* 16:1310–1317.
18. Chan JKC, Pilch BZ, Kuo TT (2005) Nasopharyngeal carcinoma. In: Barnes L, Eveson JW, Reichart P, Sidransky .
19. Chen YH, Wu LC, Wu WR,(2012) Loss of epithelial membrane protein-2 expression confers an independent prognosticator in nasopharyngeal carcinoma: a cohort study. *BMJ Open.* 5;2(2)
20. Cheung, A. and Kieff, E. (1982)Long internal direct repeat in Epstein- Barr virus DNAs. *the Journal of Virology*.**44:** 286–294.
21. Christian, M. and Moormann, A,(2008) Immune escape by Epstein–Barr virus associated malignancies. *Seminars in Cancer Biology***18:** 381–387.

22. Chu PG and Weiss (2004). Induces Epithelial Cell Proliferation and Sensitizes Transgenic Mice to Chemical Carcinogenesis. Cancer Res *61;* 6730*Clinical Cancer Research*. **10:** 803–821.
23. Curran JA, Laverty FS, Campbell D, Macdiarmid J, and Wilson JB(2001).Epstein-Barr Virus Encoded Latent Membrane Protein-Dekker, New York, pp 453–466
24. De la Chica, R., Ribas, I., Giraldo, J., Epozcue, J., Fuster, C.(2005) Chromosomal instability in amniocytes from fetuses of mothers who smoke. *TheJournal of the American Medical Association*. **10:** 293: 12-22.
25. Decker J, Goldstein JC: (1982)Risk factors in head and neck cancer. N Engl J Med 306 (19): 1151-5.
26. Elbashir S.M., (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cell*s. Nature* 411:494–49*8.*
27. Fletcher CDM (2009).Diagnostic Histopathology of Tumors, 3rd edn. Elsevier, London, pp 83–149
28. Frazer, J. E. (2003 a). The pharyngeal end of Ratlhke's pouch. .1. Anat. Physiol., Lond., 45,190-196.
29. Friedmiann, I. (2001). Electron microscopy of human biopsy material. Proc. R. Soc. Med. 54,
30. Gastpar H, Wilmes E, Wolf H. (1981)Epidemiologic, etiologic and immunologic aspects of nasopharyngeal carcinoma [NPC]. *J Med*. ;DA - 19820212(4):257-84.
31. Glanzmann C, Aberle HG, Horst W (2001). Radiotherapy results of nasopharyngeal carcinomas (41 patients)*J ClinOncol* 16:1310–1317.
32. Gullo C, Low WK, Teoh G (2008) Association of Epstein–Barr virus with nasopharyngeal carcinoma and current status of development of cancer-derived cell lines. *Ann Acad Med Singapore* 37(9):769–777.

33. Henle G, Henle W. (1976)Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. *Int J Cancer*. Jan 15 ;17(1):1-7.
34. Heussinger N, (2008.)Expression of the Epstein-Barr virus (EBV)-encoded latent membrane protein 2A (LMP2A) in EBV-associated nasopharyngeal carcinoma. J. Pathol. 2004;203:696–699.68: (19).
35. Hidayatalla (1983). Studies on nasopharyngeal carcinoma in the Sudan: Epidemiology and Aetiology. *Eur. J. Cancer Clin. Oncol*; 19 (6): 705-10
36. Hildesheim A, Wang CP (2012). Genetic predisposition factors and nasopharyngeal carcinoma risk: a review of epidemiological association studies, 2000–2011: Rosetta Stone for NPC: genetics, viral infection, and other environmental factors . Semin. *Cancer Biol.* 22 (2) , 107 – 116 .
37. HoffrandV,PetitJ,MossP.Essential.(2001) haematology.4th ed.Blackwell science.UK.149-150.
38. Hu LF, Chen F, Zhen QF, Zhang YW, Luo Y, Zheng X, Winberg G, Ernberg I, Klein G (1995). Differences in the growth pattern and clinical course of EBV-LMP1 expressing and non-expressing nasopharyngeal carcinomas. Eur. J. Cancer, 5:658-660.
39. Hu, Reymond J L,Pinel N, Zabot M T , Urban Z.(2006.) Inflammatory destruction genes .Journal of investigate dermatology. *J ClinOncol* 16:1310–1317.
40. IARC (2014). Cancer Pathology and Genetics. *Pathology and Genetics of Head and Neck Tumours.* IARC Publications, Lyon, France. www.iarc.fr/en/publications/pdfs-online/pat-gen/bb9/index.php
41. International Agency for Research on Cancer: Epstein–Barr Virus and p 2655–2700.(2007). In Knipe DM, Howley PM (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA

42. Jean Beltran PM, Cristea IM.(2014) The life cycle and pathogenesis of human cytomegalovirus infection: lessons from proteomics. Expert Rev Proteomics.10. 18:1-15.

43. Jia WH, (2005) Complex segregation analysis of nasopharyngeal carcinoma in Guangdong, China: evidence for anon-expressing nasopharyngeal carcinomas. Eur. J. Cancer, 5:658-660.

44. Jia, W.H., and Qin, H.D. (2012). Non-viral environmental risk factors for nasopharyngeal carcinoma: a systematic review. Semin. *Cancer Biol*. 22, 117–126.

45. Johnson, F. R. (1961). Characteristics of epithelia. In Recent Advances in Anatomy. Eds.a Bing Li XueZaZhi.;41(7):461-5.

46. Jones C. Cervical cancer: is herpes simplex virus type II a cofactor? Clin. Microbiol. Rev. October 1995 vol. 8 no. 4 549-556.

47. Kamil JP, Hume AJ, Jurak I, Munger K, Kalejta RF, et al (2009). Human papillomavirus 16 E7 inactivator of retinoblastoma family proteins complements human cytomegalovirus lacking UL97 protein kinase. ProcNatlAcadSci U S A. 106:16823–16828.

48. Kandouz M, Batist G.(2010) Gap junctions and connexins as therapeutic targets in cancer. Expert OpinTher Targets;14:681–92.20:508–511

49. Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8. Lyon: World Health

50. Karjala Z, Neal D, Rohrer J; (2011)Association between HSV1 seropositivity and obesity: data from the National Health and Nutritional Examination Survey, 2007-2008. PLoS One. 11;6(5).

51. KeijiTabuchi, Masahiro Nakayama, Bungo Nishimura, Kentaro Hayashi, andAkira Hara. Early Detection of Nasopharyngeal Carcinoma. International Journal of Otolaryngology Volume(2011), Article ID 638058, 6 pages.
52. Kenneth, J., Ryan, D. and George, R. *Sherris Medical Microbiology*. 4th ed.
53. Kietly C M, Sheratt M J, Shuttle CA. (2002).Elastic fibers .Journal of cell biology.
54. Knipe DM, Howley PM, Rickinson AB, Kieff E.(2007) Epstein-Barr virus, p 2655–2700. In Knipe DM, Howley PM (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
55. Kramer M (2008); Ethnic differences in HSV1 and HSV2. Eurosurveillance, 13, Issue (24).
56. LabrecqueG,BarneM,FentimanS,Griffin E.(1995) Epstein barr virus in epithelial cell tumors.*Cancer research*.55:.39-45.
57. Lei DS, Yu J, Tong XL, Wang MW, Wang K, Chen H.(2012) Diagnostic value of cytokeratin 19 fragment in nasopharyngeal carcinoma. a Bing Li XueZaZhi.;41(7):461-5.
58. Leung SF, Zee B, Ma BB, Hui EP, Mo F, Lai M, (2006)Plasma Epstein-Barr viral deoxyribonucleic acid quantitation complements tumor-node-metastasis staging prognostication in nasopharyngeal carcinoma. *J ClinOncol* 1 ;24(34):5414-8.
59. Li XM, Huang WG, Yi H, Cheng AL, Xiao ZQ (2009). Proteomic analysis to identify cytokeratin 18 as a novel biomarker of nasopharyngeal carcinoma. *J Cancer Res Clin Oncol*.;135(12):1763-75.
60. Liehr T, Kuhlenbaumer G, Wulf P, (1999)Regional localization of the human epithelial membrane protein genes 1, 2, and 3 (EMP1, EMP2, EMP3) to 12p12.3, 16p13.2, and 19q13.3. Genomics;58:106–8.

61. Little, J.(1999)*Epidemiology of childhood cancer*. IARC Scientific Publications. Vol 149, Lyon, France, IARC.
62. LM (2002). Keratin expression in human tissues and neoplasms. Histopathology, 40 : 403–439.
63. Lutzky,(2008). Biomarkers for Cancers of the Head and Neck. *Clinical Medicine: Ear, Nose and Throat*; 1.
64. Marks JE, Phillips JL, Menck HR (1998) The National Cancer Data Base report on the relationship of race and national
65. Matthew, P., Thompson and Razelle, K.(1997) Epstein-Barr Virus and Cancer. multifactorial mode of inheritance (complex segregation analysis of NPC in China). Eur J Hum Genet 13(2):248–252
66. Munoz Sanchez PJ, Capote Femenias JL, Diaz Tejeda A; The effect of 670-nm low laser therapy on herpes simplex type 1. Photomed Laser Surg. 2012 Jan;30(1):37-40. doi: 10.1089/pho.2011.3076. Epub 2011 Nov 2.
67. MustofiFK,DaviscJJ, sesterlenn 1A (1992). Pathology of the carcinoma of the prostate cancer.,70:235-53.
68. Nicholson LJ, Hopwood P, Johannessen I, Salisbury JR, Codd J, Thorley-Lawson D, Crawford DH (1997). Epstein-Barr virus latent membrane protein does not inhibit differentiation and induces tumorigenicity of human epithelial cells. *Oncogene,* 15:275-283.
69. Opstelten W, Neven AK, Eekhof J; (2008)Treatment and prevention of herpes labialis. Can Fam Physician. Dec;54(12):1683-7.
70. Parkin, D.M., Bray, F., Ferlay, J. and Pisani, P. (2005). Global cancer statistics, 2002. *CA Cancer J. Clin.*, 55:74–108.
71. Parkin, D.M., Bray, F., Ferlay, J. and Pisani, P. (2006). Global cancer statistics, 2002. *CA Cancer J. Clin.*, 55:74–108.

72. Pathmanathan R, Prasad U, Sadler R, et al (1995) Clonal proliferations of cells infected with Epstein–Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. N Engl J Med 333:693–698.
73. Paul W. Flint, Bruce H. Haughey, Valerie J. Lund, John K. Niparko, Mark A. Richardson (2010). observations on its contractile function. J. Lar. Otol. 72, 465-471.*Oncology.* 36: 280-283,
74. Petermann P, Thier K, Rahn E (2014). Entry mechanisms of Herpes Simplex Virus Type 1 into murine epidermis: Involvement of nectin-1 and HVEM as cellular receptors. J Virol. JVI.02917-14.
75. Peters, J., Preston-Martin, S., London, S., Bowman, J., Buckley, J. and Thomas, D. (1994) Processed meats and risk of childhood leukaemia. *Cancer Causes and Control*. **5**(2): 195-202..Press, Lyon, pp 83–97
76. Qian Z, Leung-Pineda V, Xuan B, Piwnica-Worms H, Yu D (2010). Human cytomegalovirus protein pUL117 targets the mini-chromosome maintenance complex and suppresses cellular DNA synthesis.*PLoSPathog.* 19;6(3):e1000814.
77. Raab-Traub N. (2002)Epstein-Barr virus in the pathogenesis of NPC. Semin. *Cancer Bio;*12:431–441.
78. Rajcáni J, Durmanová V (2000). Early expression of herpes simplex virus (HSV) proteins and reactivation of latent infection. *Folia Microbiol*(Praha).;45(1):7-28.

79. Schweizer J, Bowden PE, Coulombe PA, (2006). "New consensus nomenclature for mammalian keratins". The Journal of Cell Biology 174 (2): 169–74.

80. Serhat I, Neecmettin K, Metin K , Orhan O. Facial linear focal elastosis. *International journal of dermatology* .2010.20.

81. Shanmugaratnam K, Chan SH, de-Thé G, (1979) Histopathology of nasopharyngeal carcinoma: histology of nasopharyngeal carcinoma Cancer 83(3):582–588.Otolaryngology. 5th ed. Chapter 99. pg 1344.

82. Shedd DP, von Essen CF, Eisenberg H (1999) Cancer of the nasopharynx in Connecticut, 1935 through 1959.Global cancer statistics, 2002. *CA Cancer J. Clin*., 55:74–108.

83. Shen Y and J Nemunaitis J.(2006) Herpes simplex virus 1 (HSV-1) for cancer treatment. *Cancer Gene Therapy* 13, 975–992.

84. Shibata D,Weiss l(1992.) Epstein barr virus associated gastric adeno carcinoma. American J P.140:769-774.

85. Srinivasan M, Sedmak D, Jewell S .Effect of fixatives and tissue processing on the content and Integrity of nucleic acids. American journal of pathology. 2013.451-266.

86. Sugano H, Sakamoto G, Sawaki S, Hirayama T. (1978). Histopathological types ofnasopharyngeal carcinoma in a low-risk area: Japan. In: de The´ G, Ito Y,editors. Nasopharyngeal carcinoma: etiology and control. *IARC scientific publications no. 20. Lyon*: IARC p. 27 – 39.

87. Tan KB, Putti TC (2005) Cyclooxygenase 2 expression in nasopharyngeal carcinoma: immunohistochemical findingsStrahlentherapie 152:310–315.

88. Temple RM, Zhu J, Budgeon L, Christensen ND, Meyers C, Sample CE. (2014)Efficient replication of Epstein-Barr virus in stratified epithelium in vitro. ProcNatlAcadSci U S A.13. Tsang J, Lee VH, Kwong DL. (2014)Novel therapy for nasopharyngeal carcinoma--where are we. *Oral Oncol.*;50(9):798-801.

89. Tsao SW, Tsang CM, To KF, Lo KW. (2014)The role of Epstein-Barr virus in epithelial malignancies. *J Pathol.* 24. doi: 10.1002.

90. Ushiki T.(2002.) Collagen fibers, reticular fibers and elastic fibers a comprehensive understanding from morphological view point .*Journal of archives of histology and cytology* .

91. Vasef MA, Ferlito A, Weiss LM (1997) Nasopharyngeal carcinoma, with emphasis on its relationship to Epstein–Barrvirus. *Ann OtolRhinolLaryngol*106(4):348–356

92. Vivian Barak, Helena Goike, Katja W. Panaretakis, Roland Einarsson (2004). Clinical utility of cytokeratins as tumor markers.*Clinical Biochemistry* ; 37(7): 529–540.

93. Wadehra M, Goodglick L, Braun J. (2004)The tetraspan protein EMP2 modulates the surface expression of caveolins and glycosylphosphatidyl inositol-linked proteins. *MolBiol Cell;*15:2073–83.

94. Wadehra M, Natarajan S, Seligson DB, (2006)Expression of epithelial membrane protein-2 is associated with endometrial adenocarcinoma of unfavorable outcome.*Cancer*107:90–8.

95. Wan SK, Chan JK, Lau WH, (1995) Basaloid-squamous carcinoma of the nasopharynx. An Epstein–Barr virus-associated neoplasm compared with morphologically identical tumors occurring in other sites. *Cancer* 76:1689–1693

96. Wang CX, Wadehra M, Fisk BC,(2001)Epithelial membrane protein 2, a 4-transmembrane protein that suppresses B-cell lymphoma tumorigenicity. *Blood;*97:3890–5.

97. Wei Z, Zeng X, Xu J, Duan X, Yang J, Xie Y (2014). Prognostic value of the pretreatment serum level of cytokeratin fraction 21-1 in undifferentiated nasopharyngeal carcinoma: a study of 332 cases. *Head Neck.*;36(1):71-6.

98. Weiland LH (1985) Nasopharyngeal carcinoma. In: Barnes LSurgical pathology of the head and neck. Marcel"New consensus nomenclature for mammalian keratins". The Journal of Cell Biology 174 (2): 169–74.

99. Wenig BM (2007) Tumors of the upper respiratory tract: part A: nasal cavity, paranasal sinuses and nasopharynx. *Clinical Biochemistry* ; 37(7): 529–540.

100. Wilson JB, Weinberg W, Johnson R, Yuspa S, Levine A J (1990). Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. *Cell,* 61:1315-1327.

101. World Health Organization(2003)classification of tumors.Pathology and genetics. Head and neck tumors. IARC1064-1071.

102. Xu HM, Liang Y, Chen Q, (2011). Correlation of Skp2 overexpression to prognosis of patients with nasopharyngeal carcinoma from South China. *Chin J Cancer*;30:204–12

103. Yi-Hsien Chen, Li-Ching Wu, Wen-Ren Wu, et al.(2012) Loss of epithelial membrane protein-2 expression confers an independent prognosticator in nasopharyngeal carcinoma: a cohort study. *BMJ*;2.

104. Zeka, A., Gore, R. and Kriebel, D. (2003). Effects of alcohol and tobacco on aerodigestive cancer risks: a meta-regression analysis. *Cancer Causes Control*, 14:897–906

105. Zheng X, Hu L, Chen F, Christensson B (1994). Expression of Ki67 antigen, epidermal growth factor receptor and Epstein-Barr virus-encoded latent membrane protein (LMP1) in nasopharyngeal carcinoma. *Eur. J. Cancer B Oral Oncol.*, 30B:290-295.

106. ZurHausen, H., O'Neill, F.J., Freese, U.K., and Hecker, E. (1978). Persisting oncogenic herpesvirus induced by the tumour promotor TPA. *Nature* 272, 373–375.

Appendices

Materials and instruments

Materials and instruments used for collection and of processing of specimens include:

- Disposable syringes
- Disposable gloves
- Disposable plastic containers
- Neutral buffered formalin
- Ethanol (Absolute, 90%, 70%, 50%)
- Xylene
- Paraffin wax
- Mould
- Cassettes
- Pencil
- Forested slides (75 x 25x 2mm)
- Rotary microtome
- Water bath
- Dry oven

Materials and instruments used for staining the histopathology specimen include

- Coplin jars
- Cover glass (22x 50mm). DPX mounting media
- Mayer's Haematoxylin component (Haematoxylin , Distilled water, Potassium Alum, Sodium iodate, Citric acid, Chloral hydrate)
- Eosin component (Eosin Y, D.W)

Materials and instruments used for immunohistochemistry staining include

- Forested slides coated with (0.01% aqueous high molecular weight poly-L - lysine)
- Citrate buffer (6.8pH) component (sodium di-hydrogen orthophosphate, di-sodium hydrogen orthophosphate, cetric acid, D. W)
- Phosphate buffer (7.4pH) component (sodium di-hydrogen orthophosphate, di-sodium hydrogen orthophosphate, D.w)
- Peroxidase blocking solution component (casein, PBS, sodium azide)
- Anti CK rabbit antibody
- Anti EMA rabbit antibody
- Avidin – biotin complex
- 1 vial (25ml) Biotinated secondary antibody
- 1 vial (25ml) streptoavidin conjugated horseradish peroxidase
- DAB medium (3.3 diaminobenzidine in chromogen solution)
- DAB substrate buffer (PH 7.5) containing hydrogen peroxide and antimicrobial agent
- Mayer's Haematoxylin

Materials and instrument used for circulating tumor markers

- Precision micropipettes
- Vortex mixer
- Oscillating plat form shaker
- Aspiration system
- Gamma counter set for I^{25}

Preparation of solutions

- Mayer's Haematoxylin

Haematoxylin	1g
Distilled water	1000ml

Potassium Alum	50g
Sodium iodate	0.2g
Citric acid	1g
Chloral hydrate	50g

- Eosin

Eosin Y	1g
D.W	100ml

Citrate buffer (6.8pH)

- 72.7ml of solution A, which prepared from

  0.2M sodium di-hydrogen orthophosphate

  2.83g di-sodium hydrogen orthophosphate in 100ml D. W
- 22.8 ml of solution B, which prepare from

  0.1M cetric acid

  2.1g citric acid in 100ml D.W

- Phosphate buffer (7.4pH)
  - 9.5ml from solution A, which prepared from

    0.2 M sodium di-hydrogen orthophosphate

    3.12g di-sodium hydrogen orthophosphate in 100mL D.w
  - 40.5 ml from solution B, which prepared from

    0.2M sodium di-hydrogen orthophosphate

    2.83g di-sodium hydrogen orthophosphate in 100mL D.W
- Peroxidase blocking solution

  0.25% casein in PBS

  0.015 mol/L sodium azide