Sudan University of Science and Technology

College of Graduate Study

Frequency of Human Papilloma Virus (16 and 18) and Epstein Bar Virus among Sudanese patients with Esophageal Cancer using Immunohistochemistry and Polymerase Chain Reaction

تردد فيروس الورم الحليمى البشرى (16 و 18) وفيروس الابشتين بار المعدل مع سرطان المرئ لدى المرضى السودانين باستخدام الكيمياء النسيجية المناعية وتفاعل البلمرة المتسلسل

A thesis submitted for fulfillment of the requirement of PhD degree in medical laboratory science (histopathology and cytology)

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الآيــــه

قَالَ تعَالَىٰ:

بسم الله الرحمن الرحيم

﴿ وَيَسۡـَٔلُونَكَ عَنِ ٱلرُّوحِۖ قُلِ ٱلرُّوحُ مِنۡ أَمۡرِ رَبِّي وَمَآ أُوتِيتُم
مِّنَ ٱلۡعِلۡمِ إِلَّا قَلِيلٗا ٨٥ ﴾

صدق الله العظيم

سورة الإسراء الآيات (85)

I dedicate this work to all Muslims all over the world

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Abstract

This study was conducted as retrospective case study aimed to determine the frequency of HPV (16 and 18) and EBV among Sudanese with esophageal cancer using immunohistochemistry and PCR**.** The study included one hundred and two 102 paraffin blocks from patients previously diagnosed as esophageal cancer from Khartoum Sudan, in Ibn Sina hospital, khartoum hospital, soba teaching hospital, military hospital and national health laboratory. Their age ranged from 21 to 98 years old with mean age 59 years. 46 (45 %) were female and 56(55%) were male. The data were collected from examination of biopsies and patients records. All esophageal cancer biopsies were examined and classified into histopathological pattern using hematoxylin & eosin standard method. 91(89.2%) samples were esophageal squamous cell carcinoma and 11(10.8%) samples were adenocarcinoma. Regarding IHC staining for HPV (16 and 18), positive findings were revealed 14 (13.7%) and couldn't be disclosed in 88 (86.3%) of the study subjects, the highest positive results were found in age group 66-75 years, of the 56 males with esophageal cancer, 7 (12.5%) samples were identified with HPV16, 18 infection and the remaining 49(87.5%) samples found without HPV (16 and 18) infection. Of 46 females positive revealed in 7(13%) samples and the remaining 39(87 %) samples were found negative for HPV (16 and 18).

IHC staining for EBV, positive findings were revealed 22 (21.5%) and 80 (78.5%) of the study subjects were negative. Of the 56 males with esophageal cancer, 15/56 (27%) were identified with EBV infection and the remaining 41/56(73%) without EBV infection. Of the 46 females with esophageal cancer, 7/46(13%) were found positive for EBV immunostaining and the remaining 39/46(87 %) were found negative for EBV, with age distribution, the highest positive results were found in age group 56-65 years, representing 7/23(32%).

Of 102 subjects, 25(24.5%) were found positive for HPV 16 by PCR while 77 (75.5%) were found negative, HPV 18 reveals no positivity.

Of 102 subjects, 6/102(6%) were found positive for EBV by PCR while 96 (94%) were found negative.

The study shows that there was moderate frequency of HPV16 and EBV in esophageal cancer although no evidence indicates the frequency of HPV 18 with esophageal cancer.

The sensitivity and specificity of IHC method for HPV (16 and 18) when PCR regarded as the gold standard method was 28% and 90.9% respectively.

The majority of cancers were squamous cell carcinoma, Aggressiveness of cancer increased with age.

It could be concluded that there was obvious frequency of HPV16 and EBV in esophageal cancer among Sudanese patients, therefore further studies with wide scope in this topic were recommended.

مستخلص

هذه دراسة صممت كدراسة تراجعية لدراسة حالة هدفت لتحديد تردد فيروس الورم الحليمى البشرى (16 و 18) وفيروس الابشتين بار المعدل مع سرطان المرئ لدى السودانين باستخدام الكيمياء النسيجية المناعية وتفاعل البلمرة المتسلسل. اشتملت هذة الدراسة على 102 كتلة برافين من مرضى مشخصين مسبقا" بسرطان المرئ فى الخرطوم-السودان بمستشفى الخرطوم، مستشفى ابن سينا، مستشفى سوبا، المستشفى العسكرى والمعمل القومى للصحة العامة. اعمارهم فى المدى بين 21 – 98 سنة بمتوسط عمرى 59 سنة. 46(45%) كانوا اناثا" 56(55%) هم الذكور. المعلومات جمعت من تشخيص الخزعات وسجلات المرضى. كل خزعات سرطان المرئ تم تشخيصها و تصنيفها للانماط النسيجية باستخدام صبغة الهماتوكسلين والايوسين بالطريقة القياسية. 91(89.2%) هم مصابين بسرطان المرئ ذى الخلايا الظهارية، و11(10.8%) هم مصابين بسرطان المرئ الغدى.

ما يخص صبغة الكيمياء النسيجية المناعية لفيروس الورم الحليمى البشرى (16و 18) العينات الايجابية كانت فى 14(13.7%) ولم تظهر ايجابية فى 88(86.3%) من عينات الدراسة. أعلى ايجابية كانت فى المجموعة العمرية بين 66 – 75 سنة. من 56 ذكور مصابين بسرطان المرئ 7(12.5%) كانت ايجابية لفيروس الورم الحليمى البشرى(16و18) والمتبقى 49(87.5%) كانت سلبية. من 46 اناث مصابين بسرطان المرئ 7(13%) كانت نتائجهم ايجابية لفيروس الورم الحليمى البشرى والمتبقى 39(87%) كانت نتائجهم سلبية. صبغة الكيمياء النسيجية المناعية لفيروس الابشتين بار المعدل الايجابية ظهرت فى 22(21.5%) بينما80(78.5%) من عينات الدراسة كانت سلبية. من 56 ذكور مصابين بسرطان المرئ 15(27%) كانوا ايجابين للابشتين بار المعدل والمتبقى 41(73%) لم تظهر عليهم الاصابة. من اجمالى 46 اناث مصابين بسرطان المرئ 7(13%) كانوا ايجابين لفيروس الابشتين بار المعدل بصبغة الكيمياء الهستولوجية المناعية والمتبقى 39(87%) كانوا سلبين. مع التوزيع العمرى اعلى ايجابية كانت فى المجموعة العمرية بين 56 – 65 سنة وتمثل 7(32%). من 102 مريض 25(24,5%) كانوا ايجابين لفيروس الورم الحليمى البشرى 16 بتفاعل البلمرة المتسلل فى حين 77(75.5%) كانوا سلبين. أما فيروس الورم الحليمى البشرى 18 لم يظهر ايجابية بواسطة تفاعل البلمرة المتسلل. من 102 مريض 6(6%) كانوا ايجابين للابشتين بار المعدل بتفاعل البلمرة المتسلل و96(94%) كانوا سلبين.

الدراسة اوضحت ان هناك تردد متوسط لظهور فيروس الورم الحليمى البشرى 16 وفيروس الابشتين بار المعدل مع سرطان المرئ، مع انه ليس هناك مؤشرات تردد ظهورلفيروس الورم الحليمى البشرى 18 مع سرطان المرئ.

الحساسية والنوعية للكيمياء النسيجية المناعية عند اعتبار تفاعل البلمرة المتسلسل هو الطريقة الذهبية القياسية كانت 28% و 90.9% تتابعا" لفيروس الورم الحليمى البشرى. معظم العينات كانت من نوع سرطان المرئ ذى الخلايا الظهارية ، خطورة المرض تزيد مع زيادة العمر.

يمكن التلخيص الى ان هناك تكرار واضح لفيروس الورم الحليمى البشرى 16 فيروس الابشتين بار المعدل مع سرطان المرئ لدى المرضى السودانين. لذا نوصى لمزيد من الدراسات برؤى اوسع.

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Chapter one

1.1 Introduction:

Esophageal cancer is the eighth most common cancer worldwide, with nearly 456,000 new cases diagnosed in 2012 representing 3% of the total cancer. The highest incidence rates were reported from Eastern Asia and the lowest were found in Western Africa (Ferlay, *et al*. 2013). Worldwide Malawi had the highest rate of esophageal cancer, followed by Turkmenistan and Kenya. About 81% of esophageal cancer cases occurred in less developed countries (Ferlay, *et al*. 2013). The majority of esophageal cancers are squamous cell carcinoma (SCC) followed by adenocarcinoma (AC) (Cook, *et al*. 2010). In Sudan, the magnitudes of esophageal cancers were recognized since the mid 70s when it was reported together with other alimentary tract malignancies. Esophageal cancer was found to be the commonest gastrointestinal malignant tumor making about 19.4% of alimentary tract malignancies, and 1.3% of all cancer registered. Esophageal cancer is gaining an elevated in Sudan since progressively increasing in incidence with varying demographic features (Malik, *et al*. 1976). However, the newest report from Sudan has reported that esophageal cancer is fourth common cancer in Sudan (Elamin, *et al*. 2015).

The use of tobacco and alcohol are strong risk factors for esophageal cancer. Cigarette smoking is associated with a 10-fold increase in risk for SCC and a 2- to 3-fold increase in risk for AC (Cook, *et al*. 2010). Obesity has been linked with increased risk for AC but reduced risk for SCC. Obesity increases the risk of gastro-esophageal reflux disease (GORD), in turn increasing the risk of Barrett's esophagus which strongly related with esophageal Ac (Abrams, *et al*. 2011). Also there are link between esophageal cancer and tooth loss. A family history of hiatus hernia is a risk factor for esophageal, and some people appear to have a genetic predisposition to develop some types of gastro-esophageal cancers (Jiang, *et al*. 2014). An etiologic role of different microorganisms such as fungi, bacteria and viruses has also been proposed for the pathogenesis of esophageal cancer. Of these microorganisms, HPV,

cytomegalovirus (CMV) and EBV have been investigated (Lambert and Hainaut. 2007). The role of HPV in the etiology of ECC has been debated for the past 30 years, however; EBV role is still unclear (Reed and Johnston, 1993). HPV infection has been recognized as a contributing factor to esophageal cancer. However, there were some reported limited serologic evidence of an association between esophageal cancer and HPV (Sitas, *et al*. 2012). The study could not exclude the possibility that certain HPV types may be involved. HPV detection rates in esophageal carcinomas are highly variable in different geographical areas of the world, being significantly higher in high risk areas than in low risk regions. Of the several thousands of carcinomas analyzed, HPV detection rates are 23% using in situ hybridization and 15% by the polymerase chain reaction (Syrjänen, *et al*. 2002). EBV-associated tumorigenesis seems to be rather restricted to gastric cancer whereas the role of EBV in other gastrointestinal carcinomas such as esophageal carcinomas or small and large bowel cancers seems to be negligible (Cho, *et al*. 2001; Wong, *et al*. 2003;Von Rahden, *et al*. 2006).

Dysphagia and odynophagia are the most common symptoms of esophageal cancer. Dysphagia is the first symptom in most patients. Odynophagia may also be present. Substantial weight loss is characteristic of reduced appetite and poor nutrition and, pain, often of a burning, heartburn-like nature, may be severe, present it almost daily, and is worsened by swallowing any form of food. The presence of the tumor may disrupt normal peristalsis, leading to nausea and vomiting, regurgitation of food, coughing and an increased risk of aspiration pneumonia. The tumor surface may be fragile and bleed, causing hematemesis. Compression of local structures occurs in advanced disease, leading to such problems as upper airway obstruction and superior vena cava syndrome. Fistulas may develop between the esophagus and the trachea, increasing the pneumonia risk, this condition is usually heralded by cough, fever or aspiration (Enzinger and Mayer, 2003).

Although an occlusive tumor may be suspected on a barium swallow or barium meal, the diagnosis is best made with esophagogastroduodenoscopy (EGD, endoscopy);

this involves the passing of a flexible tube down the esophagus and visualizing the wall. Biopsies taken of suspicious lesions are then examined histologically for signs of malignancy. Additional testing is usually performed to estimate the tumor stage. Computed tomography (CT) of the chest, abdomen and pelvis, can evaluate whether the cancer has spread to adjacent tissues or distant organs (especially liver and lymph nodes). EDG-PET (positron emission tomography) scan is also being used to estimate whether enlarged masses are metabolically active, indicating faster-growing cells that might be expected in cancer. Esophageal endoscopic ultrasound (EUS) can provide staging information regarding the level of tumor invasion, and possible spread to regional lymph nodes. The location of the tumor is generally measured by the distance from the teeth. Histopathology diagnose most tumors of the esophagus are malignant and few are benign. A very small proportion (under 10%) is leiomyoma or gastrointestinal stromal tumor (GIST). Malignant tumors are generally adenocarcinomas, squamous cell carcinomas, and occasionally small-cell carcinomas (Stewart, *et al*. 2003).

1.2 Rationale:

Esophageal cancers were one of the health problems worldwide; represent sixth most common cancer among men and ninth most common among women (WHO & IARC, 2008). Tobacco and alcohol use are well recognized as the main risk factors for esophageal cancer (Castellsague*, et al*, 2000; Adami*, et al*, 2002). Recent studies have shown a strong association between esophageal cancers with human papillomavirus (HPV) and Epstein-Barr virus this is mainly found in many countries including Mexican and Shantou China. In Sudan, there were considerable numbers of esophageal cancers, most of them are attributed to unknown risk factor, according to my knowledge there were few if any previous study highlighted the association between HPV and EBV with esophageal cancer, this study investigate the frequency of HPV and EBV in esophageal cancer patients in Sudan. Since immunohistochemistry technique is one of the methods used for identifying these viruses it was been used. Hence PCR is innovational technique it was been used here to confirm positive immunohistochemistry.

1.3 Objectives:

1.3.1 General objective:

To study the frequency of high risk HPV 16, 18 and Epstein Bar virus in esophageal cancer among Sudanese patients.

Specific objectives.3.2.1

1- To detect for high risk HPV (16 and 18) group and EBV by immunohistochemistry and PCR in esophageal cancer.

2- To determine the frequency HPV (16 and 18) and EBV with esophageal cancer subtypes.

3- To correlate the presence of HPV (16 and 18) and EBV in esophageal cancer with age and sex.

4- To compare between IHC results and PCR results for HPV and EBV detection.

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Chapter two

2 Literature Review

2.1 Scientific Background:

The esophagus (American English) or oesophagus (British English), commonly known as the food pipe or gullet, is an organ in vertebrates which consists of a fibro muscular tube through which food passes, aided by peristaltic contractions, from the pharynx to the stomach. In humans, the esophagus is usually 18–25 centimeters (cm) long. During swallowing the epiglottis tilts backwards to prevent food from going down the larynx. The esophagus travels behind the trachea and heart, passes through the diaphragm and empties into the cardia of the stomach. The word *esophagus* derives from the Greek word *oisophagos*, which means "to carry to eat (Harper and Douglas, 2014).

The wall of the esophagus from the lumen outwards consists of mucosa, sub-mucosa (connective tissue), layers of muscle fibers between layers of fibrous tissue, and an outer layer of connective tissue. The mucosa is a stratified squamous epithelium (multiple layers of cells topped by a layer of flat cells) which contrasts to the single layer of columnar cells of the stomach. The transition between these two types of epithelium is visible as a zig-zag line. Most of the muscle is smooth muscle although striated muscle predominates in its upper third. It has two muscular rings or sphincters in its wall, one at the top and one at the bottom. The lower sphincter helps to prevent reflux of acidic stomach content. The esophagus has a rich blood supply and vascular drainage. Its smooth muscle is innervated by involuntary nerves (sympathetic nerves via the sympathetic trunk and parasympathetic nerves via the vagus nerve) and in addition voluntary nerves (lower motor neurons) are carried in the vagus nerve to innervate its striated muscle (Drake, *et al*. 2005).

2.2 Microanatomy and histology:

The human esophagus has a mucous membrane consisting of a tough stratified squamous epithelium without keratin, a smooth lamina propria, and a muscularis mucosae (Kuo*, et al.* 2006) The epithelium of the esophagus has a relatively rapid

turnover, and serves a protective function against the abrasive effects of food. In many animals the epithelium contains a layer of keratin, representing a coarser diet *(*Ross and Pawlina, 2011). There are two types of glands, with mucus-secreting esophageal glands being found in the submucosa, and esophageal cardiac glands, similar to cardiac glands of the stomach, located in the lamina propria and most frequent in the terminal part of the organ (Ross and Pawlina, 2011; Takubo and Kaiyo, 2007). The mucus from the glands gives a good protection to the lining (*Yang, et al.* 2015) The submucosa also contains the submucosal plexus, a network of nerve cells that is part of the enteric nervous system (Ross and Pawlina, 2011). The muscular layer of the esophagus has two types of muscle. The upper third of the esophagus contains striated muscle, the lower third contains smooth muscle, and the middle third contains a mixture of both (Kuo*, et al.* 2006). Muscle is arranged in two layers: one in which the muscle fibers run longitudinal to the esophagus, and the other in which the fibers encircle the esophagus. These are separated by the myenteric plexus, a tangled network of nerve fibers involved in the secretion of mucus and in peristalsis of the smooth muscle of the esophagus. The esophagus also has an adventitia, but not a serosa. This makes it distinct from many other structures in the gastrointestinal tract (Kuo*, et al.* 2006).

2.3 Benign esophageal disorder:

2.3.1 Inflammation:

Inflammation of the esophagus is known as esophagitis. Reflux of gastric acids from the stomach, infection, substances ingested (for example, corrosives), some medications (such as bisphosphonates), food allergies, and can all lead to esophagitis. Esophagitis can cause painful swallowing and is usually treated by managing the cause of the esophagitis - such as managing reflux or treating infection (Colledge*, et al*. 2010).

2.3.2 Esophageal Varices:

Esophageal varices refer to engorged blood vessels present within the esophageal walls. These blood vessels are engorged more than normal, and in the worst cases

may partially obstruct the esophagus. These blood vessels develop as part of a collateral circulation that occurs to drain blood from the abdomen as a result of portal hypertension, usually as a result of liver diseases such as cirrhosis. This collateral circulation occurs because the lower part of the esophagus drains into the left gastric vein, which is a branch of the portal vein. Because of the extensive venous plexus that exists between this vein and other veins, if portal hypertension occurs, the direction of blood drainage in this vein may reverse, with blood draining from the portal venous system, through the plexus. Veins in the plexus may engorge and lead to varices (Patti, *et al*. 1997; Kuo, *et al*. 2006)

2.3.3 Esophageal Achalasia:

Is characterized by difficulty in swallowing, regurgitation, and sometimes chest pain. Diagnosis is reached with esophageal manometry and barium swallow radiographic studiesThe most common form is primary achalasia, which has no known underlying cause. It is due to the failure of distal esophageal inhibitory neurons. However, a small proportion occurs secondary to other conditions, such as esophageal cancer or Chagas disease (an infectious disease common in South America) (Spiess and Kahrilas, 1998). Achalasia affects about one person in 100,000 per year (Spiess and Kahrilas, 1998; Lake and Wong, 2006). There is no gender predominance for the occurrence of disease (Francis and Katzka, 2010).

2.3.4 Barrett's esophagus:

Sometimes called Barrett syndrome, Barrett esophagus, or columnar epithelium lined lower oesophagus (CELLO), refers to an abnormal change (metaplasia) in the cells of the lower portion of the esophagus. It is characterized by the replacement of the normal stratified squamous epithelium lining of the esophagus by simple columnar epithelium with goblet cells. The medical significance of Barrett's esophagus is its strong association (about 0.5% patient per year) with esophagealadenocarcinoma, a very often deadly cancer,(Koppert, *et al*. 2005; Shaheen and Richter, 2009) because of which it is considered to be a premalignant condition. The main cause of Barrett's esophagus is thought to be an adaptation to chronic acid exposure from

reflux esophagitis. Diagnosis requires endoscopy more specifically, esophagogastroduodenoscopy, the cells of Barrett's esophagus, after biopsy, are classified into four general categories: nondysplastic, low-grade dysplasia, high-grade dysplasia, and frank carcinoma. High-grade dysplasia and early stages of adenocarcinoma can be treated by endoscopic resection and new endoscopic therapies such as radiofrequency ablation, whereas advanced stages (submucosal) are generally advised to undergo surgical treatment. Nondysplastic and low-grade patients are generally advised to undergo annual observation with endoscopy, with radiofrequency ablation as a therapeutic option. In high-grade dysplasia, the risk of developing cancer might be at 10% per patient-year or greater (Shaheen and Richter, 2009).

2.3.5 Esophageal rupture: is a rupture of the esophageal wall. Iatrogenic causes account for approximately 56% of esophageal perforations, usually due to medical instrumentation such as an endoscopy or paraesophageal surgery. In contrast, the term Boerhaave's syndrome is reserved for the 10% of esophageal perforations which occur due to vomiting. Spontaneous perforation of the esophagus most commonly results from a full-thickness tear in the esophageal wall due to a sudden increase in intraesophageal pressure combined with relatively negative intrathoracic pressure caused by straining or vomiting (effort rupture of the esophagus or Boerhaave's syndrome). Other causes of spontaneous perforation include caustic ingestion, pill esophagitis, Barrett's esophagus, infectious ulcers in patients with AIDS, and following dilation of esophageal strictures. A related condition is Mallory-Weiss syndrome which is only a mucosal tear. In case of iatrogenic perforation common site is cervical esophagus just above the upper sphincter where as spontaneous rupture as seen in Boerhaave's syndrome perforation commonly occurs in the lower (1/3)rd of esophagus (Rosen, *et al*. 2010).

2.4 Esophageal cancer:

Esophageal cancer (or oesophageal cancer) is cancer arising from the esophagus the food pipe that runs between the throat and the stomach. Symptoms often include

difficulty in swallowing and weight loss. Other symptoms may include pain when swallowing, a hoarse voice, enlarged lymph nodes (glands) around the collarbone, a dry cough, and possibly coughing up or vomiting blood(Ferri, 2013). The two main sub-types of the disease are esophageal squamous-cell carcinoma which is more common in the developing world, and esophageal adenocarcinoma (EAC), which is more common in the developed world. A number of less common types also occur (Montgomery, *et al*. 2014). Squamous-cell carcinoma arises from the epithelial cells that line the esophagus (Kelsen and David, 2007). Adenocarcinoma arises from glandular cells present in the lower third of the esophagus, often where they have already transformed to intestinal cell type (a condition known as Barrett's esophagus) (Whittemore, *et al*. 2006; Montgomery, *et al*. 2014).

2.4.1 Squamous cell carcinoma:

The two major risk factors for esophageal squamous-cell carcinoma are tobacco (smoking or chewing) and alcohol (Montgomery, *et al*. 2014). The combination of tobacco and alcohol has a strong synergistic effect (Prabhu, *et al*. 2014*)*. Some data suggest that about half of all cases are due to tobacco and about one-third to alcohol, while over three-quarters of the cases in men are due to the combination of smoking and heavy drinking (Montgomery, *et al*. 2014). Risks associated with alcohol appear to be linked to its aldehyde metabolite and to mutations in certain related enzymes (Pennathur, *et al*. 2013). Such metabolic variants are relatively common in Asia (Montgomery, *et al*. 2014). High levels of dietary exposure to nitrosamines (chemical compounds found both in tobacco smoke and certain foodstuffs) appear to be a relevant risk factor (Pennathur, *et al*. 2013). Unfavorable dietary patterns seem to involve exposure to nitrosamines through processed and barbecued meats, pickled vegetables, etc., and a low intake of fresh foods (Montgomery, *et al*. 2014). Other associated factors include nutritional deficiencies, low socioeconomic status, and poor oral hygiene (Pennathur, *et al*. 2013). Chewing betel nut (areca) is an important risk factor in Asia (Akhtar, 2013). Physical trauma may increase the risk

(Hunter, *et al*. 2009). This may include the drinking of very hot drinks (*Zhang*, 2013).

2.4.2 Adenocarcinoma:

Male predominance is particularly strong in this type of esophageal cancer, which occurs about 7 to 10 times more frequently in men. This imbalance may be related to the characteristics and interactions of other known risk factors, including acid reflux and obesity (Rutegård, *et al*.2011). The long-term erosive effects of acid reflux (an extremely common condition, also known as gastroesophageal reflux disease or (GERD) have been strongly linked to this type of cancer. Longstanding GERD can induce a change of cell type in the lower portion of the esophagus in response to erosion of its squamous lining. This phenomenon, known as Barrett's esophagus, seems to appear about 20 years later in women than in men, maybe due to hormonal factors (De Jonge, *et al*. 2014). Having symptomatic GERD or bile reflux makes Barrett's esophagus more likely, which in turn raises the risk of further changes that can ultimately lead to adenocarcinoma (Pennathur, *et al*. 2013). The risk of developing adenocarcinoma in the presence of Barrett's esophagus is unclear, and may in the past have been overestimated (Montgomery, *et al*. 2014).

Being obese or overweight both appear to be associated with increased risk (Turati, *et al*. 2013). The association with obesity seems to be the strongest of any type of obesity-related cancer, though the reasons for this remain unclear. Abdominal obesity seems to be of particular relevance, given the closeness of its association with this type of cancer, as well as with both GERD and Barrett's esophagus. This type of obesity is characteristic of men (Lagergre, 2011*)*. Physiologically, it stimulates GERD and also has other chronic inflammatory effects (De Jonge, *et al*. 2014).

EAC has one significant protective factor reducing risk for both sexes. Although Helicobacter pylori infection, which has affected over half of the world's population, is a cause of GERD and a risk factor for gastric cancer, it seems to be associated with a reduced risk of esophageal adenocarcinoma of as much as 50% (Falk, 2009;

Lagergren and Lagergren. 2013). The biological explanation for a protective effect is somewhat unclear (Falk, 2009). One explanation is that some strains of H. pylori reduce stomach acid, thereby reducing damage by GERD (Harris and Randall, 2013). The decreasing rates of H. pylori infection in Western populations in recent decades have been suggested as a factor in the great increase in esophageal adenocarcinoma over the same period. The decrease is caused by better hygiene, for example through increased refrigeration of food and less crowded households, and has also been associated with an increase in stomach cancer. Female hormones may also have a protective effect, as EAC is not only much less common in women but develops later in life, by an average of 20 years. Although studies of many reproductive factors have not produced a clear picture, risk seems to decline for the mother in line with prolonged periods of breastfeeding. Tobacco smoking increases risk, but the effect in esophageal adenocarcinoma is slight compared to that in squamous cell carcinoma, and alcohol has not been demonstrated to be a cause (Lagergren and Lagergren. 2013).

2.4.3 Epidemiology of esophageal cancer:

Esophageal cancer is one of the most serious malignant diseases, owing to its rapid development and fatal outcome in most cases (Kollarova, *et al*. 2007). It is the 6th leading cause of death from cancer and the 8th most common cancer in the world. The 5-year survival is around 15%-25% and the best results are related to early diagnosis, which is commonly known as "early stages (Pennathur, *et al*.2013), with an incidence rate of 11.5/100 000 (Cancer*Mondial*. 2004; Kollarova, *et al*. 2007). The incidence of the tumor increases with age with the highest incidence in the age group 50–70 years. The disease is diagnosed more frequently in males than in females with an approximate ratio of 3–5:1. The most frequent histological type is squamous cell carcinoma. The proportion of Adenocarcinoma has increased from 3.5 % in 1985 to 17.0 % by the year 2000 according to reviewed data from several countries (Kollarova, *et al*. 2007). However, In Western Europe and the U.S.A., the proportion of Adenocarcinoma is almost 50 %. Cummings and Cooper (2008),

conducted a study in USA aimed to compare incidence rates of esophageal adenocarcinoma (EAC) and Squamous cell carcinoma (ESCC) by race. The study revealed that African-Americans had higher ESCC incidence than whites (5.0 versus1.3 cases/100,000/year). However, whites had higher EAC incidence (3.3 versus 0.8 cases/100,000/year) (Cummings and Cooper, 2008).

One of the features of Squamous cell carcinoma of the esophagus is thefragmentation of its incidence into low risk and high risk areas, based on geographical location. Some of the low risk areas include North America, countries in Western Asia, and Northern and Southern Europe, where the incidence rates range from 1.5 to 6.0/100,000. And well defined high risk areas include South Africa, China, Iran and countries in Eastern Africa, where the incidence rates range from 10 to 25/100,000 (Cummings and Cooper, 2008). Furthermore, within these high risk areas, there are regions, such as the Transkei region in South Africa, and northern parts of China, where the incidence rates are substantially higher (Matsha,*et al*, 2007).

In the United States, an estimated 16,910 cases of esophageal cancer will be diagnosed each year and 15,690 deaths are expected from the disease (Siegel, *et al*.2016).

Esophageal cancer represents 6–8% of all malignancies in Egypt. Affected patients have a mean age of 58.7 years and the male to female ratio is 1.9. Data from the Gharbeya population-based registry conducted in 2002 showed that approximately 40% of the tumors are found in the lower third of the esophagus, 40% at the gastroesophageal junction (GEJ), 13% in the middle esophagus, and 7% in the upper esophagus. Histologically, 53% of the tumors are SCC and 18% are adenocarcinomas (Enzinger and Mayer, 2003).

2.4.3.1 Esophageal cancer in Sudan:

In Sudan, the magnitude of esophagus cancer was recognized since the mid 70s when 106 cases were reported together with other alimentary tract malignancies (Malik, *et al*. 1976). In 1993, Ahmed had reported an increase in the relative incidence of cancer esophagus of 4.6%, in Sudan compared to studies done in the 70s. During the

same period, Hamo 1993 had found that esophagus cancer was diagnosed in 2.1% of 5086 patients who underwent upper GI endoscopy for different reasons (Hamo, 1993). The last report from national cancer registry the esophageal cancer is seventh cancer in Sudan with (rate = 5.8 per 100,000) (Intisar, *et al*.2014).

2.4.3.2 Worldwide mortality rates of esophageal cancer:

Mortality rates represent roughly 90 % of the incidence rates of the highest number of cases in males is reported in Ethiopia, Kenya, and China, with standardized mortality rates around 27 per 100.000 in the year 2002. In females, the highest numbers are observed in Mongolia, Iran, Kenya and China, where the standardized mortality rates are around 16 per 100.000. Among European countries, the highest mortality rates in males are in Hungary (9.1) and the United Kingdom (9.0 per 100.000). In females, the United Kingdom is in the top position with a standardized mortality rate of 4.1 per 100.000, as well as the Netherlands with the standardized mortality rate 2.2 per 100.000 (CANCERMondial, 2002).

2.4.3.3 Risk factors of esophageal cancer:

The etiologies of adenocarcinoma of the esophagus and squamous cell cancer are different. While squamous cancer is associated with alcohol and tobacco use, esophageal adenocarcinoma develops as a consequence of gastroesophageal reflux disease (Lagergren, *et al*. 1999).

2.4.3.3.1 Gender and race:

Squamous cell carcinoma is the most frequent histological type in black individuals and white women, while adenocarcinoma is predominant in white men (Zhang, 2013). The incidence of esophageal cancers is generally higher in men than women in most countries (Wheeler and Reed, 2012).

2.4.3.3.2 Smoking:

Smoking is one of the major risk factor for developing esophageal squamous carcinoma. Smokers have a 5-fold risk of developing this disease compared to non-smokers (Wheeler and Reed, 2012), tobacco is a known risk factor for EAC, relative to non-smokers (Oze, *et al*. 2012).

2.4.3.3.3 Alcohol:

Alcohol is a clear risk factor for squamous carcinoma although it is not strongly related to EAC. The relative risk increases with the amount of alcohol ingested varying between 1.8 and 7.4 depending on the weekly volume (Wheeler and Reed, 2012). In Northern China, alcohol is not consumed regularly and therefore the risk associated with this habit is not relevant (Wheeler and Reed, 2012).

2.4.3.3.4 Diet and nutrients:

Tea, mate and coffee have been extensively studied as potential risk factors associated with esophageal carcinoma and its geographical distribution, particularly in regions of South America. There is little evidence for carcinogenicity relationship through its components except for mate, which has been linked for both amount consumed and temperature (Wheeler and Reed, 2012).

2.4.3.3.5. Gastroesophageal reflux disease and Barrett's esophagus:

The prevalence of gastroesophageal reflux disease (GERD) in the Western population is about 10%-20%, and about 30 to 60 million people in the United States. This entity is capable of producing esophageal adenocarcinoma directly or, more commonly, through an intermediate pre-neoplastic lesion, the Barrett's esophagus (BE). The increased incidence of BE in the last 30 years, is correlated with an increased incidence of adenocarcinoma in the same period. Barrett's esophagus is a pre-malignant lesion that develops in 6%-14% of patients with GERD and of which, around 0.5%-1% will develop adenocarcinoma (Wheeler and Reed, 2012). In a study performed in Spain, the incidence of adenocarcinoma during follow-up of patients with BE was 0.48% per year (95%CI: 0.006%-2.62%), for an incidence of 1 per 210 patient-years (Alcedo, *et al*. 2009). The largest study is a nationwide, population-based, cohort study conducted in Denmark, involving all patients with BE during the period from 1992 through 2009, using data from the Danish Pathology Registry and the Danish Cancer Registry. The study included 11028 patients with BE for a median of 5.2 years. The incidence rate for adenocarcinoma was 1.2 cases per 1000 person-years (95%CI: 0.9-1.5). As compared with the risk in

the general population, the RR of adenocarcinoma among patients with BE were 11.3 (95%CI: 8.8-14.4). However; the annual risk of esophageal adenocarcinoma was 0.12% (95%CI: 0.09-0.15). Current surveillance guidelines assume a risk for adenocarcinoma of 0.5%-1%, far from the results obtained in this study. Detection of low-grade dysplasia was associated with an incidence rate for adenocarcinoma of 5.1 cases per 1000 person-years compared to 1.0 case per 1000 person-years among patients without dysplasia. These data question the rationale for ongoing surveillance in patients who have Barrett's esophagus without dysplasia (Hvid, *et al*. 2011).

2.4.3.3.6 Obesity:

Esophageal squamous cell carcinoma (ESCC) is clearly linked to a low socioeconomic status. The increasing prevalence of obesity in the Western world is thought to add to the rising incidence of esophageal adenocarcinoma. More specifically, it has been postulated that obesity increases intraabdominal pressure and gastroesophageal reflux by a specific mechanism, although some studies provided contradictory results. On the other hand, adipose tissue itself influences tumor development. Adipocytes and inflammatory cells secrete adipokines and cytokines which are known to promote tumor development. The abundant availability of lipids from adipocytes in the tumor microenvironment, supports tumor progression and uncontrolled growth. Given that adipocytes are a major source of adipokines and energy for the cancer cell, understanding the mechanisms of metabolic symbiosis between cancer cells and adipocytes, should reveal new therapeutic possibilities (Lagergren and Lagergren, 2013; Löfdahl, *et al*. 2013).

2.4.3.3.7 Drugs:

Observational studies with a large number of patients showed that the use of non-steroidal anti-inflammatory drugs, proton pump inhibitors and statins in patients with BE, reduced the progression to adenocarcinoma (Wheeler and Reed, 2012).The most studied agents have been acid suppressants. A systematic review with meta-analysis of studies evaluating the association between PPIs and histamine receptor

antagonists (H2RA) and risk of esophageal adenocarcinoma or high-grade dysplasia (HGD) in patients with BE has been recently published. Considerable heterogeneity was observed. Two studies reported the association between H2RA use and risk of esophageal adenocarcinoma and/or HGD and both studies did not show a significant effect (Singh, *et al*.2014). The largest study was published short after and challenged these results. In such nationwide case-control study carried out in Denmark, no cancer-protective effects from PPI's were seen. In fact, among 9883 patients with a new diagnosis of BE the authors identified 140 cases with incident esophageal adenocarcinomas and/or high-grade dysplasia, with a median follow-up time of 10.2 years. Based on these above results and until the results from future studies can elucidate what the association might be, continuous PPI therapy might not be necessary in all patients and could be directed at symptom control (Hvid, *et al*. 2014).

2.4.3.3.8. Genetic aspects:

The genetic and molecular changes underlying the development of esophageal cancer remain poorly understood. Genetic analysis of these cancers reveals frequent chromosomal losses (4q, 5q, 9p, and 18q), chromosomal gains (8q, 17q, and 20q), and occasional gene amplifications (7, 8, and 17q) (Enzinger and Mayer, 2003).

The minor allele frequency (maf) of a SNP is different among different populations. Since it is ethnicity related, more information is needed to know the demographic information of the patient and the control group.

Zhuo et al, 2012. reported that homozygous AA alleles might elevate esophageal cancer risk among Asians, but not Caucasians.

CCND1 G870A polymorphism might be a low-penetrant risk factor for esophageal carcinoma, particularly among Asians. (Yang, *et al.*2015).

2.4.3.4 Virus role in esophageal cancer:

Many viruses have been shown to have oncogenic potential, such as hepatitis B virus linked to cancer of the liver, Epstein-Barr virus to nasopharyngeal carcinoma, and human papilloma virus (HPV) to carcinoma of the cervix. An association between

viral infection and the development of esophageal carcinoma has been reported, particularly the HPV and EBV (Zhong,*et al*. 2013).

The evidence of HPV infection in esophageal SCC is mixed and shows geographical variations among high prevalence areas. Studies from some high prevalence regions (China and Trukomestan) report integrated HPV- DNA in up to 71% of esophageal SCC, but most studies from North America and northern Europe show little or no HPV DNA in esophageal squamous cell malignancies (White,2005).

2.4.3.4.1 Human Papilloma virus in esophageal cancer:

HPVs are a large group of small, non-enveloped, double-stranded DNA viruses. Infection with HPV typically leads to benign epithelial proliferations; however, a growing number of viral subsets have been associated with epithelial cancers. Malignant transformation, if it occurs, tends to occur after a long latency period, reflecting that infection with HPV is necessary but not sufficient for the development of HPV-associated cancers (Madkan, *et al*. 2007).

The frequency of detection of HPV DNA in ESCC is highly variable, ranging from 0 to 70% in different geographic areas (Lambot, *et al*. 1998; Sur and Cooper.1998). The great variation in the association between HPV and ESCC worldwide may be due to environmental and geographic factors, genetic susceptibility to esophageal HPV infections, or to variations in the sensitivity of techniques used in the detection of the virus and in the methodology for processing the tumor tissues (Zhang,*et al*.2000; Matsha, *et al*. 2007). Studies from China identified detection rates of HPV DNA in esophageal cancer, ranging from 40 to 60% (Shen,*et al*.2002; Chang, *et al*.2000). Other high-incidence areas, including far east and South Africa (Cooper, *et al.* 1995; Lavergne, *et al*. 1999). Also implicated HPV infection as a risk factor in the development of ESCC (Poljak, *et al*. 1998; Syrjanen, *et al*. 2002; *Zhong, et al.200*3) carried out an experiment to verify the role played by HPV in carcinogenesis of ESCC; they induced immortalized esophageal epithelial cells by E6 and E7 genes of HPV type 16m 18 and followed their biological behavior. They

concluded that genes E6/E7 of the HPV18 were capable of inducing immortalization in fetal esophageal epithelial cells. The immortal phenotype requires both activation of telomerase and genetic alterations that alter normal differentiation and promote cellular proliferation.

Molecular pathogenesistransforming proteins E6 and E7 from the high risk sub-types 16 and 18,interact with p53 protein and ribosomal protein respectively, leading to loss of function of these tumor suppressor gene products. These interactions further lead to inactivation of the growth suppressive effects of the p53 and ribosomal proteins, resulting in abnormal proliferative states. P53 protein expression has been found in both HPV-positive and - negative tumors, indicating that HPV and p53 protein expression are not mutually exclusive and can occur together in the same tumor. It has been observed that HPV plays a more significant role in esophageal carcinogenesis in geographic areas with a high prevalence of the disease. Esophageal carcinogenesis is a complex multistep process with a multifactorial etiology. Infection with oncogenic HPV types may be an integral part in a multistep process that leads to ESCC (Matsha, *et al*.2007).

2.4.3.4.2 EBV role in esophageal cancer:

EBV is a herpesvirus with which most of the world's population is infected. Epithelial cells of the oropharynx are primary sites of infection and viral replication. The virus usually establishes latent infection in the subsequently infected B cells and these cells probably serve as a reservoir for later infections of epithelial cells (Baumforth, *et al.* 1999). EBV is associated with infectious mononucleosis and a number of human malignancies including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, T cell lymphomas and gastric carcinoma (Baumforth, *et al.* 1999).

The viral genome codes various proteins whose activities may be relevant to carcinogenesis such as proteins which down regulate the immune response (Rousset*et al.,* 1992), inhibit apoptosis (Oudejansm*et al.* 1995) and associate with retinoblastoma and p53 proteins (Szekely, *et al.* 1993). The LMP1 viral protein can

transform rodent fibroblast cell lines (Wang, *et al.* 1985). B cells and esophageal epithelial cells both express CD21, a purported receptor for EBV (Wang,*et al.*1990). The possible association of EBV with cancer of the esophagus has been investigated in various low risk and high risk populations during the past years. In short, EBV was not found in the cancerous samples of most of the studies. The virus was identified in Taiwanese and also in cancerous tissues of European origin. In one of the studies on European subjects, EBV was detected at the same frequency in cancerous and non-cancerous subjects and the association with cancer was therefore deemed to be insignificant (Morgan, *et al.* 1997).

2.4.3.5 Screening and early detection of esophageal cancer:

Although several potential preventive measures exist, none has been proven to decrease the risk of esophageal carcinoma in prospective well-designed trials (Pennathur, *et al*. 2013). The relatively low incidence of esophageal cancer, the absence of early symptoms, and the rarity of a hereditary form of the disease make population-based screening untenable except in certain high-risk areas of the world (Enzinger and Mayer, 2003).

Patients who are found to have Barrett's esophagus, however, may be candidates for regular endoscopic surveillance, since the incidence of low-grade dysplasia, high-grade dysplasia, and cancer is approximately 4 percent, 1 percent, and 0.5 percent per year, respectively, among such patients (Enzinger and Mayer, 2003). Whether endoscopic screening programs to detect Barrett's esophagus in patients with chronic reflux disease symptoms are useful has been debated. Critics point out the high number of people in the general population who have reflux symptoms and the fact that at least 40% of patients with Barrett's esophagus do not have reflux symptoms, and question the cost-effectiveness of screening. Proponents of screening for Barrett's esophagus point to the clear associations between reflux, Barrett's esophagus, and esophageal adenocarcinoma, and suggest that the rising incidence of esophageal adenocarcinoma justifies screening. No definitive data are available on whether endoscopic screening for Barrett's esophagus is associated with a reduction

in cancer-related mortality and, therefore, screening is not routinely recommended. However, some experts have recommended that endoscopy be performed every three to five years in patients who have Barrett's esophagus in the absence of epithelial dysplasia and more frequently if they are found to have low-grade dysplasia. Diagnostic endoscopy for early detection can be conducted in 2 steps: at first detection of an abnormal area through changes in relief, in color or in the course of superficial capillaries; then characterization of the morphology of the lesion. Then treatment decision offers 3 options according to histologic prediction: abstention, endoscopic resection, surgery. The rigorous quality control of endoscopy will reduce the miss rate of lesions and the occurrence of interval cancer (Lambert, 2012)

2.4.3.6 Diagnosis of esophageal cancer:

An esophagogram (i.e., a barium-swallow examination) is usually the initial diagnostic study obtained and typically shows a stricture or ulceration of the esophagus. Patients with esophageal cancer that is thought to be restricted to the esophagus may benefit from further evaluation with the use of endoscopic ultra-sonography. This technique can be used to predict the depth of tumor invasion (the tumor stage) in 80 to 90 percent of patients and the extent of lymph-node involvement by metastatic disease (the node stage) in 70 to 80 percent of patients (Van Dam,1997). The ability to detect regional lymph-node involvement may be further enhanced by the use of endoscopic, ultrasonographically guided fine-needle aspiration, which has an accuracy of more than 90 percent at many centers. Endoscopic ultrasonography and biopsies are useful for determining the correct stage (prognosis i.e adenocarcinoma or squamous cell carcinoma and even differentiation of cancer cells involved) and accurately identifying superficial lesions, which are best treated with surgery alone (Vazquez, *et al*. 2001). In contrast, standard tumor markers, such as CEA, cancer antigen (CA) 19-9, and CA 125, have a low sensitivity and specificity in esophageal cancer and are therefore thought to be

of little value for screening, detecting recurrences, or predicting the response to therapy or the likelihood of survival (Mealy, *et al*. 1996).

2.4.3.7 Pathological patterns of esophageal cancer:

2.4.3.7.1 Location:

Twenty percent of tumors occur in the upper third, 50% in the middle third, and 30% in the lower third (Chen, *et al*. 2005).

2.4.3.7.2 Configuration:

Like other GI malignancies, ESCC grow in three patterns: deep irregular ulcer (25%), fungatingexophytic growth (60%) , and plaque like stenosing mural thickenings (15%) (Chen, *et al*. 2005; Sami, *et al*. 2002).

2.4.3.7.3 Clinical picture:

Asymptomatic course and late presentation of most esophageal malignancies; are the major causes of the deadly outcome in most cases (Sami, *et al*. 2002; Chen, *et al*. 2005). Progressive dysphagia may not be obvious until two thirds of the lumen is obliterated by the tumor. Oesophageal obstruction will result in malnutrition, weight loss, regurgitation, and occasionally aspiration (Sami, *et al*. 2002).

Growth of esophageal cancer (ESCC or EAC) occurs by intra-esophageal spread, direct extension, and lymphatic or hematogenous spread. ESCC more typically invades adjacent structures than EAC. Distant metastasis may be present in 25- 30% of ESCC patients at the time of diagnosis and in up to 50% of patients at the time of diagnosis and in up to 50% of patients at autopsy. The liver (32%), lungs (21%), and bones are the most frequent sites (Sami, *et al*. 2002).

2.4.3.8 Pathological staging of esophageal cancer:

Esophageal cancer is classified according to the 2002 American Joint Committee on Cancer tumor node metastasis (TNM) classification system, which takes into account the characteristics of the primary tumor, regional nodal metastases (Mealy, *et al*. 1996), and distant metastases.

2.5.3.9 Prognosis of esophageal cancer:

The prognosis of esophageal cancer is generally unfavorable, even when thetumor is surgically removed at its early and operable stage. The most reliableprognostic indicators are the degree of penetration of the wall, and the involvement oflymph nodes. The overall five-year survival rate is less than 5%(Chen, *et al*. 2005; Sami, *et al*. 2002; Kollarova, *et al*. 2007).

2.5.3.10 Management of esophageal cancer:

Both squamous cell carcinoma and adenocarcinoma of the esophagus are responsive to chemotherapy. Shrinkage of the tumor by at least 50 percent may occur in 15 to 30 percent of patients who are treated with fluorouracil, a taxane (paclitaxel or docetaxel), or irinotecan (Enzinger, *et al*. 1999; Enzinger ,*et al*.2000). Localized esophageal cancer is most commonly resected with the use of either a right transthoracic or a transhiatal approach (Hulscher,*et al.*2002).

Chapter three

3. MATERIALS AND METHODS

3.1 Study design:

Case study retrospective descriptive hospital based.

3.2 Study area:

The study was conducted in Khartoum state from 2012 to 2016, in Ibn Sina Hospital, Khartoum Hospital, Soba teaching hospital, Military hospital and National health laboratory.

3.3 Sampling:

The study included One hundred and two (102) formalin fixed paraffin embedded biopsies of esophageal cancer taken from endoscopic biopsy and patients underwent surgical operation treatment. Clinical and demographic data were collected from hospital registration records.

3.4 Sample size:

The sample were 102 subject, the actual cases that were found in above hospitals record was 340 subject from year 2008 to 2014, while the available was about 170 subject, from them just 102 subjects were fulfilling our study requirement.

3.5 Sample collection and processing:

From each paraffin blocks three sections were cut two into 4μm thickness, sections were floated into preheated 40°C using water bath and were placed in coated slide for immunohistochemistry and the third into 20μm from same biopsies were taken in to tubes for DNA extraction using QIAGEN QIAamp DNA formalin fixed paraffin embedded (FFPE) tissue Kits for molecular purpose.

3.5.1 Immunohistochemistry:

Immunohistochemistry (Dako kit) for HPV 16 and 18 E6 early proteins was performed following the manufacturer's instructions and EBV LMP1 from the same supply. Briefly, paraffin-embedded sections were dewaxed with two changes of xylene then rehydrated through descending alcohol (ABS for 3 min, 90% for 2 min 70% for 2 min), antigen retrieval was performed by heating the sections for 30

minutes in phosphate buffer saline. Endogenous peroxides activity was blocked with 3% hydrogen peroxides for 10 minutes, then washed in phosphate buffer (PBS) for 2 minutes , then section was incubated with primary antibodies for HPV and next sections for EBV for 30 minutes at room temperature in a moisture chamber, and then rinsed in phosphate buffer saline for 2 minutes. Sections were incubated with primary antibody enhancer for 15 minutes, and then washed in phosphate buffer for 2 minutes, and then secondary antibody labeled with horse raddish peroxidase was applied for 15 minutes. Sections were incubated in diaminobenzidine tetra hydrochloride to produce the characteristic brown stain for the visualization of the antibody/enzyme complex for 3 minutes, and then washed in phosphate buffer for 2 minutes.

Sections were counter stained with Mayer's Hematoxylin for 50 seconds and blued in running tap water for 5 minutes and dehydrated in ascending ethanol, then cleared in xylene 2 minutes for each, finally mounted using DPX . (The immunohistochemistry dark-brown signals scattered in the infected tumor cells). Positive and negative controls for HPV and EBV were stained parallel with test sections.

3.5.2 Isolation of DNA from FFPE tissue sections:

3.5.2.1 Principle of extraction

The QIAamp DNA FFPE Tissue procedure consists of 6 steps:

- Removal: paraffin is dissolved in xyline and removed
- Lysis: sample is lysed under denaturing conditions with proteinase K
- Heating: incubation at 90°C reverses formalin cross linking.
- Binding: DNA binds to the membrane and contaminants flow through
- Washing: residual contaminants are washed away
- Eluting: pure, concentrated DNA is eluted from the membrane

Preparing buffer AW1**:** 25 ml ethanol (96–100%) added to the bottle containing 19 ml Buffer AW1 concentrate.

Preparing buffer AW2**:** 30 ml ethanol (96–100%) added to the bottle containing 13 ml Buffer AW2 concentrate.

The check box on the bottle label was ticked to indicate that ethanol has been added. Reconstituted buffers stored at room temperature (20°C)

Note: Before starting the procedure, reconstituted buffers mixed by shaking.

3.5.2.2 Extraction procedure:

Using a scalpel, excess paraffin of sample block trimmed, then sections 20 µm thick were placed in a microcentrifuge tube and 2 ml xylene was added to the sample. The lid closed and vortexed vigorously for 10 second. Incubated at room temperature for 5minutes. Then centrifuge at full speed for 2 min at room temperature, the supernatant was carefully removed by pipetting. 2 ml ethanol (96–100%) was added to the pellet, and mixed by vortexing, then Incubated at room temperature for 5mins (The ethanol extracts residual xylene from the sample), then centrifugation at full speed for 2 min at room temperature, and carefully ethanol removed using a fine pipette tip. The tube opened and incubated at room temperature (15–25°C) until all residual ethanol had evaporated. The pellet suspended in 180 µl buffer ATL. And 20µl proteinase K added and mixed by vortexing and incubation at 56°C for 1 h in water bath until the sample had been completely lysed. The 1.5 ml tube briefly centrifuged to remove drops from the inside of the lid.200 µl buffer AL added to the sample, and mixed thoroughly by vortexing. Then 200 µl ethanol (96–100%), and mixed again thoroughly by vortexing. (The sample, buffer AL, and ethanol were mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution). The 1.5 ml tube briefly centrifuged to remove drops from the inside of the lid. The entire lysate carefully transferred to the QIAampMinElute column (in a 2 ml collection tube) without wetting the rim, the lid closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAampMinElute column placed in a clean 2 ml collection tube, and the collection tube containing the flow-through discharded. The QIAamp Min Elute column carefully opened and 500 µl buffer AW1added without wetting the rim. Centrifugation at 6000 x g (8000 rpm) for 1

min. the QIAamp Min Elute column placed in a clean 2 ml collection tube, and the collection tube containing the flow-through discarded then carefully the QIAampMin Elute column opened and 500 µl buffer AW2 was added without wetting the rim. Centrifugation at 6000 x g (8000 rpm) for 1 min. The QIAamp Min Elute column placed in a clean 2 ml collection tube, and the collection tube containing the flow-through discarded and centrifugation at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely. This step was necessary, since ethanol carryover into the elute may interfere with some downstream applications. The QIAamp Min Elute column was placed in a clean 1.5 ml micro centrifuge tube and the collection tube containing the flow-through discarded.

3.5.2.3 PCR primers

The preparation of primers based on gene sequences from previous studies and confirmed from viruses map. Nucleotide sequences of the primers are shown below:

3.5.2.3.1 Primers sequence for HPV 16 and 18

16A	5'-TCAAAGCCACTGTGTCCTG-3'		
16B	5'-CGTGTTCTTGATGATCTGCAA-3'	E6	271 pb
18A	5'-TGGTGTATAGAGACAGTATACCCCA-3		
18B	5'-GCCTCTATAGTGCCCAGGTATGT-3'	E6	247 pb

3.5.2.3.2Primers sequence for EBV:

Gene target: EBNA gene 1

EBV1 : 5' GTG TGC GTC GTG CCG GGG GCA GCC AC-3

EBV2 : 5' ACC TGG GAG GGC CAT CGA AAG CTC C-3

3.5.2.4 Polymerase chain reaction (PCR):

Maxime PCR preMix (i-Taq; for 20µl rxn) 96 tubes.

Component in 20 µl reaction were used.

Table 3.1 PCR cycling parameters

PCR cycles		Temperature °C	Time
Initial denaturation		94	2 min
35 cycle	Denaturation	94	20 sec
	Annealing	55	10 sec
	Extension	65	20 sec
Final extention		72	5 min
Final		10	10 sec

3.5.2.5PCR protocol

Template DNA and primers were added into Maxime PCR PreMix tubes (iTaq). And then distilled water was added into the tubes to a total volume of 20µl. The pellet was easily dissolved by standing at RT for 1-2min after adding water, and then PCR performed.

3.4.2.5.1 PCR reaction mixture

Table 3.2 PCR reaction premix

Reagent	**volume**
Template DNA	2µl
Primer (F: 10pmol/µl)	1µl
Primer (R: 10pmol/µl)	1µl
Distilled water	16 µl
Total reaction volume	20 µl

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3.4.2.5.2 Electrophoresis of amplified DNA:

3.4.2.5.2.1 Agarose gel electrophoresis protocol

Agarose gel prepared as (1.2%). The gel poured when the agarose has cooled to about 55˚ C. Proper comb was inserted for the particular gel rig. Then the comb carefully removed and gel placed in the gel rig with the wells closest to the cathode (black) end, covered with 1X TAE running buffer. Total PCR product volume loaded on agarose gel without adding a loading-dye, 6 µl of 1 Kb ladder was placed at end of the series of samples. The gel electrophoresis performed at 100 volts for 1 h. Then the gel visualized with UV light and photographed with a polaroid Photo documentation camera.

3.4.2.5.2.2Viruses detection:

During visualization, a 271 bp product for the positive control, no amplified product for the negative control of HPV 16, while the length of positive control product for HPV 18 was 247 bp. EBV amplified positive control was 375 bp.

3.5 Statistical analysis:

Statistical analysis was performed by the SPSS version 20.0. The results were tested by chi-square. P value lower than 0.05 was considered statistically significant. We used frequency and percentage to show descriptive data of demographic variables of sex, age and type of tumor.

3.6 Ethical consideration:

The study was approved by academic research board committee of faculty for any obligations, and then the specimens were be retrieved after the laboratory consent was singed from the above hospitals.

Chapter four

4 Results

In this retrospective case study 102 patients with esophageal cancer were assessed for viral frequency. Of the 102 study subjects 56 were males and 46 were females their age ranging from 21 to 98 years old with a mean age of 59 years. The highest frequency of the study populations were at the age range 66-75 years constituting 26 patients followed by age ranges, both < 45 and 56-65 years representing 24 patients. Moreover, 16 patients were identified among age group 46-55 years, hence only 12 patients were found among age group 76+ years as indicated in Table 1.

In regard to the sex, the age distribution was relatively similar. Most of males were found at age group <45 years representing 13 patients, hence most of females were found at age group 66-75 representing 14 patients. For males, 12 patients were represented by each of age group 56-65 and 66-75, followed by 46-55 and 76+ years constituting 10 and 8 patients in this order. For females, 11 patients were represented by <45 and 56-65 for each followed by 6 and 4 patients for age groups 46-55 and 76+, respectively as indicated in Table1. When calculating the proportion of males and females within each age group, variable percentages can be revealed. For males the highest percentage were found in age group 76+ representing 8/12(66.7%) followed by 46-55, <45, 56-65 and 66-75 constituting 10/16(62.5%), 13/24(54%), 12/23(52%) and 12/26(46%) percapta. For females the highest percentage were found in age group 66-75 years constituting 14/26(54%) followed by 56-65, <45, 46-55 and 76+, representing 11/23(48%), 11/24(46%), 6/16(37.5%) and 4/12(33.3%), respectively as shown in Fig 1.

Table 2. Summarized the distribution of the cancer type by demographical factors. However, most of the patients were diagnosed as having squamous cell carcinoma representing 89% followed by adenocarcinoma representing 11%. Of the 91 patients with squamous cell carcinoma, 53% were males and 47% were females. Of the 11 patients with adenocarcinoma 73% were males and 27% were females, as indicated in Table 2. The highest percentage of patients with squamous cell carcinoma were

found at age group 56-65 years followed by <45, 66-75, 46- 55 and 76+ representing (24) 26.4% , (22) 24.3%, (21) 23%, (14) 15.3% and (10) 11% respectively, as shown in Fig 2. Regarding adenocarcinoma the highest percent was in age range 66 – 75 years representing (5) 45.6% followed by <45, 46-55 and 76+ constituting (2)18.2% for each, as shown in table 2 figure3.

Regarding IHC staining for HPV 16 and 18, positive findings were revealed in 14 (13.7%) and couldn't be disclosed in 88 (86.3%) of the study subjects, hence, none of the cases was identified with HPV18. The risk of HPV 16 with esophageal cancer and the Odd Ratio (OR) and the 95% confidence interval was 31(1.81-528), P <0.017. Of the 56 males with esophageal cancer, 7(12.5%) were identified with HPV16 infection and the remaining 49(87.5%) found without HPV16 infection. Of the 46 females with esophageal cancer, 7(15%) were found positive for HPV 16 immunostaining and the remaining 40(85 %) were found negative for HPV16. The association of HPV 16 risk with sex was OR (CI) = 0.97(0.30-3.12), P <0.9.

According to cancer type, of the 91 cases of the squamous cell carcinoma, 11 (12%) were found with positive HPV16 and the remaining 80 (88%) were negative. Of the 11 cases of the Adenocarcinoma, 3(27.3%) were found with positive HPV16 and the remaining 8(72.7%) were negative. The association of HPV 16 risk with Adenocarcinoma was OR (CI) = 0.37(0.084-1.56), P <0.18, as indicated in Table 3, Fig 4.

Concerning IHC staining results of HPV subtypes 16, 18 with age distribution, the highest positive results were found in age group 66-75 years, representing 6(23%), followed by (<45& 56-65) and 46-55 constituting 3(12.5%) and 2 (6.5%) respectively, where's; the groups with the highest negative results were 56-65 years, representing 21(23.6%) followed by (<45 & 66-75),46-55 and 76+ constituting 20 (22.5%), 15 (16.8%) and 12(13.5%) in this order, as shown in Table 3, Fig 5.

Of 102 subjects, 25(24.5%) were found positive for HPV 16 by PCR while 77 (75.5%) were found negative. Of the 56 males 17/56 (30.3%) were found positive and 39/56(69.7%) were found negative. Of the 46 females, 8/46 (17.4%) were found

positive and 38/46(82.6%) were found negative. The risk associated with squamous cell carcinoma was and OR (CI) = 0.85(0.21-3.48), P = 0.82.

Of the 91 squamous cell carcinoma 22/91 (24%) were found positive and 69/91(76%) were found negative IHC staining for HPV16. Of the 11 Adenocarcinoma, 3/11 (27%) were found positive and 8/11(73 %) were found negative. The risk associated with male sex was and OR (CI)= 2.13(0.82-5.5), P = 0.12. As indicated in Table 3, Fig 8.

Concerning PCR results of HPV subtype 16 with age distribution, the highest positive results were found in age group 66-75 years, representing 9/25(36%), followed by 56-65, <45, 46-55 and 76+, constituting 6/25(24%), 5/25(20%), 4/25(16%) and 1/25(4%) respectively, where's; the groups with the highest negative results were <45 years, representing 19/77(24.7%) followed by 56-65, 66-75,46-55 and 76+ constituting 18/77 (23.4%), 16/77 (20.7%), 12/77(15.6%) and 11/77(14.3%) in this order, as shown in Table 3, Fig 9.

Regarding IHC staining for EBV, positive findings were revealed in 22 (21.5%) and 80(78.5%) of the study subjects were negative. Of the 56 males with esophageal cancer, 15/56 (27%) were identified with EBV infection and the remaining 41/56(73%) found without EBV infection. Of the 46 females with esophageal cancer, 7/46(13%) were found positive for EBV immunostaining and the remaining 39/46(87 %) were found negative for EBV. The association of EBV risk with male sex was OR (CI) = 2.08(0.77-5.7), P =0.145.

According to cancer type, of the 91 cases of the squamous cell carcinoma, 19/91 (21%) were found with positive EBV and the remaining 72/91(79%) were negative. Of the 11 cases of the Adenocarcinoma, 3(27.3%) were found with positive EBV and the remaining 8 (72.7%) were negative, as indicated in Table 4. The association of EBV risk with Squamous cell carcinoma was OR (CI) = 0.70(0.17-2.9),P =0.63 , as indicated in Table 4, Fig6.

Concerning IHC staining results of EBV with age distribution, the highest positive results were found in age group 56-65 years, representing 7/23(32%), followed by

46-55, <45, 66-75 and 76+ constituting 6/22(27.3%), 4/22(18.2%),3/22(13.5%) and 2/22(9%) respectively, whereas; the groups with the highest negative results were 66-75 years, representing 23/80(28.8%) followed by <45,56-65 and (46-55& 76+) constituting 20/80 (25%), 17/80 (21.2%), and 10/80(12.5%) for each, respectively, as shown in Table 4, Fig 7.

Of 102 subjects, 6/102(6%) were found positive for EBV by PCR while 96 (94%) were found negative. Of the 56 males 5/56 (9%) were found positive and 51/56(91%) were found negative. Of the 46 females, 1/46 (2.2%) were found positive and 45/46(97.8%) were found negative. The risk associated with male sex was and OR (CI) = 4.5(0.51-39.99), P = 0.18.

Of the 91 squamous cell carcinoma 5/91(5.5%) were found positive and 86/91(94.5%) were found negative. Of the 11 Adenocarcinoma, 1/11(9%) was found positive and 10/11(91%) were found negative. The risk associated with Squamous cell carcinoma OR (CI) = 0.58(0.06-5.48), P = 0.64. As indicated in Table 4, Fig 10.

Concerning PCR results of EBV with age distribution, the highest positive results were found in age groups (66-75 &76+ years), representing 2/6(33.3%) for each and followed by (56-65& 46-55, constituting 1/6(16.7%), for each, where's; the groups with the highest negative results were (<45 &66-75 years), representing 24/95(25.3%) for each, followed by 56-65,46-55 and 76+ constituting 22/95 (23.2%), 15/95 (15.9%), and 10/95(10.3%) respectively, as shown in Table 4, Fig 11.

When comparing IHC HPV 16 demonstration with PCR detection, 7/25 (28%) cases were found positive for both detection methods hence 70/76(92 %) were found negative by both methods. Moreover, 18/25(72%) were found positive with PCR but negative with IHC, whereas, 7/14(50%) were positive by IHC but negative with PCR, as shown in Table 5.

In regard to the age 6/20(30%), 3/15(20%),3/21(14.2%) and 1/1 5(6.7%), of the positive cases by IHC were identified among age groups, 66-75, <45, 56-65 and 46-

55, in this order, whereas,9/16(56.2%),6/18(33.3%), 5/19(26.3%), 4/12(33.3%) and 1/11(9%) of age groups, 66-75, 56-65,<45, 46-55 and 76+ respectively were classified as positive by PCR HPV16.(see Fig12).

When comparing IHC EBV demonstration with PCR detection, 3/6 (50%) cases were found positive for both detection methods hence 76/95(80%) were found negative by both methods. Moreover, 3/6(50%) were found positive with PCR but negative with IHC, whereas, 19/22(86.4%) were positive by IHC but negative with PCR, as shown in Table 6.

In regard to the age 7/24(29%), 6/16(38%),4/24(17%),3/26(11.5)% and 2/12(17%), of the positive cases by IHC were identified among age groups, 56-65, 46-55, <45, 66-75 and 76+, respectively, whereas, 2/12(17%),2/26(7.7%), 1/16(6.3%), and 1/23(4.3%) of age groups, 76+, 66-75, 46-55 and 56-66, respectively were classified as positive by PCR for EBV.(see Fig13).

If PCR results were used as the gold standard for HPV 16 and EBV the sensitivity and specificity of IHC was 28 % and 90.9%, respectively,and the positive predictive value of IHC was only 50 % and the negative prediction value was 79.5% for HPV.

Table (4.1) Distribution of the study population by age and sex.

Age group	Males	Females	Total	Percentage
< 45 years	13	11	24	23.5
46-55	10	6	16	15.7
56-65	13	11	24	23.5
66-75	12	14	26	25.5
76+	8	4	12	11.8
Total	56	46	102	100

Table (4.2) Distribution of the cancer type by demographical factors

Category	Variable	Squamous carcinoma	Adenocarcinoma	Total
Sex	Males	48	8	56
	Females	43	3	46
	Total	91	11	102
Age				
	<45 years	22	2	24
	46-55	14	2	16
	56-65	24	0	24
	66-75	21	5	26
	76+	10	2	12
	Total	91	11	102

Table (4. 3) Distributions of HPV16 detection methods (IHC and PCR) by sex age and cancer type

Variable	Category	HPV 16 (IHC)		HPV 16 (PCR)	
		+ve	-ve	+ve	-ve
Sex					
	Males	7	49	17	39
	Females	7	39	8	38
	Total	14	88	25	77
Age					
	<45 years	4	20	5	19
	46-55	2	15	4	12
	56-65	3	21	6	18
	66-75	6	20	9	17
	76+	0	12	1	11
	Total	14	88	25	77
Cancer type					
	Squamous carcinoma	11	80	22	69
	Adenocarcinoma	3	8	3	8
	Total	**14**	**88**	**25**	**77**

Table (4.4) Distributions of EBV detection methods (IHC and PCR) by sex age and cancer type

Variable	Category	EBV (IHC)		EBV (PCR)	
		+ve	-ve	+ve	-ve
Sex					
	Males	15	41	5	51
	Females	7	39	1	45
	Total	22	80	6	96
Age					
	<45 years	4	20	0	24
	46-55	6	10	1	15
	56-65	7	17	1	22
	66-75	3	23	2	24
	76+	2	10	2	10
	Total	22	88	6	96
Cancer type					
	Squamous carcinoma	19	72	5	86
	Adenocarcinoma	3	8	1	10
	Total	**22**	**80**	**6**	**96**

Table (4.5) Description of HPV 16 results by IHC and PCR detection methods.

		HPV 16 PCR		Total
HPV 16 IHC		Positive	Negative	
	Positive	7	7	14
	Negative	18	70	88
	Total	**25**	**77**	**102**
P. value = 0.07				

Table (4.6) Description of EBV results by IHC and PCR detection methods.

		EBV PCR		Total
EBV IHC		Positive	Negative	
	Positive	3	19	22
	Negative	3	77	80
	Total	**6**	**96**	**102**
P.value = 0.084				

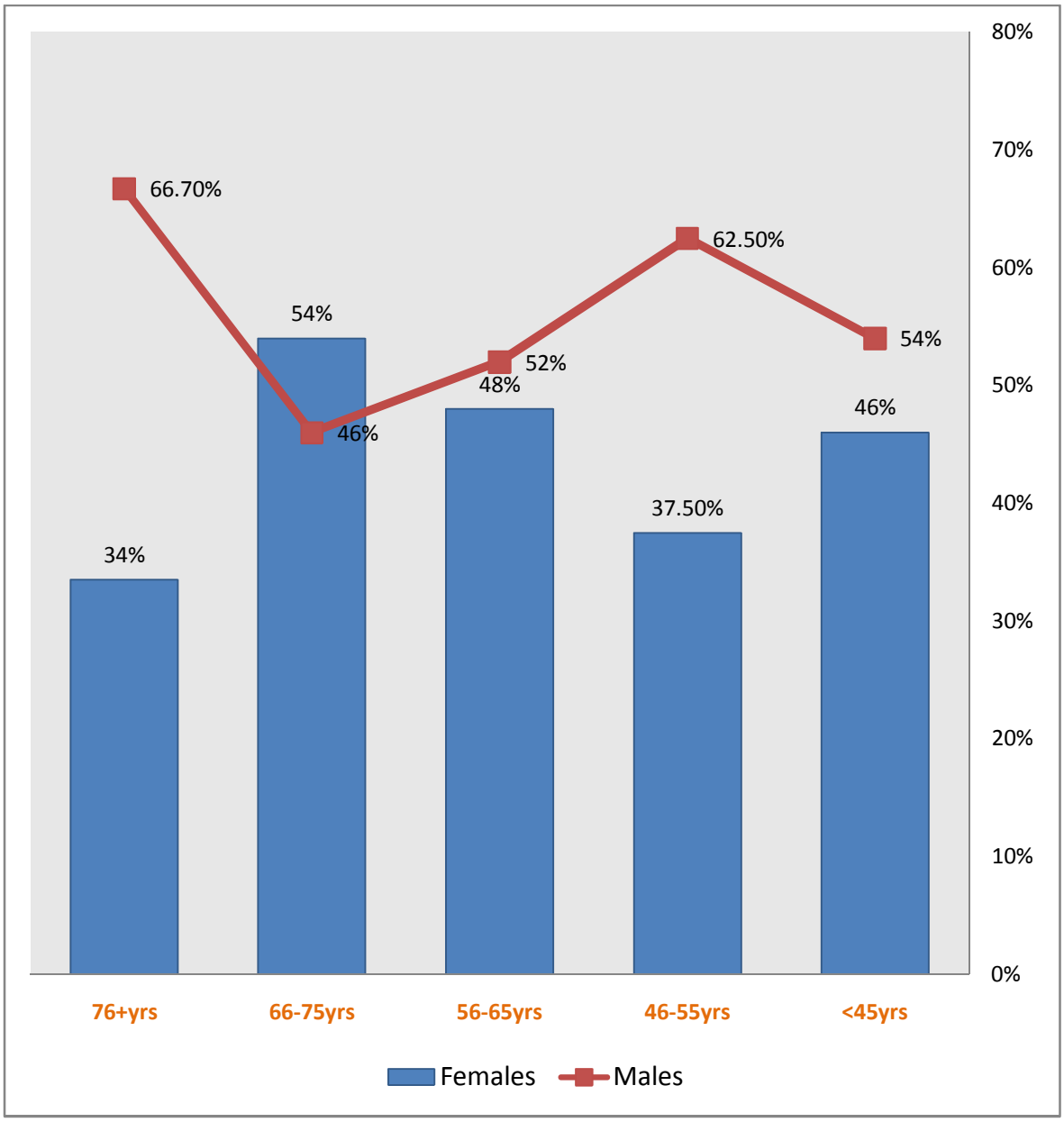


Figure (4.1) Description of the study subjects by age and sex.

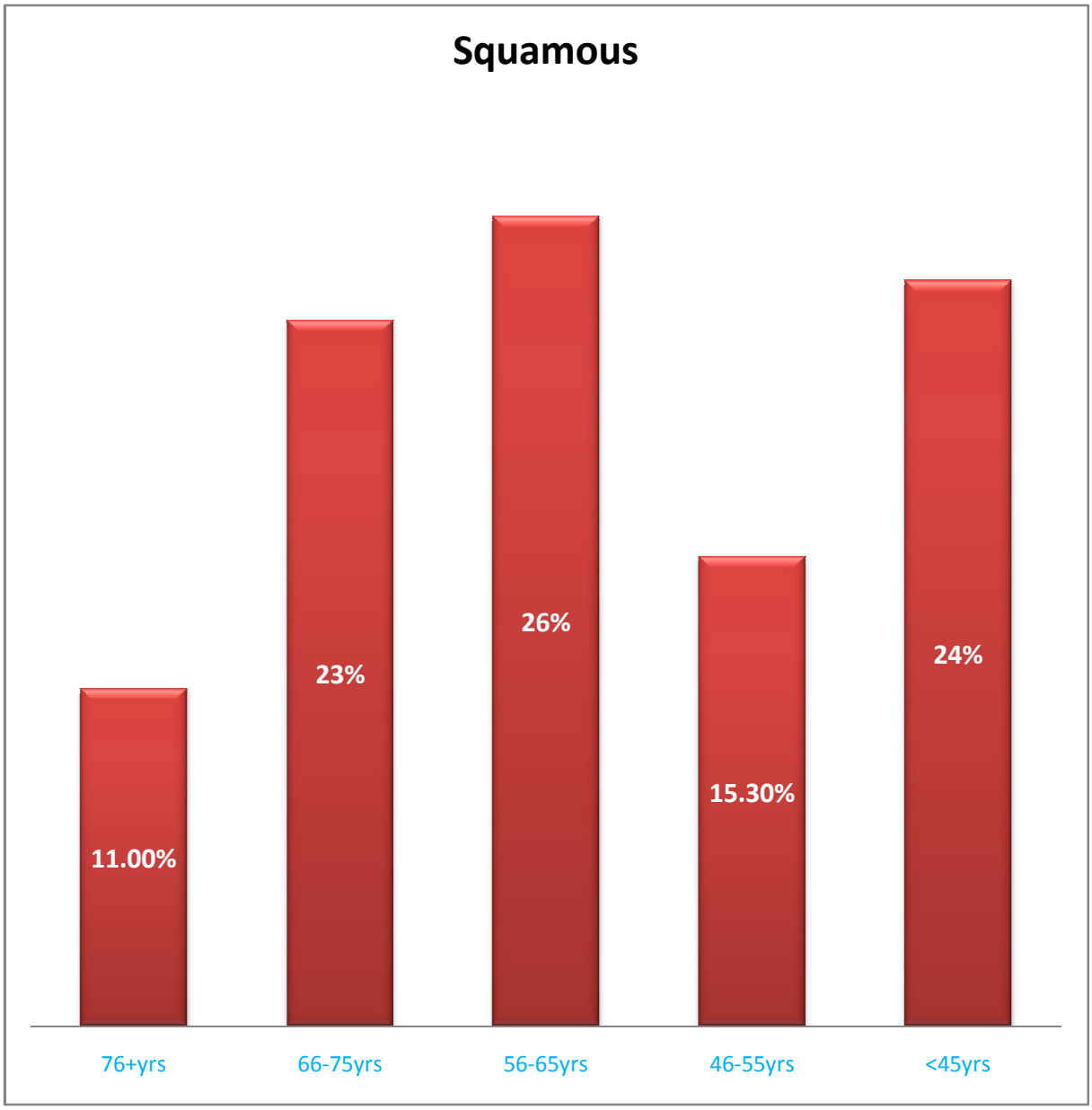


Figure (4.2) Description of Squamous cell carcinoma among different age groups.

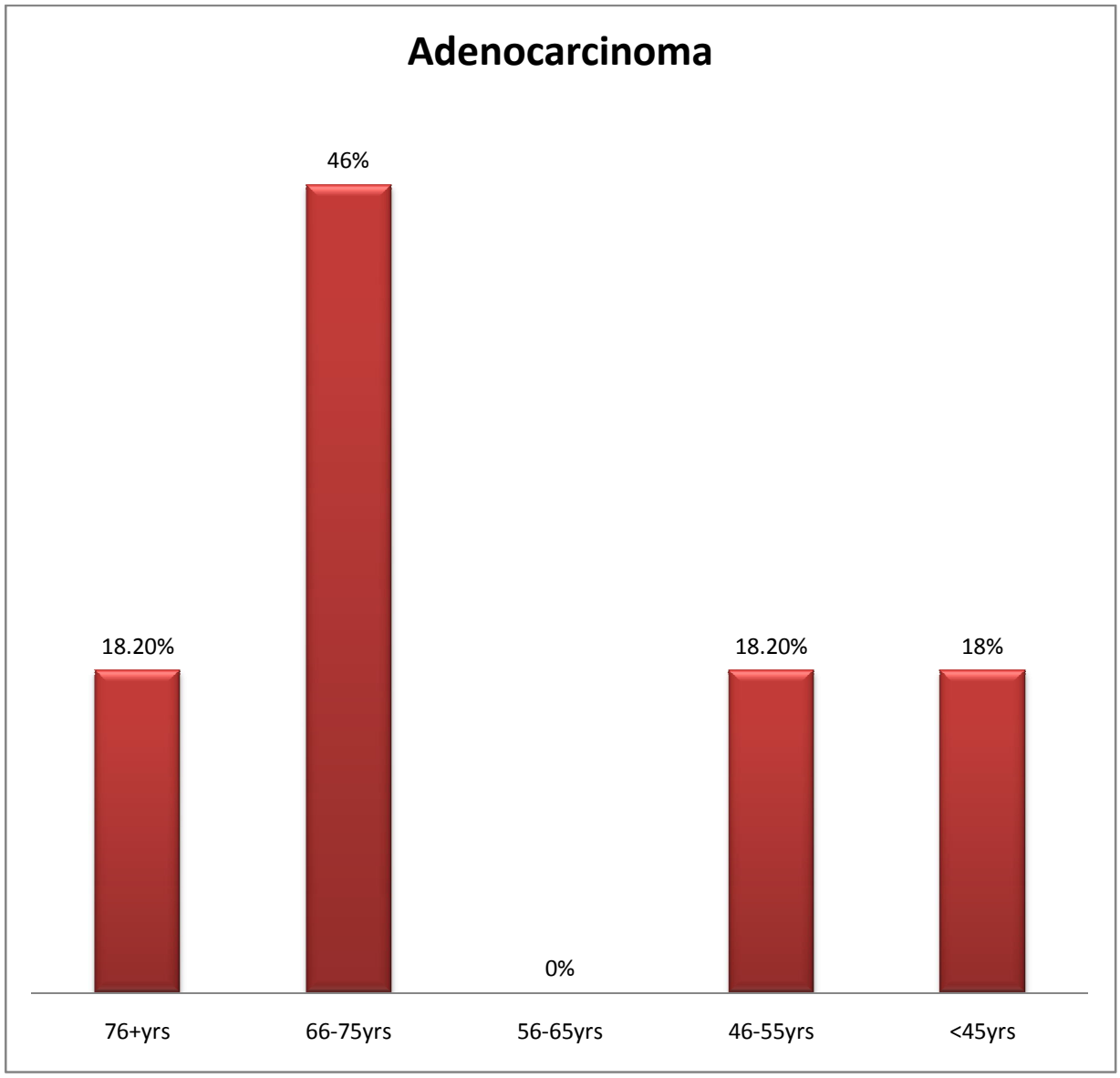
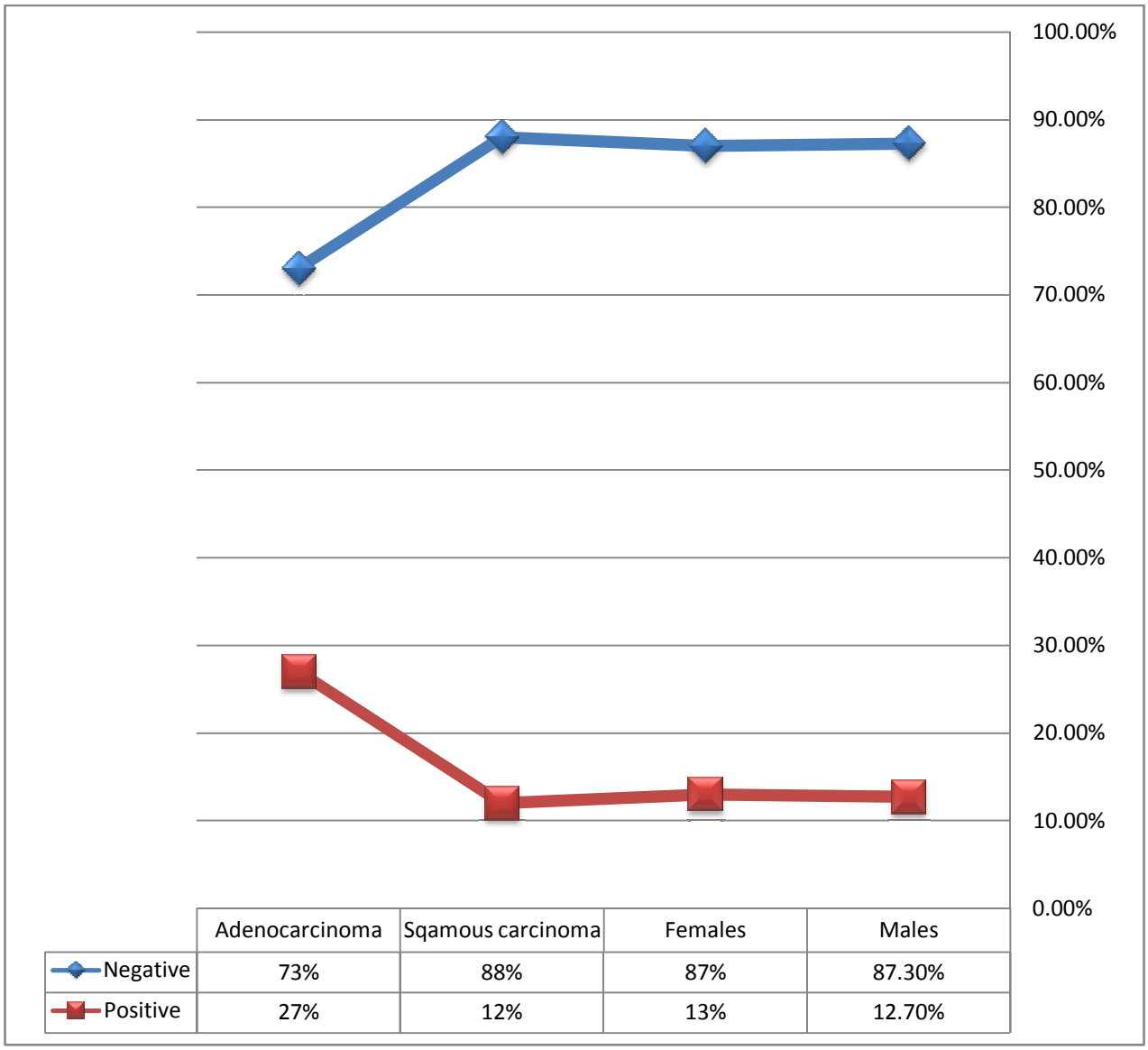


Figure (4.3) Description of Adenocarcinoma among different age groups.



	Adenocarcinoma	Sqamous carcinoma	Females	Males
Negative	73%	88%	87%	87.30%
Positive	27%	12%	13%	12.70%

Figure (4.4) Description of IHC results of HPV subtypes 16, 18 by sex and cancer type.

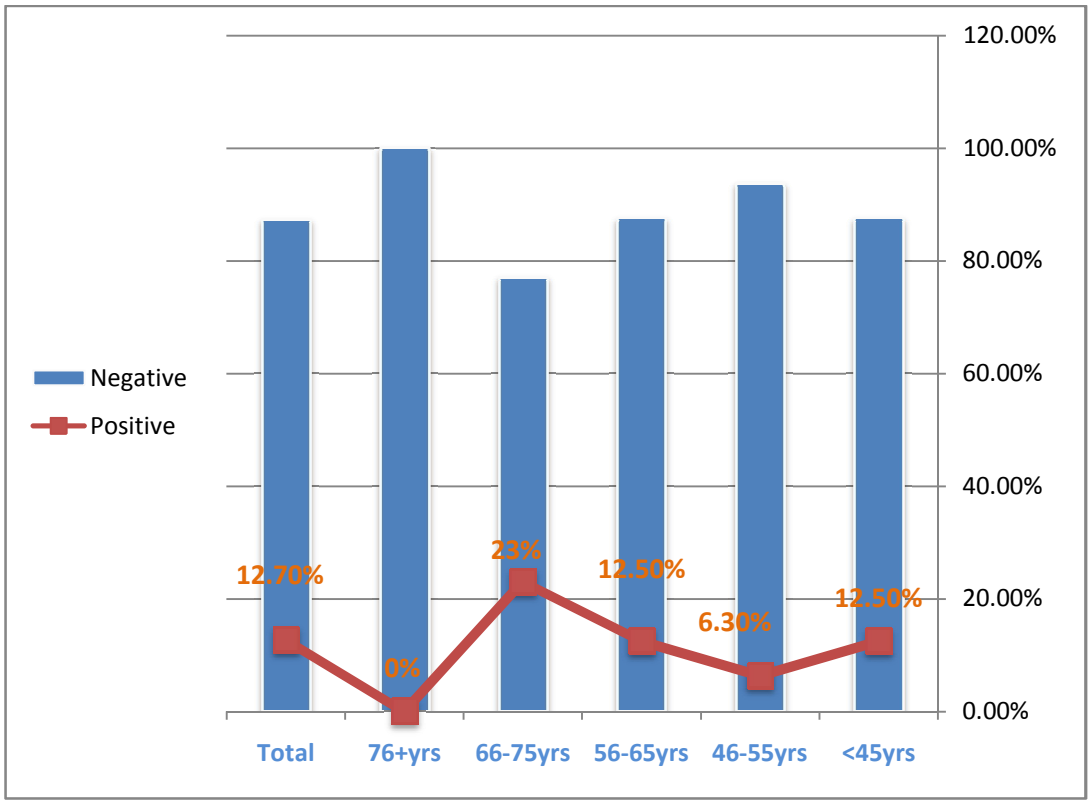


Figure (4.5) Description of IHC results of HPV subtypes 16, 18 by age

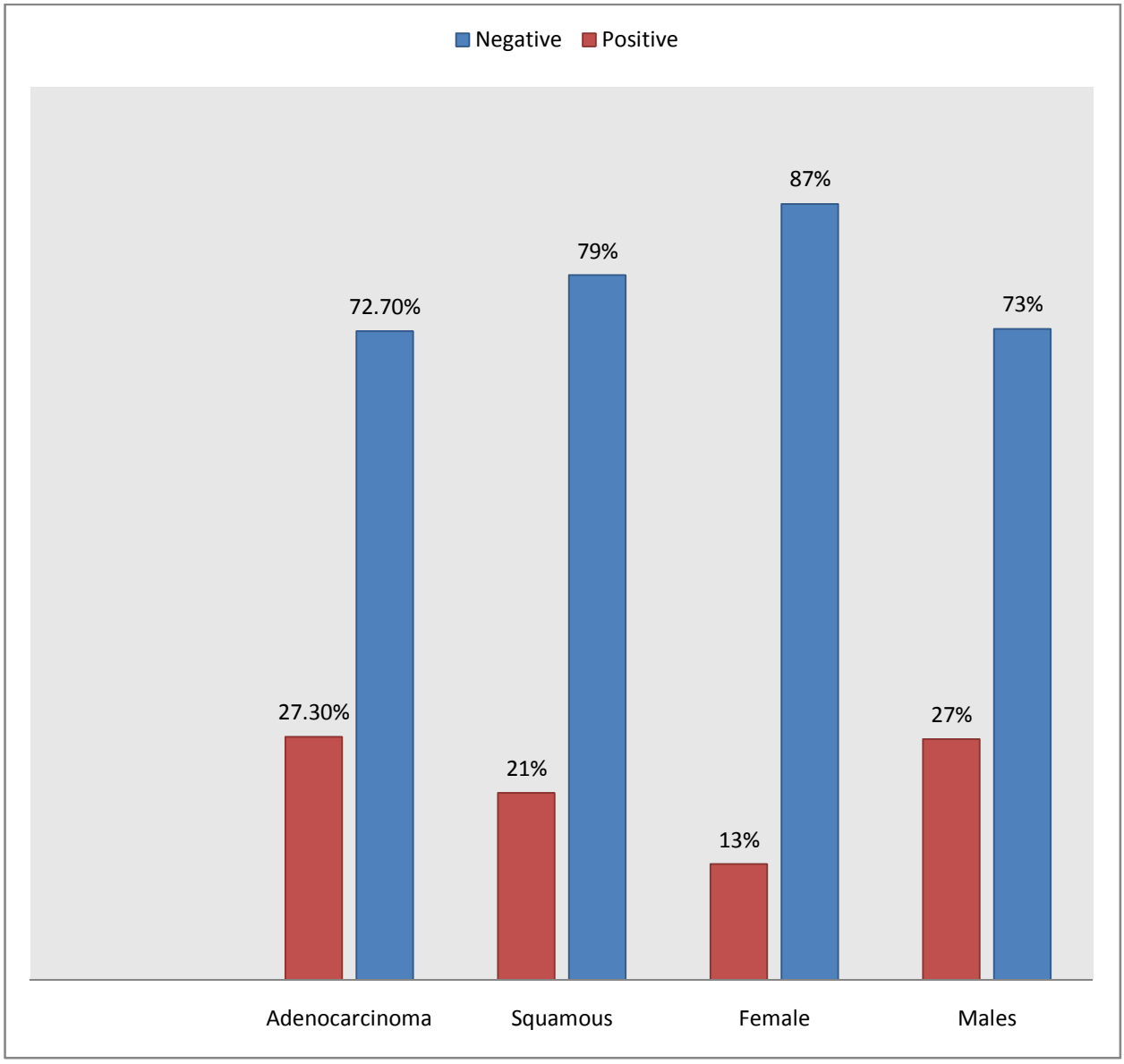


Figure (4.6) Description of IHC results of EBV by sex and cancer type

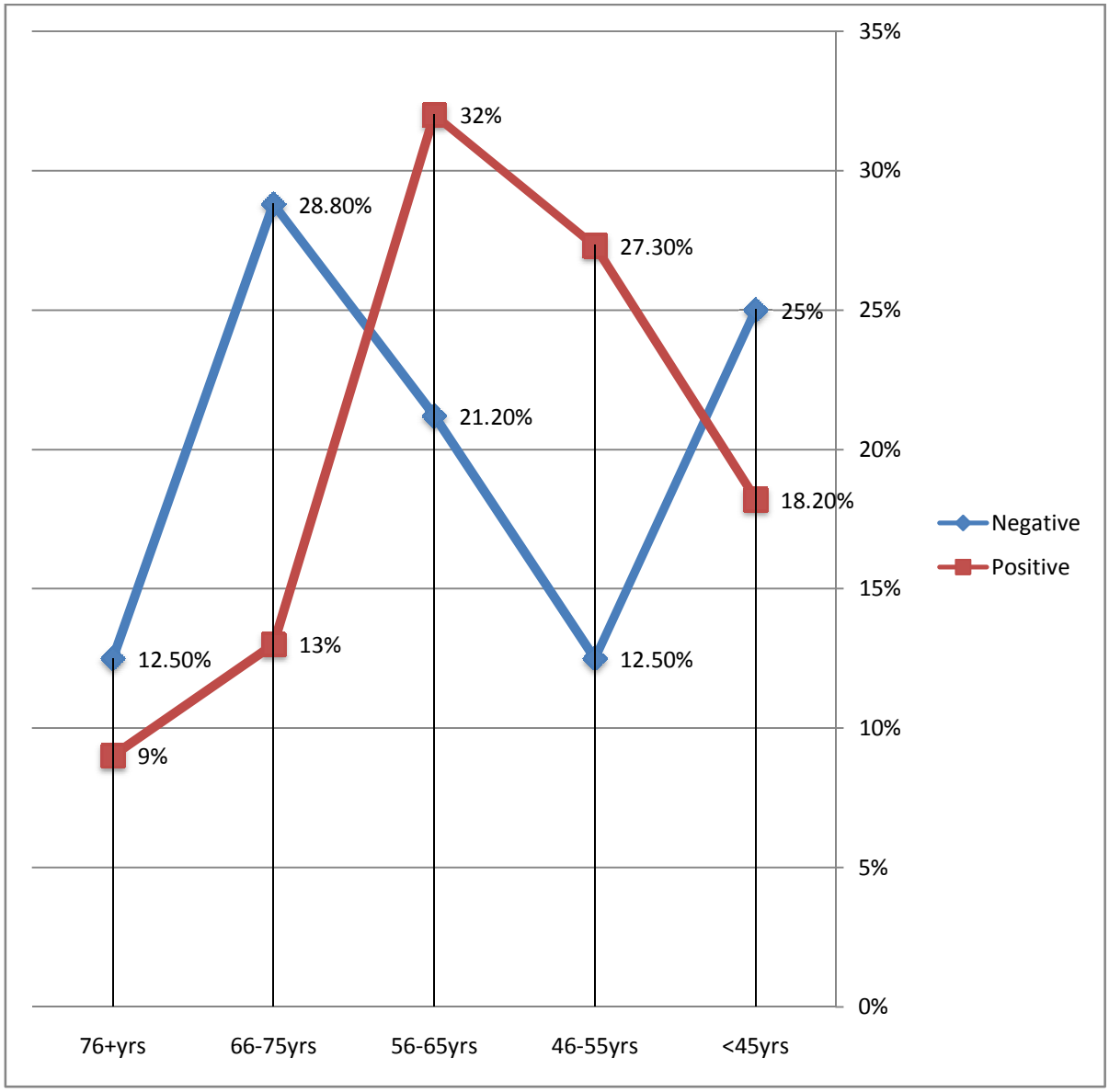


Figure (4.7) Description of IHC results of EBV by age

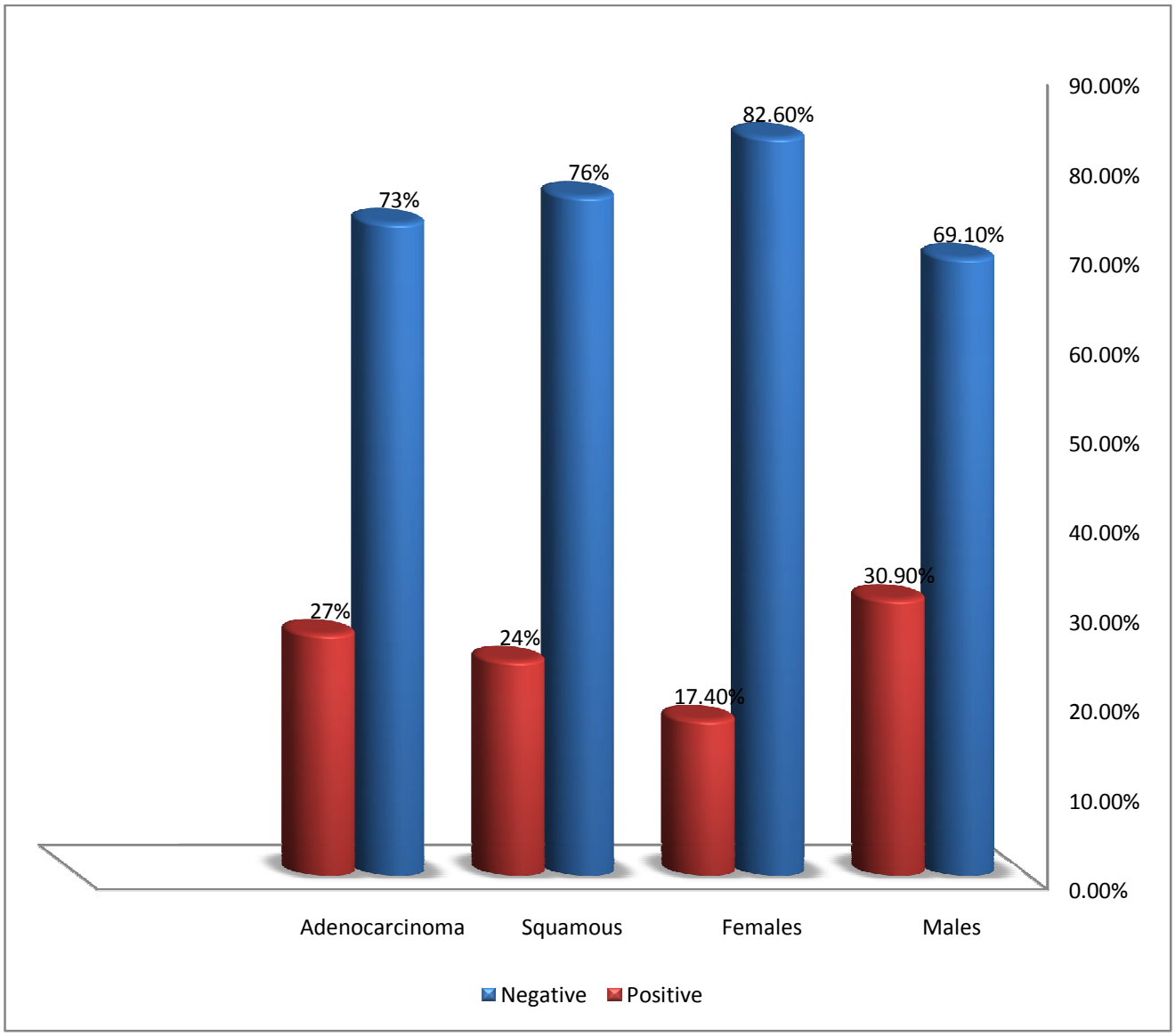


Figure (4.8) Description of HPV16 PCR by sex and cancer type.

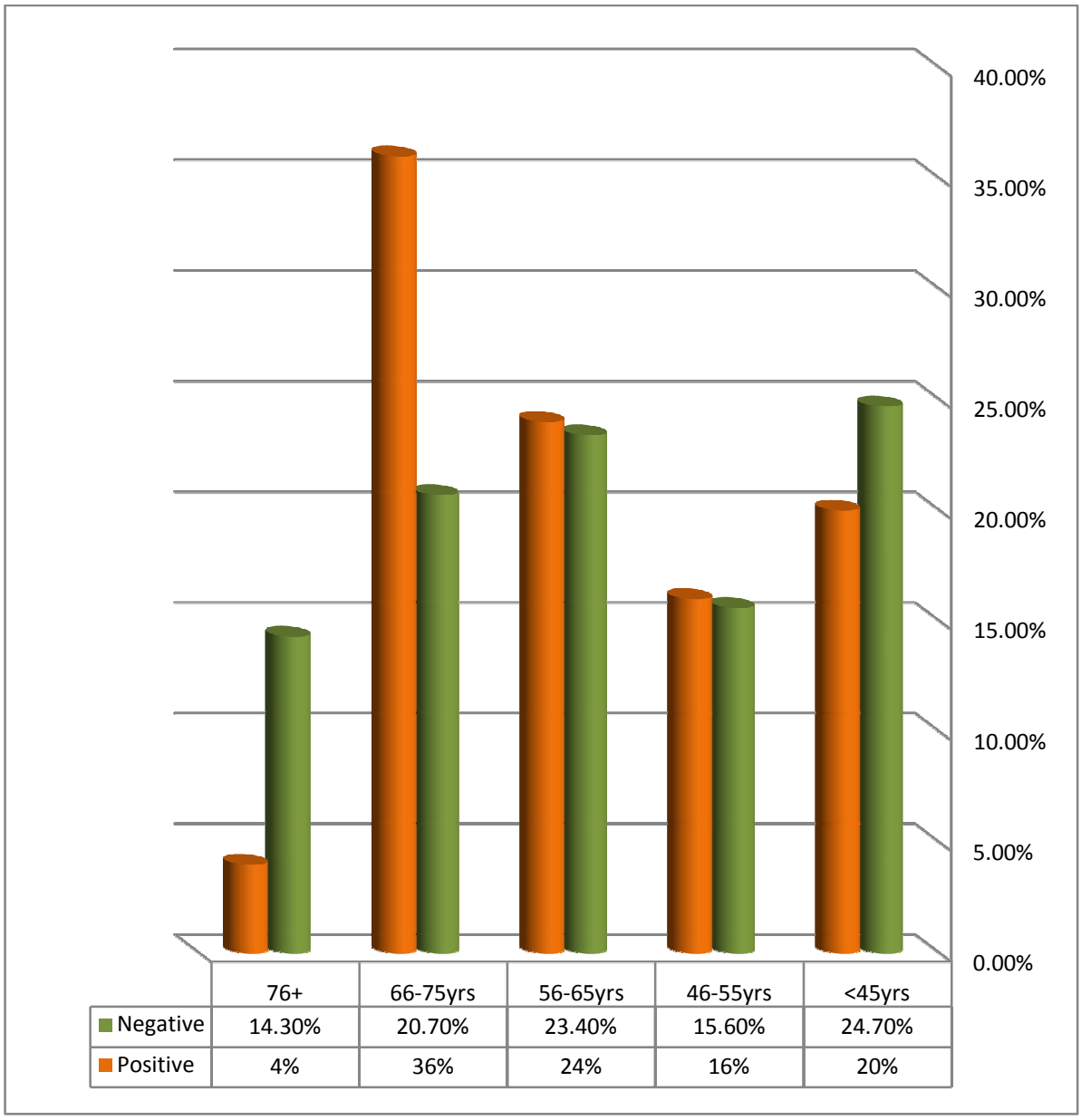


Figure (4.9) Description of PCR results of HPV subtype 16 by age

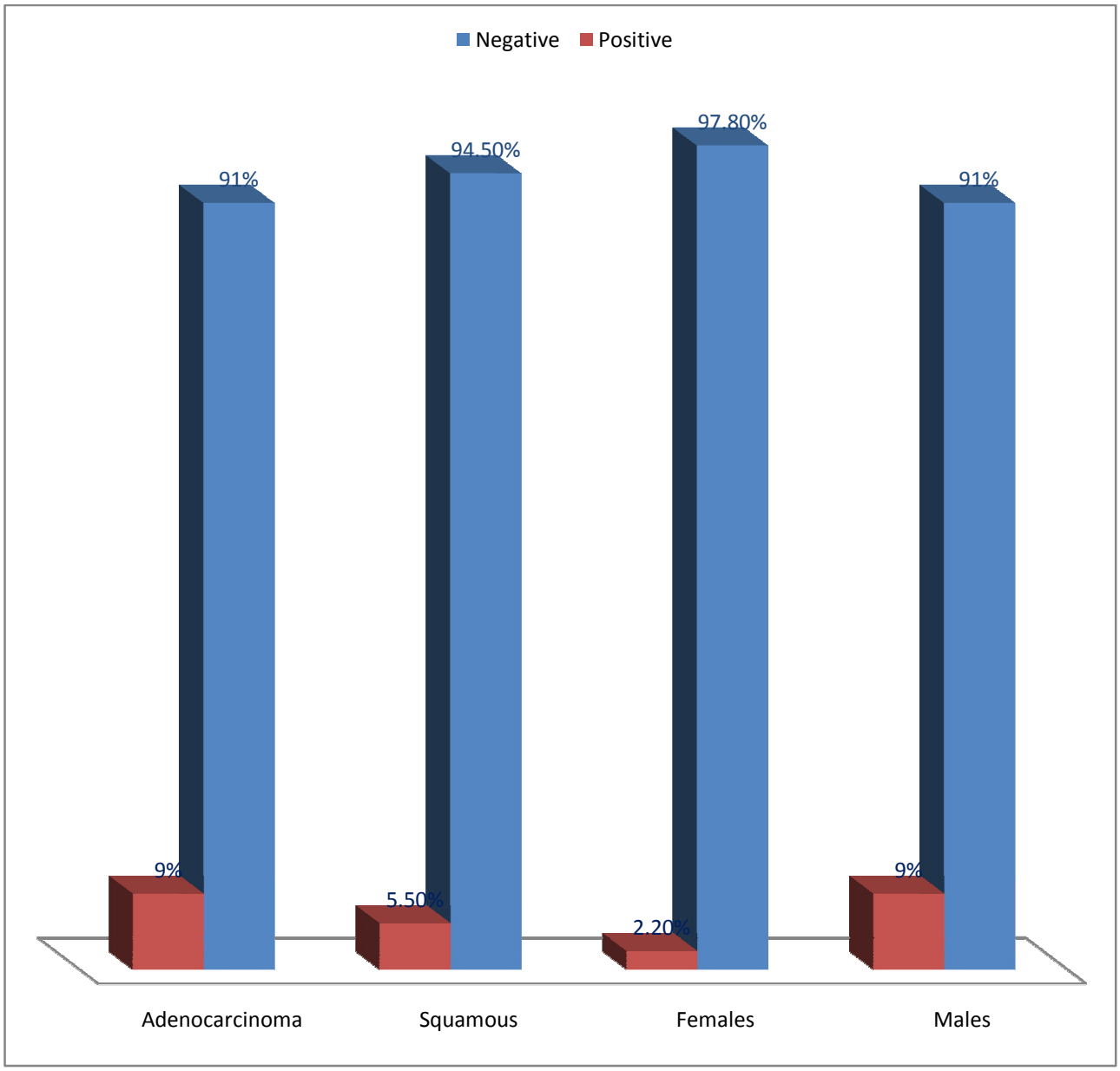


Figure (4.10) Descriptions EBV positive with PCR among sex and cancer types.

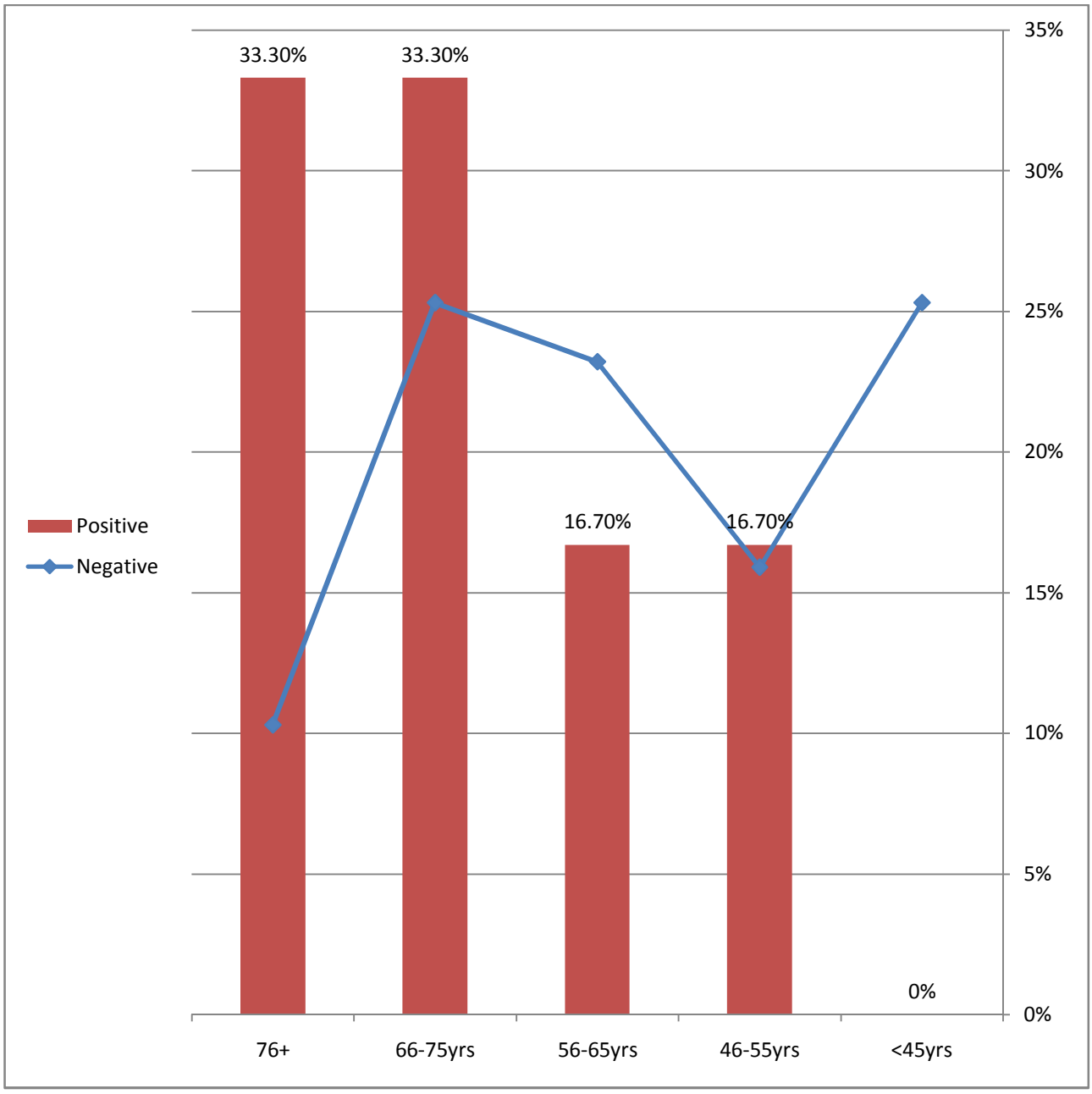


Figure (4.11) Description of PCR results of EBV by age.

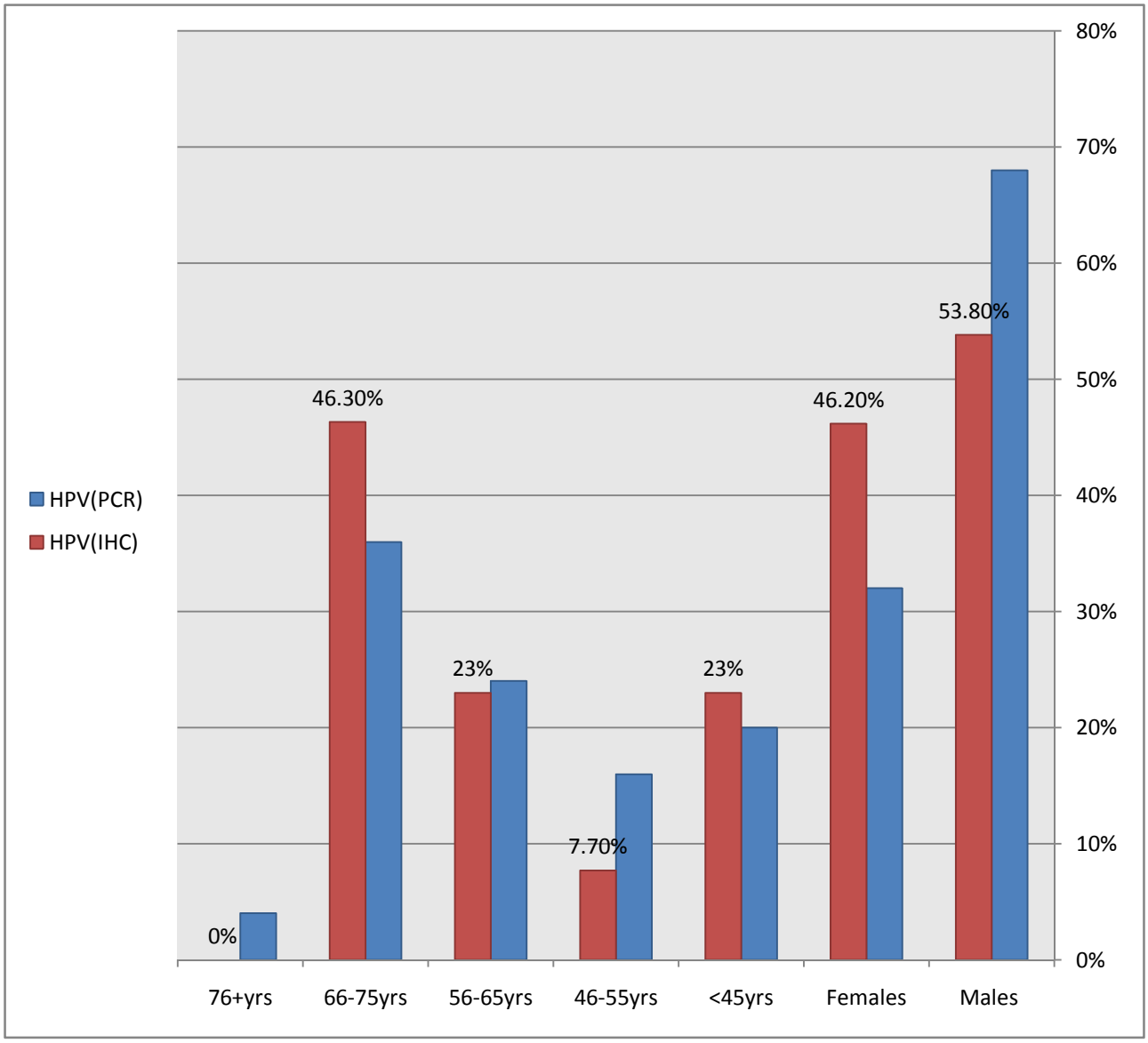


Figure (4.12) Comparison of HPV 16 positive results (both IHC and PCR) by demographical factors

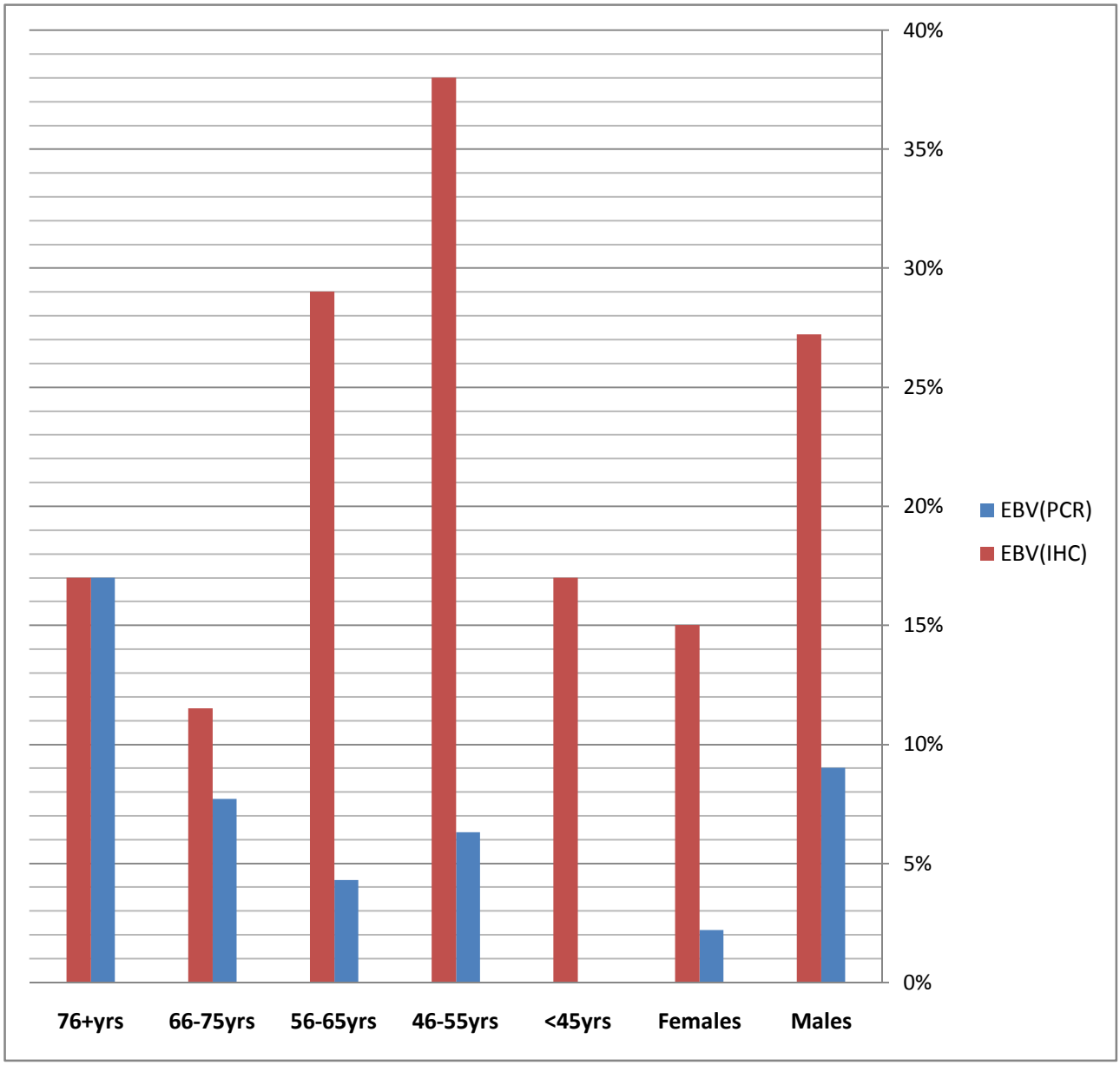
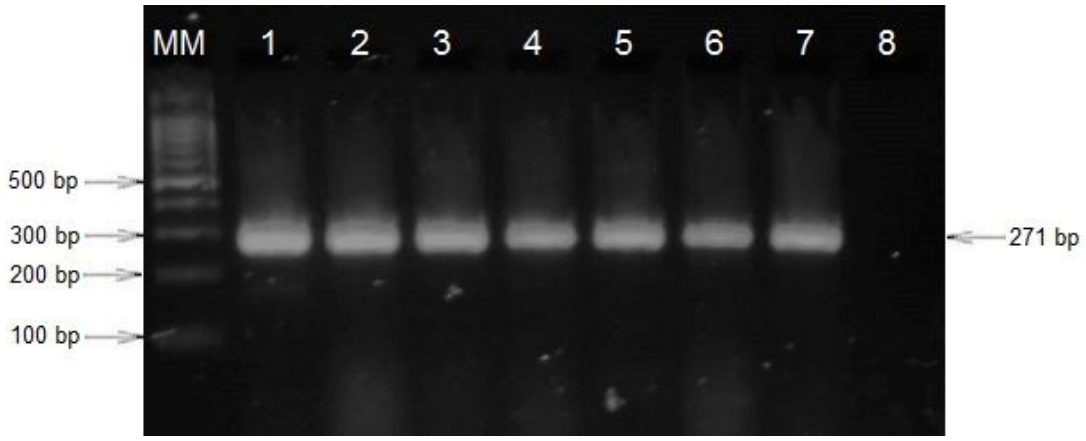
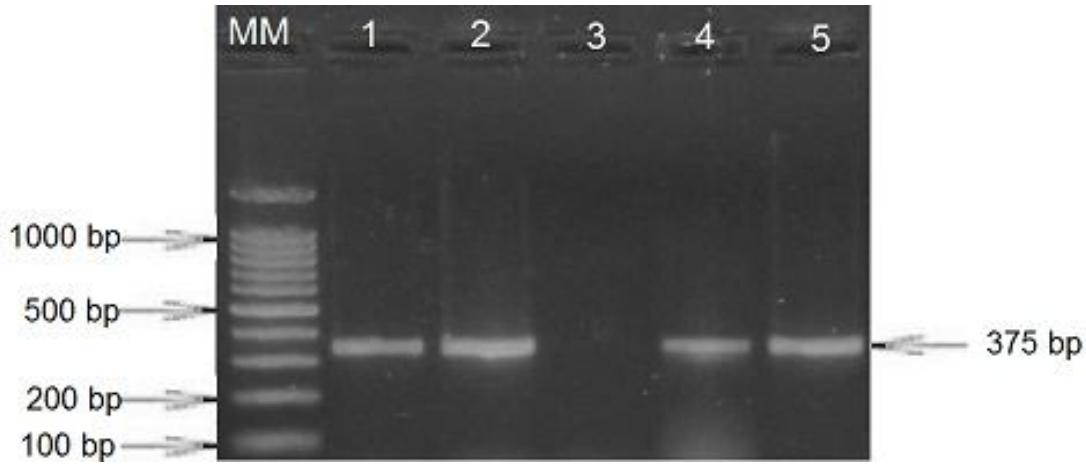


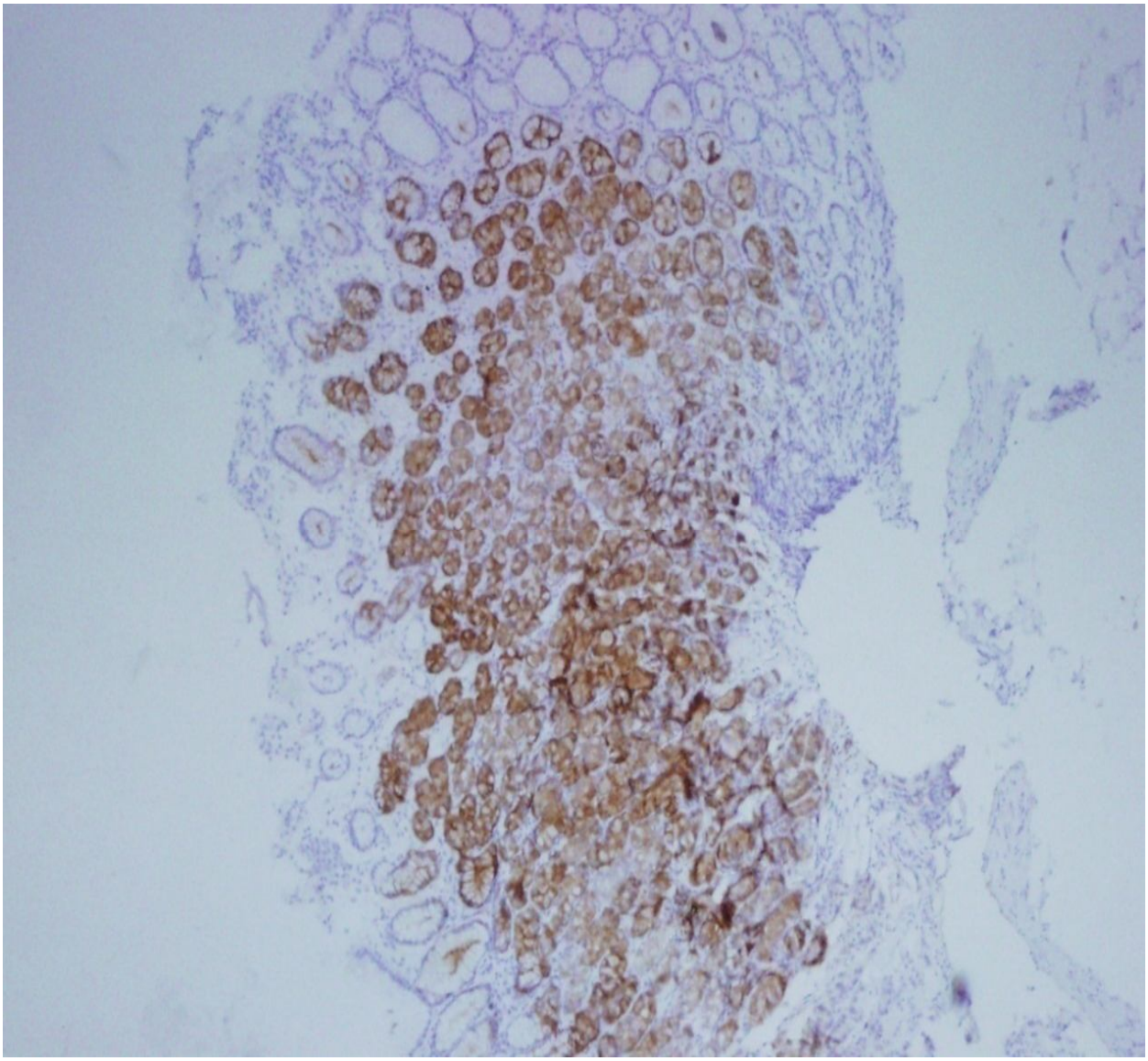
Figure (4.13) Comparison of EBV positive results (both IHC and PCR) by demographical factors



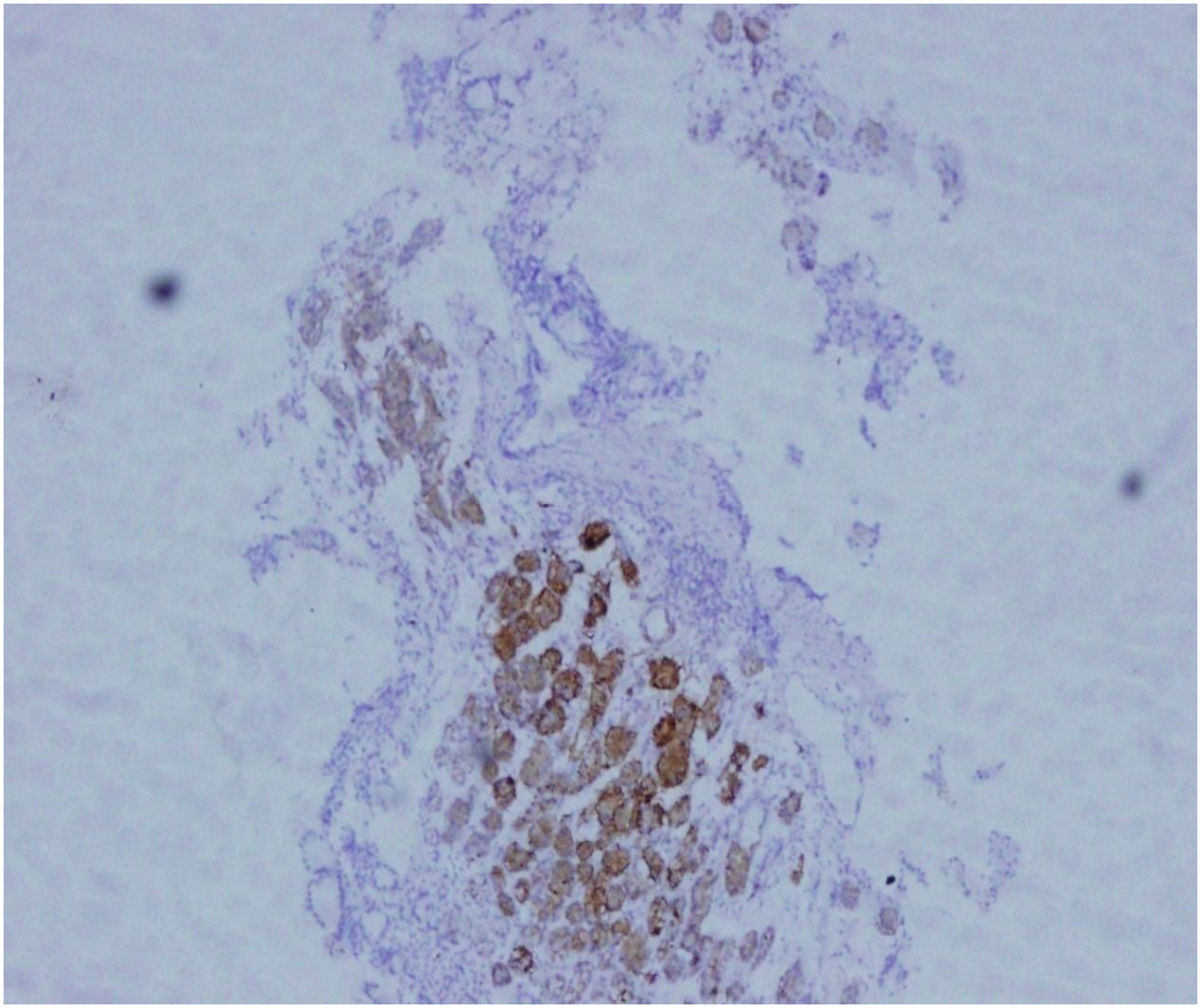
Microphotograph (4.1) Agarose gel electrophoresis of PCR products. M, 100 bp DNA ladder; sample 1, positive control and 2 to 7 were show positive result for HPV 16. And 8 was negative control.



Microphotograph (4. 2) Agarose gel electrophoresis of PCR products. M, 100 bp DNA ladder; samples 1and 2 are positive controls, sample 3 was negative control and 4 and 5 were show positive for EBV.



Microphotograph (4.3) Esophageal carcinoma showing positive HPV16 expression (Immunohistochemical staining), En Vision_ System. X100.



Microphotograph (4.4) Esophageal carcinoma showing positive EBV expression (Immunohistochemical staining), En Vision_ System HRP. X100.

Chapter five

5 Discussions

Esophageal cancer is one of the most common malignancies in Eastern Africa, but the occurrence of EC in Sudan has rarely been described in the scientific literature. Some studies from Sudan have shown that the number of EC patients has increased in recent years, approximately in many cancer centers (Gasmelseed*, et al*. 2015). Due to the absence of a unique cancer registry centre, no center can capture all EC patients. Therefore, a population- based cancer registry would provide more complete data required to better understanding EC patterns and however, many etiological factors have been linked to the etiology of EC including viral agents. The most frequent viruses that suspected to contribute to etiology of esophageal cancer are HPV particularly type 16 and EBV. Consequently in the present study we evaluated the frequency of HPV 16, HPV18 and EBV can be as risk factors of EC in Sudan.

Since the first reports in 1982 suggesting an etiological role for human papillomavirus (HPV) in a subset of esophageal squamous cell carcinomas (ESCC), the literature reporting HPV detection in ESCC has expanded rapidly.

In the present study we found a prevalence of 24.5% for HPV 16 within the investigated samples. HPV related esophageal SCC detection rates are greatly variable across different countries. Geographic location is one of a majority of the variation in HPV prevalence, with high-incidence regions including Asia reporting significantly higher HPV related esophageal SCC infection rates compared with low-incidence regions such as Europe, North America, and Oceania (Ludmir, *et al*. 2015). Studies have shown that Asia is high-risk region (Syrjänen, 2013; Hardefeldt, *et al*. 2014). In a meta-analysis study, an overall HPV prevalence of 30.6% was calculated, with a region-specific infection rate of 10.1% for Canada and the United States (Syrjänen, 2013). However, we didn't found any recent literature regarding the relationship between esophageal carcinoma and HPV reported from Sudan.

In our study the detected positive cases of HPV infections were infected with HPV16 when further confirmed with molecular tests. Such findings were reported in several studies. In a study investigated 8990 esophageal squamous cell carcinoma (SCC) patients and 174 esophageal adenocarcinomas patients, the prevalence of HPV in esophageal SCC was 22.2%, HPV16 was the most frequently observed subtype with a summarized prevalence of 11.4% . With respect to esophageal adenocarcinoma, HPV prevalence was 35.0% (95% CI, 13.2-65.7%) and HPV-16 prevalence was 11.4%. Significant association was observed between HPV 16 infection and esophageal SCC. According to HPV16, the strength of the association was found to be 3.52 (95% CI, 2.04- 6.07) (Li X, *et al*. 2014). Although HPV18 was not detected in this present study but studies have reported low prevalence rates in the highly infected regions (Liu, *et al*. 2013). In a study to determine the prognostic importance of high-risk HPV in patients with EC. A total of 105 consecutive patients who underwent esophagectomy in 2008 were included, all specimens with EC were tested by in situ hybridization for HPV16/18 and immunohistochemistry. HPV was detected in 29 of the 105 patients (27.6%) with EC (Cao, *et al.* 2014).

In our study no significant correlation between HPV16 infection with age, sex and cancer types. Study has found that HPV16 infection in ESCC was detected by genotype-specific polymerase chain reaction. HPV16 DNA was detected in 55 of 150 ESCC samples (36.7%) and 24 of 150 corresponding normal esophageal mucosa samples (16%) with significant differences ($P < 0.001$, odds ratio = 3.039, 95% confidence interval: 1.756-5.260). No statistically significant correlations were found between HPV16 infection and the age or gender of patients, tumor site, tumor cell differentiation, or lymph node metastasis ($P > 0.05$), which agreed with our finding in current study (Hu*, et al.* 2013).

In our study when PCR used to genotyping HR HPV IHC result no HPV 18 positive result obtained while HPV 16 frequency was represent 25/102(24.5%). In studies regarding HPV 18 reporting either very low contribution or absence of infection in several EC series. In population-based study to verify the association of HPV with

esophageal cancer and to investigate possible confounding factors. A nationwide study in Sweden of HPV16 and HPV18 infection and risk of esophageal squamous cell carcinoma or esophageal/gastroesophageal Adenocarcinoma was performed. About 121 case subjects with esophageal squamous cell carcinoma and 173 case subjects with adenocarcinoma of the esophagus. They conclude that no evidence of a positive association between HPV18 infection and either form of esophageal cancer (Lagergren, *et al*. 1999). And also in study conducted in Iran they report that the prevalence of HPV16 was significantly higher in ESCC cases than that in controls (P = 0.05), but there was no statistically significant difference in the prevalence of HPV18 between cases and controls. This implies that only HPV16, but not HPV18, may be a risk factor for ESCC (Farhadi*, et al*. 2005).

Many reports showed different finding concerning EBV association with esophageal cancer, some reveal strong association while other with low or even no association.

However, in the present study we found a prevalence of 21.5% of infection of EBV among Sudanese patients with EC. Relatively higher prevalence rates were previously reported in some studies. In study from German investigated 37 patients with esophageal carcinoma (Esophageal squamous cell carcinomas (n=23) and adenocarcinomas (n=14)) for the presence of human EBV DNA, EBV was detected in 35% of squamous cell carcinomas and 36% of adenocarcinomas (Awerkiew, *et al*. 2003). Nevertheless in the present study EBV positive was found in 27.3% of the cases of the adenocarcinoma and 21% of the cases of SCC. In another study an association between EBV infection and the development of esophageal carcinoma has been reported in 35.5% of the cases (Wang, *et al*. 1999). Although, it is strongly suspected that the EBV plays a role in the genesis of nasopharyngeal carcinoma, but some studies reported that, EBV is infrequently associated with esophageal cancer, and may appear through tumor-infiltrating lymphocytes in some advanced lesions (Yanai, *et al*. 2003; Chen, *et al*. 2003). EBV-associated tumori-genesis appears to be rather restricted to gastric cancer while the role of EBV in other parts such as

esophageal carcinomas appears to be insignificant in most parts of the globe (Lee, et al. 2009).

To the best of our knowledge there no study investigated the role of the EBV frequency in EC from Sudan. Most of the studies in this context from Sudan tested the association between EBV and nasopharyngeal carcinoma. Almost all of these studies found some degrees of association between EBV and nasopharyngeal carcinoma. In one study from Sudan, EBV genes were detected in 92/150 (61.3%) of patients with nasopharyngeal carcinoma (Ahmed, *et al*.2015).

In regard to the sex the prevalence of EBV infection in the current study was 27% in males and 13% in females. Since there are only small studies in regard to the relationship between EBV and EC, the relationship of sex and EBV can't be strongly, but studies on the association of EBV and nasopharyngeal carcinoma indicates similar findings to our series.

In regard to the age in our study most of cases with positive EBV expression were found among age 56-65 years. Although cancer in general accumulate at elderly people, but this need further research in regard to the start of infection and initiation of carcinogenesis.

In conclusion HPV 16 and EBV seemed to have reasonable frequency in esophageal cancer that can expected to have a role in occurrence of esophageal cancer in Sudan. Crucial preventive strategies are extremely needed to reduce the burden of esophageal cancer in Sudan.

Chapter six

6.1 Conclusion:

On the base of this study we conclude that the majority of affected people are male in spite females were with significant percentage. Greater part of cases was squamous cells carcinoma, while adenocarcinoma represent bare minimum. The most affected groups were those in old age.

HPV16 and EBV could be regarded one the most common viruses repeatedly associate with esophageal cancer, although HPV18 was not detected in each biopsy that indicate no or minimum association with esophageal cancer. IHC staining method revealed low sensitivity in comparing with PCR which may lead to missing of many positive subjects in HPV diagnosis.

6.2 Recommendations:

A further study with wide scope in this topic is recommended among Sudanese suffering of esophageal cancer, considering other factors such as environmental, habitual, biological factors and nuclear genetic alterations which may contribute as risk factors with major roles in esophageal carcinogenesis in addition to HPV high risk and low risk types. More study should be carry on this task using more sensitive method than IHC for viral detection i.e. insitu hyperdization and real time PCR. And the high incidence of cancer invites wide popular awareness about risk factors through health education that importance for early diagnosis and even incorporate screening program for viruses hence facilitate treatment and yet vaccination for the most vulnerable groups.

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6.4 Appendices:

A. Hematoxylin & Eosin procedure

i. Take sections to water.
ii. Place sections in haematoxylin for 8 minutes.
iii. Wash in tap water.
iv. Blue sections in lithium carbonate or tap water.
v. Wash in tap water.
vi. Place sections in 1% acid alcohol for a few seconds.
vii. Wash in tap water.
viii. Stain with eosin for 1 minute.
ix. Wash in tap water.
x. Dehydrate, clear.
xi. Mount sections in DPX