

**Sudan University of Science and Technology College
of Graduate Studies**

**Detection of CDX2 Tumor Marker and Human Papilloma Virus in
Esophageal Tumors**

الكشف عن الواسمة الورمية CDX2 و فيروس الورم الحليمي البشري فى اورام المريء

**A dissertation submitted for partial fulfillment for the requirement for M.Sc degree in
Medical laboratory Science (Histopathology and cytology)**

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قال الله تعالى

وَقُلْ اَعْمَلُوا فَسَيَرَى اللَّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ ۖ وَسَتُرَدُّونَ

إِلَىٰ ۖ عَالَمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُمْ بِمَا كُنْتُمْ تَعْمَلُونَ

صدق الله العظيم

Dedication

To my father....

My mother....

My brothers....

My friends...

Eslam, 2014

Acknowledgement

All great thanks are firstly to Allah.

I would like to send thanks to my supervisor Dr. Mohamed Siddige Abdalaziz for his guidance and continuous assistance.

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Eslam, 2014

Abstract

This is a descriptive retrospective study conducted at Ibn Seina hospital and Sudan University of Science and Technology during the period from April 2014 to October 2014. The study aimed at detecting CDX2 and association of Human Papilloma Virus (HPV) in esophageal tumor among Sudanese patients using immune-histochemistry and polymerase chain reaction. Samples from 30 patients previously diagnosed with esophageal tumor (20 with esophageal cancer and 10 with benign). Their ages ranging between 8 to 82 years with mean age 37.

From each block two slides were cut one for CDX2 and other for HPV detection, also 10 μ was cut from each block in eppendorf tube for detection of HPV type 18 gene using PCR. SPSS version 16 computer program was used to analyze the data. Mean, frequency and Chi square were calculated.

From thirty patients twenty were male (66.7%) and ten were female (33.3%). CDX2 was detected in four (13.3%) and twenty six samples (86.7%) were show negative result.

Seven cases (23.3%) were positive for Human papilloma virus and 23 (76.7%) were negative by using immune-histochemistry. The detection of HPV type 18 was done for 7 samples by using PCR only 5 samples were gave positive result.

On the basis of these findings the study concluded that, there is no association between CDX2 expression and esophageal cancer ($P= 0.129$). There is no relation between HPV expression and esophageal cancer ($P= 0.222$).

المستخلص

اجريت هذه الدراسة الوصفية التراجعية في مستشفى ابن سينا وجامعة السودان للعلوم والتكنولوجيا خلال الفترة ما بين ابريل 2014 إلى اكتوبر 2014. هدفت الدراسة إلى الكشف عن CDX2 وارتباط فيروس الورم الحليمي البشري (HPV) في اورام المريء لدى المرضى السودانيين باستخدام كيمياء ومناعة الانسجة وتفاعل البلمرة المتسلسل، تم جمع العينات من 30 مريضا مشخصة مسبقا باورام المريء 20 عينات مشخصة سرطان المريء و 10 عينات مشخصة اورام حميدة. تراوحت اعمارهم بين 8 إلى 82 سنة، بمتوسط عمر 37.

من كل قالب قطع مقطعين واحد لتحديد CDX2 والآخر للكشف عن فيروس الورم الحليمي البشري، كما تم قطع $10\mu\text{m}$ من كل قالب في أنبوب ابدادورف للكشف عن نوع الورم الحليمي البشري 18 الجيني باستخدام تفاعل البلمرة المتسلسل .

تم تحليل البيانات التي تم الحصول عليها باستخدام برنامج التحليل الاحصائي (الحزم الاحصائية للدراسات الاجتماعية).

من ثلاثين مريضا عشرون كانوا من الذكور (66.7%) وعشرة من الإناث (33.3%). اعطي ظهور ال CDX2 نتيجة ايجابية في أربعة عينات (13.3%) وستة وعشرين عينة كانت سالبة (86.7%). سبع حالات (23.3%) نتيجة إيجابية لفيروس الورم الحليمي البشري و 23 (76.7%) كانت سلبية بواسطة تقنية كيمياء و مناعية الانسجة.

تم الكشف عن ايجابية وجود الفيروس في 7 حالات (التي اعطت نتيجة ايجابية بتقنية كيمياء ومناعة الانسجة) باستخدام تفاعل البلمرة المتسلسل وجدت فقط 5 حالات ايجابية لهذ الفيروس.

علي أساس هذه النتائج، خلصت الدراسة إلى أنه، لا يوجد ارتباط بين التعبير النسيجي ل CDX2 وسرطان المريء ($P = 0.129$) ولا توجد علاقة بين فيروس الورم الحليمي البشري وسرطان المريء ($P = 0.222$) .

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1.1. Introduction:

Esophageal cancer (Oesophageal cancer) is cancer arising from the food pipe known as the esophagus that runs between the throat and the stomach (Montgomery *et al.*, 2014). Carcinoma of esophagus cause trouble swallowing and weight loss)pain with swallowing, a hoarse voice, enlarged lymph nodes around the clavicle, a dry cough, and possibly coughing up blood or vomiting blood)Ferri and Fred, 2012).

As of 2012, esophageal cancer is the eighth-most common cancer globally with 456,000 new cases (WHO, 2014(. It caused about 400,000 deaths that year, up from 345,000 in 1990 (WHO, 2014; Lozano *et al.*, 2012). Rates vary widely between countries, with about half of all cases occurring in China, it is around three times more common in men than women)WHO, 2014(.

According to radiation and isotope center of Khartoum (RICK) record, the esophageal cancer is the forth most common cancer among Sudanese males, and fifth most common among females (Hamad, 2011).

There are many types of esophageal cancer squamous cell cancer, which is more common in the developing world, and adenocarcinoma, which is more common in the developed world) WHO, 2014). The causes of the squamous cell carcinoma variety are: smoking tobacco, drinking alcohol, drinking hot drinks, and a poor diet. The causes of the adenocarcinoma variety are smoking tobacco, obesity, and gastroesophageal reflux disease (Zhang *et al.*, 2012) Often, the changes of Barrett esophagus are present before adenocarcinoma begins (WHO, 2014(. Squamous cell cancer which arise from the skin cells that line the esophagus (Kelsen and David, 2007). Adenocarcinoma which arise from glandular cells present in the lower third of the esophagus (WHO, 2014; David *et al.*, 2006).

Diagnosis include, physical exam: The exam is rarely helpful in diagnosing esophageal cancer. Enlarged lymph nodes in the neck or fluid collection in the chest or abdomen may be found late in the disease. Direct observation of the upper gastrointestinal tract using a flexible optical instrument (endoscope) may reveal a growth on the walls of the esophagus (*American Cancer Society*, 2004).

Tests which include a complete blood count (CBC) may be done to determine if the individual has low amounts of hemoglobin in the blood (anemia). Liver function tests (SGOT, alkaline phosphatase, and bilirubin) may be performed to determine if the cancer has metastasized to the liver. The esophagus may be visualized by x-ray after swallowing an opaque dye (barium

swallow x-ray) or by using an endoscope (endoscopy). Both of these tests may help to know the location of the tumor and the degree of obstruction within the esophagus. Also, during endoscopy, a biopsy of the tumor can be performed followed by microscopic examination of the tissue to determine if it is cancerous. An endoscopic examination of the upper respiratory tract (bronchoscopy) may determine if the tumor has spread into the upper airways. High-frequency sound waves (endoscopic ultrasound) may be used in the early stages of the disease to determine how deep the tumor has spread into the esophageal tissue. The degree of metastasis can also be determined using harmless, low-energy radio waves (MRI) or computer analysis of x-ray data (*American Cancer Society*, 2004).

Prevention includes stopping smoking and a healthy diet) WHO, 2014(. Treatment includes surgery alone with the hope of a cure, in most other cases, chemotherapy with or without radiation therapy is used along with surgery (Stahl *et al.*, 2013). Larger tumors may have their growth slowed with chemotherapy and radiation therapy)WHO, 2014(. In those with extensive disease and in many of those who are not healthy enough for surgery, palliative care or supportive care is recommended (Stahl *et al.*, 2013) .

The human Cdx2 gene is a member of the caudal-type homeobox gene family, whose members are homologs of the caudal gene of *Drosophila melanogaster*. The gene product is important in the early differentiation and maintenance of intestinal epithelium via regulation of intestine-specific gene transcription. The cdx2 gene is expressed throughout the small and large intestine, with the proximal limit occurring at the gastroduodenal junction. Cdx2 also has been shown to be present in metaplastic intestinal-type epithelium within the stomach. Cdx2 gene expression occurs in the development of intestinal metaplasia of the esophagus and its encoded protein might be a marker for the histopathologic diagnosis of Barrett's esophagus (Philips *et al.*, 2003).

One possible risk factor for esophageal squamous cell carcinoma is infection with oncogenic human papillomavirus (HPV) types. HPV type 16 (HPV16) is known to cause the majority of squamous cell carcinomas of the cervix and is strongly associated with subgroups of squamous cell carcinomas at other anogenital sites, and with cancers of the head and neck, particularly the oropharynx, based on histological findings that suggested possible associations between HPV and both malignant and benign squamous cell lesions of the esophagus (Freddie *et al.*, 2012).

1.2. Rationale

Esophageal cancers represent the eighth cancers worldwide. The majority of esophageal carcinoma has been related to smoking, tobacco, and obesity. Human papillomavirus (HPV) virus is one cause of esophageal cancer, because the focusing on detection of HPV in esophageal cancer in Sudan very little. In this study concentration will be on it and also we want to show the role of cdx2 which has implicated in the pathogenesis of barrett's esophagus. **1.3. Objectives**

General objective:

To study the expression of cdx2 and association of HPV18 with esophageal tumor among Sudanese patient.

Specific objectives:

1. To detect HPVs using immunohistochemistry in esophageal tumor patients.
2. To detect CDX2 by using immunohistochemistry in esophageal tumor sample.
3. To correlate between the presence of CDX2 and both benign and malignant tumors.
4. To correlate between the presence of HPV and both benign and malignant tumors.
5. To detect HPV18 using polymerase chain reaction.
6. To correlate between the presence of CDX2 and HPV18 in esophageal tumor.

2. Literature review:

2.1. Anatomy, histology and physiology of esophagus:

The esophagus is one of the upper parts of the gastrointestinal system (Drake *et al.*, 2005) a hollow muscular tube that connect the throat to the stomach ,it lies behind the trachea(windpipe)and in front of the spine, food and liquid ,that are swallowed travel through the inside of the esophagus to reach the stomach ,the esophagus usually between 10 and 13 inches in adult .It The esophagus has a mucosa consisting of a tough stratified squamous

epithelium without keratin, a smooth lamina propria, and a muscularis mucosa (Kuo *et al.*, 2006). The mucosa which has three parts, the epithelium forms the inner most lining of the esophagus and is made up of flat, thin, cells called squamous cell, the lamina propria is a thin layer of connective tissue right under the epithelium and muscularis mucosa is a very thin layer of muscle under the lamina propria. The submucosa layer of connective tissue just below the mucosa that contains blood vessels and nerves, muscularis propria is a thick band of muscle under the submucosa, this layer of muscle contracts in a coordinated, rhythmic way to push food along the esophagus from the throat to the stomach. The adventitia which is the outer layer of the esophagus that connects to the stomach is called gastroesophageal junction (American Cancer Society, 2012). **2.2. Pathology of esophagus:**

The pathogenesis of esophageal cancer remains unclear, oxidative damage from factors such as smoking or gastroesophageal reflux, which cause inflammation, esophagitis, and increased cell turnover, may initiate the carcinogenic process. Once cancer develops, it may spread rapidly (Peter *et al.*, 2003)

2.2.1. Esophagitis

Is inflammation of the esophagus. Substances ingested (for example, corrosives), some medications (such as bisphosphonates), food allergies, infection, and reflux of gastric acids from the stomach 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. Prolonged esophagitis plays a role in the development of Barrett's esophagus. In this condition, the lining of the esophagus changes from multiple layers of flat cells to a single layer of taller, secretory cells. The new cells are known as cuboidal epithelia. Barrett's esophagus is thought to be one of the main contributors to the development of esophageal cancer (Davidson's, 2010).

2.2.2. Cancer of esophagus

Esophageal cancer: esophageal cancer is more common in people who already have Barrett's esophagus, and occurs in the cuboidal cells. In its early stages, esophageal cancer may not have any symptoms at all. When severe, esophageal cancer may eventually cause obstruction of the esophagus, making swallowing of any solid foods very difficult and causing weight loss. The progress of the cancer is staged using a system that measures how far into the esophageal wall

the cancer has invaded, how many lymph nodes possess the cancer, and whether there are any metastases in different parts of the body (Davidson's, 2010).

2.3. Classification of esophageal cancer:

Esophageal cancers are typically carcinomas which arise from the epithelium, or surface lining, of the esophagus. Most esophageal cancers fall into one of two classes: squamous cell carcinomas, which are similar to head and neck cancer in their appearance and association with tobacco and alcohol consumption, and adenocarcinomas, which are often associated with a history of gastroesophageal reflux disease and Barrett's esophagus (oesophagogastric junctional adenocarcinoma). A general rule of thumb is that a cancer in the upper two-thirds is a squamous cell carcinoma and one in the lower one-third is an adenocarcinoma.

Rare histologic types of esophageal cancer are different variants of the squamous cell carcinoma, and non-epithelial tumors, such as leiomyosarcoma, malignant melanoma, rhabdomyosarcoma, lymphoma and others (Shield *et al.*, 2005; Halperin *et al.*, 2008).

2.4. Risk factors of esophageal cancer:

Barrett's esophagus is considered to be a risk factor for esophageal adenocarcinoma. There are a number of risk factors for esophageal cancer. Some subtypes of cancer are related to particular risk factors. Such as age, most patients are over 60, and the median in US patients is 67 (Enzinger and Mayer, 2003). And sex, the disease is more common in men than women. Also heredity, it is more likely in people who have close relatives with cancer. Tobacco smoking and alcohol use increase the risk, Tobacco and alcohol account for approximately 90% of all esophageal squamous cell carcinomas. Tobacco smoking is also linked to esophageal adenocarcinoma though no scientific evidence has been found between alcohol and esophageal adenocarcinoma (Lubin *et al.*, 2012). Gastroesophageal reflux disease (GERD) and its resultant Barrett's esophagus increase esophageal cancer risk due to the chronic irritation of the mucosal lining. Adenocarcinoma is more common in this condition (Lagergren *et al.*, 1999) . A consequence of GERD is increased exposure of the esophagus to bile acids; and bile acids have been implicated as causal agents in esophageal adenocarcinoma (Bernstein *et al.*, 2009) . Human papillomavirus (HPV) (Syrjänen, 2002). Corrosive injury to the esophagus by swallowing strong alkalines (lye) or acids Particular dietary substances, such as nitrosamines A. Radiation therapy for other conditions in the mediastinum (Enzinger and Mayer , 2003). Coeliac disease predisposes towards squamous cell carcinoma (Green , 2003) Obesity increases the risk of

adenocarcinoma fourfold (Merry , 2007) Alcohol consumption in individuals predisposed to alcohol flush reaction (Brooks , 2009) Achalasia (Park and Vaezi, 2005).

2.5. Epidemiology of esophageal cancer:

The American Cancer Society estimated that during 2007, approximately 15,560 new esophageal cancer cases will be diagnosed in the United States (American Cancer Society, 2006). In the United States, squamous cell carcinoma of the esophagus usually affects African American males with a history of heavy smoking or alcohol use. Until the 1970s, squamous cell carcinoma made up the vast majority of esophageal cancers in the United States. In recent decades, incidence of adenocarcinoma of the esophagus (which is associated with Barrett's esophagus) steadily rose in the United States to the point that it has now surpassed squamous cell carcinoma in this country. In contrast to squamous cell carcinoma, esophageal adenocarcinoma is more common in Caucasian men (over the age of 60) than it is in African Americans. Multiple reports indicate esophageal adenocarcinoma incidence has increased during the past 20 years, especially in non-Hispanic white men. Esophageal adenocarcinoma age-adjusted incidence increased in New Mexico from 1973 to 1997. This increase was found in non-Hispanic whites and Hispanics and became predominant in non-Hispanic whites. (Kenneth *et al.*, 2000). Esophageal cancer incidence and mortality rates for African Americans continue to be higher than the rate for Caucasians. However, incidence and mortality of esophageal cancer has significantly decreased among African Americans since the early 1980s, whereas with Caucasians, it has slightly increased.

(National Cancer Institute, 2006).

2.6. Signs and symptoms of esophageal cancer:

Dysphagia (difficulty swallowing) and odynophagia (painful swallowing) are the most common symptoms of esophageal cancer.. Substantial weight loss is characteristic as a result of reduced appetite, poor nutrition and the active cancer. Pain in the epigastrium, another sign may be an unusually husky, raspy, or hoarse-sounding cough, because the tumor affect the laryngeal nerve. The presence of the tumor may disrupt normal peristalsis (the organized swallowing reflex), leading to nausea and vomiting, regurgitation of food, coughing and an increased risk of aspiration pneumonia (Enzinger and Mayer, 2003).

2.7. Diagnosis of esophageal cancer: Medical history and physical exam symptoms

that might be caused by esophageal cancer, medical history to check for possible risk

factors and to learn more about patients symptoms, many test may be done to look for esophageal cancer and other health problems., may also be referred to a gastroenterologist for further tests and treatment (American cancer Society, 2014).

Lab testing of biopsy samples

An area seen on endoscopy or on an imaging test may look like cancer, but the only way to know for sure is to do a biopsy. For a biopsy, removes small pieces of tissue from an abnormal area. This is most often done during an endoscopy examination, then looks at the tissue under a microscope to see if it contains cancer cells. Other test include CBC, occult blood,liver ,heart and lung function(American cancer society, 2014).

Barium swallow (also called an esophagram). The patient swallows a liquid containing barium and then a series of x-rays are taken. Barium coats the surface of the esophagus, making a tumor or other unusual changes easier to see on the x-ray (American Society of Clinical Oncology, 2014)

Upper endoscopy (also called esophagus-gastric-duodenoscopy, or EGD). This test help to see the lining of the esophagus. A thin, flexible tube with a light and video camera on the end, called an endoscope, is passed down the throat and into the esophagus while the patient is sedated. If there is an abnormal finding, a biopsy will be performed to find out if it is cancerous. An endoscopy using an inflatable balloon to stretch the esophagus can also help widen the blocked area so that food can pass through until treatment begins (American society of clinical oncology, 2014).

Molecular testing of the tumor. Use to identify specific genes, proteins, and other factors unique to the tumor. This test help to determine the treatment (American Society of Clinical Oncology, 2014).

2.8. Treatment:

The treatment is based on the stages of cancer location, person general condition and individual preferences. Small localized squamous cancers may be treated with surgery alone. Chemotherapy and radiation therapy is used along with surgery (Stahl *et al.*, 2013) .larger tumor may have their growth slowed with chemotherapy and radiation therapy (Montgomery *et al.*, 2014).in the presence of extensive disease or if the affected person is not fit enough to undergo surgery, palliative care is often recommended (Stahl *et al.*, 2013). **2.6. 2.9.Human papilloma virus (HPV):**

Is a DNA virus from the papilloma virus family which have ability of infecting humans. Like all papilloma viruses, HPVs establish productive infections only in keratinocytes of the skin or mucous membranes. Most HPV infections are subclinical and will cause no physical symptoms; however, in some people subclinical infections will become clinical and may cause benign papillomas (such as warts [verrucae] or squamous cell papilloma), or cancers of the cervix, vulva, vagina, penis, oropharynx and anus .HPV has been linked with an increased risk of cardiovascular disease (Kuo and Fujise , 2011). In addition, HPV 18 infection are a cause of a unique type of oropharyngeal (throat) cancer (Gillison, 2004; Megan , 2013).

In 2014 Zhang et al found that HR-HPV infection and integration related to telomere elongation and DNA methylation of human telomerase reverse transcriptase may be a potential biomarker of prognosis in patients with ESCC.

In 2012 Freddy et al found limited serological evidence of an association between esophageal squamous cell carcinoma and HPV in the populations studied. Although HPV does not appear to be an important risk factor for esophageal squamous cell carcinoma.

In 2010 Xueqian et al they tested Two hundred and forty four of 435 samples (56.1%) tested positive for HPV L1. Significant differences in detection rate were observed neither among the three areas of China nor between China and the US. HPV6, 16, 18, 26, 45, 56, 57, and 58 were identified in L1 positive samples. HPV16 and 57 were the most common types in all regions, followed by HPV26 and HPV18.

In 2010 Xueqian, et al found HPV infection is common in esophageal carcinoma independent of region and ethnic group of origin. HPV is involved in esophageal carcinogenesis.

In 2011 Goto et al said that, Among the geographic areas surveyed, Kagoshima exhibited a significantly higher prevalence of HPV infection in cases of esophageal carcinoma. Subtypespecific ISH was also performed, which identified the high-risk HPV types 16/18 in the majority of the patients with esophageal cancer positive for HPV.

In 2011 Zhang et al found that the distribution of HPV genotypes in patients from high to low proportion was HPV-16, -58, -18, -33, -31 and -11. Infection with multiple HPV genotypes mainly included HPV-16/-18 and HPV-16/-33.

2.10.1 Tumor markers:

Identification of biomarkers for oesophageal cancer in serum is attractive because of the ease of obtaining samples for analysis and would be an ideal method for screening (Bird and Fitzgerald,

2009)), standard tumor markers, such as carcinoembryonic antigen, 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 (peter *et al.*, 2003).

2.10.2. Homeobox protein CDX-2:

Is a protein that in humans is encoded by the *CDX2* gene (German *et al.*, 1995) a homeobox transcription factor is the protein encoded by this gene. CDX2 is the gene that directs early embryogenesis in mice. It is required to form the placenta (Chawengsaksophak *et al.*, 2004).

CDX2 ectopic expression was reported more than 85% of the human patients with acute myeloid leukemia (AML). Ectopic expression of CDX2 in murine bone marrow induced AML in mice and upregulate hox genes in bone marrow progenitors (Rawat *et al.*, 2004; scholl *et al.*, 2007). CDX2 has been implicated in the pathogenesis of Barrett's esophagus where it has been shown that components from gastroesophageal reflux such as bile acids are able to induce the expression of an intestinal differentiation program through up-regulation of NF- κ B and cdx2 (Debruyne *et al.*, 2006). CDX2 is also used in diagnostic surgical pathology as a marker for gastrointestinal differentiation, especially colorectal (Liu *et al.*, 2007).

CDX2 expression has been implicated in the development of intestinal metaplasia, normal esophageal squamous epithelium does not appear to express cdx2, low levels of cdx2 mRNA are detectable in biopsy samples of squamous epithelium from patients with gastroesophageal reflux disease, although high grade dysplasia and invasive esophageal adenocarcinoma are reported to have reduced, or total loss of cdx2 protein, selective cdx2 expression may be seen in well differentiated tumor (Nadine *et al.*, 2009).

In 2005 H Kazumori *et al.* said that CA activates cdx2 promoter via NF κ B and stimulates production of cdx2 protein in esophageal keratinocytes with production of intestinal-type mucin. This may be one of the mechanisms of metaplasia in Barrett's esophagus.

In 2006 Philip *et al.* said that transformation associated with reflux at the gastroesophageal junction reflects activation by bile acid and acid of a transcriptional program involving NF- κ B and cdx2, which mediate intestinal metaplasia and ectopic expression of GC-C.

In 2004 Gabriel *et al.* said that cdx2 is a highly sensitive marker for Barrett's esophagus. It is also expressed in a significant minority of cases of columnar-lined esophagus without goblet cells, suggesting that it may detect intestinal phenotypic modifications in the absence of goblet cells.

Accordingly, cdx2 immunostaining could help identify patients with Barrett's metaplasia in cases where no goblet cells are visible in biopsies from the columnar-lined esophagus. Finally, lack of cdx2 expression in the 'columnar blues' suggests that these cells are not diagnostic of intestinal metaplasia.

In 2007 Yingchuan et al said that secondary bile acid stimulation upregulates *CDX2* gene expression in both normal and cancer cell lines. They further support the role of bile acids in the pathogenesis of Barrett's esophagus and link the clinical evidence of a high prevalence of luminal bile acids in Barrett's to expression of the gene thought to be responsible for the phenotypic expression of intestinal metaplasia.

3. Materials and method:

3.1. Study design:

This is a descriptive retrospective study, aimed to detect the expression of CDX2 and association of HPV 18 in esophageal cancer.

3.2. Study area:

This study was conducted at Ibn seina hospital and Sudan University of Science and Technology Collage of Medical laboratory Science during the period of April 2014 to October 2014.

3.3. Sampling:

Thirty paraffin wax blocks were obtained from patient archive of Ibn seina hospital, twenty of them were previously diagnosed as esophageal cancer and ten of them diagnosed as benign were randomly selected.

3.4. Sample collection and preparation:

From each paraffin block two sections of 3µm thick were cut. The two sections was placed on coated slides for immunohistochemistry staining, and also 10 µ was cut in eppendorf tube for PCR.

3.5. Immunohistochemical procedure for CDX2 and HPV:

Dewaxation:

The slides were dewaxed in oven at 60°C for 2 and half hours and cleared in xylene.

Rehydration:

All slides were rehydrated in descending concentration of ethyle alchol 100, 90, 70 and 50% and D.W for 2mins in each changes.

Antigen retrieval:

Antigen was retrieved using heat, sections were placed in (citrate buffer PH 9.9) in water bath at 95°C for 30mins, then cooled for 5mins, after that the slides washed in phosphate buffer PH 7.2.

Peroxidase blocking:

The endogenous peroxidase activity was blocked by 3% H₂O₂ for 10 min, then the slides were washed in buffer.

Staining procedure:

The primary antibody (CDX2) was added to one section and the other primary antibody to (papilloma virus 16 and 18, E6 Early protein) was also added to the second section for 30 min, then the slides were washed in buffer for 2 min, after that the primary antibody enhancer was added for 15 min, then all slides were washed in buffer for 2 min. The sections were treated with HRP for 15 min and washed in buffer for 2 min after that DAB chromogen was added for 3 min, then sections were counter stained in Mayers haematoxylin for 30 seconds, then blueing was done for 5 min after that sections were dehydrated, cleared and mounted in DPX.

3.6. Polymerase chain reaction:

3.6.1. DNA extraction:

DNA was extracted from esophagus according to commercial kits (Sacace Biotechnologies, Italy) used for DNA extraction according to manufactures instruction (see appendix).

3.6.2. PCR amplification:

DNA was amplified according to commercial kits (Sacace Biotechnologies, Italy (see appendix)) then the tube was closed and transferred to the thermalcycler when the temperature was reached 95C, then the programe was started.

3.6.3. Gel electrophoresis:

The DNA amplified by PCR was separated electrophoretically in 3% agarose gel in tris borate EDTA running buffer, agarose gel was prepared by dissolving 3 gm of agarose was in 75ml of TBE buffer, ethidium bromide was added, 10µ of the PCR product were loaded into the wells 4 microliters a 100 bp DNA ladder, was loaded into the first well of the gel.

3.7. Result interpretation:

The obtained result, as well as all clinical information data were entered a computer, SPSS (version16) program was used to analyze data. Mean,frequency,and Chi square were calculated.

3.8. Ethical consideration:

The study was performed with approval from the ethics committee of Sudan University of Science and Technology and Khartoum Health ministry board (see appendix).

4. Results

The study involved 30 subjects, twenty out of them were males (66.7%) and ten were females (33.3%). table No. 1 summarize this data.

The study subjects age was ranged from 8 to 82 years, all subjects were grouped into two age groups less than or equal to 60 years and more than 60 years. Table No. 2 shows this.

When CDX2 was investigated in 30 samples, 4(13.3%) samples were positive, and 26(86.7%) were negative. As indicated in table No. 3. .All positive samples were detected in poorly and moderate differentiated adenocarcinoma, this result confermate that CDX2 expression occur just in intestinal metaplasia of the esophagus

Also human papilloma virus was tested immunohistochemically in 30 samples, 7 (23.3%) were positive. And 23(76.7%) were negative .As indicated in table No. 4.

When we compared the expression of CDX2 in malignant and benign individual there was no significant differences between malignant and benign samples ($P= 0.129$). Table No. 5.

And when we compared the expression of HPV in both malignant and benign samples we found that, there was no significant differences between them ($P= 0.222$). Table No. 6.

Out of 7 samples which were positive with expression of human papilloma virus by immunohistochemistry 5 of them were positive by polymerase chain reaction.

Table 4.1. Sex distribution among the study population

Sex	Frequency	Percent
Male	20	66.7
Female	10	33.3
Total	30	100

Table 4.2. Age of the study population

Age	Frequency	Percent
Equal to or Less than 60	14	46.7
More than 60	16	53.3
Total	30	100

Table 4.3. CDX2 immunohistochemical result

CDX2	Frequency	Percent
Positive	4	13.3
Negative	26	86.7
Total	30	100

Table 4.4. HPV immunohistochemical result

HPVI	Frequency	Percent
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Positive	7	23.3
Negative	23	76.7
Total	30	100

Table 4.5. Relationship between CDX2 and diagnosis of esophageal tumors

	Diagnosis		Total
	Malignant	Benign	

CDX2	Positive	4	0	4
	Negative	16	10	26
Total		20	10	30

Table 4.6. Relationship between HPV and diagnosis of esophageal tumors

		Diagnosis		Total
		Malignant	Benign	
	Positive	6	1	7

HPV	Negative	14	9	23
Total		20	10	30

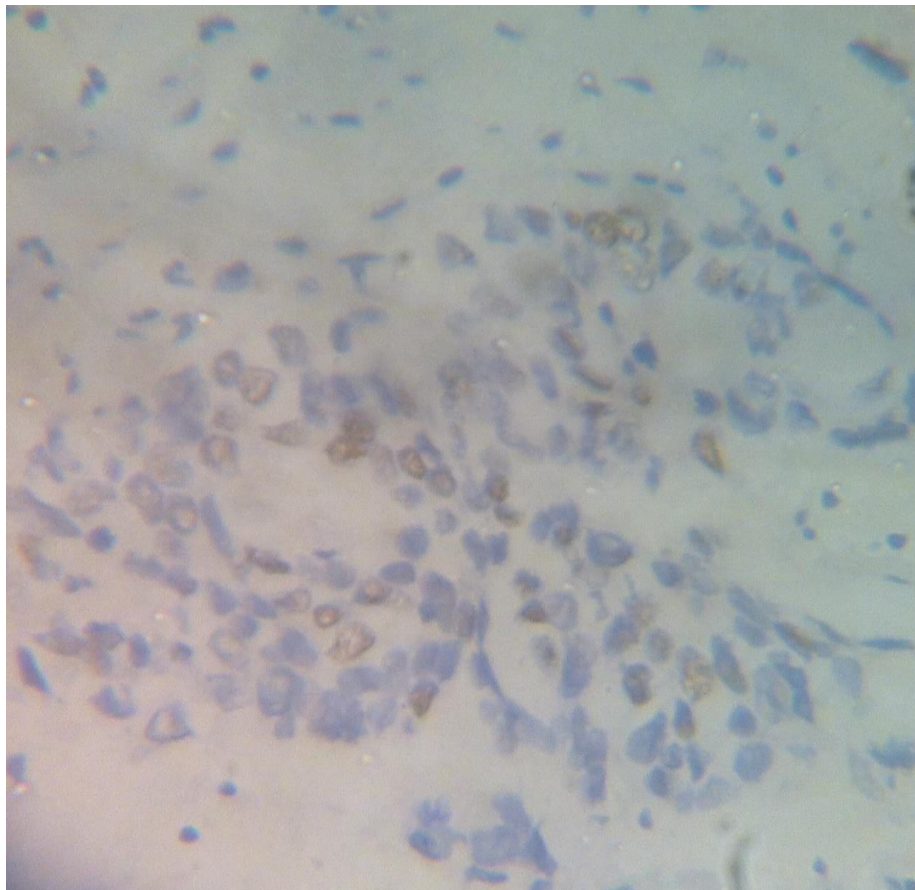


Fig. 4.1: Positive immunohistochemical staining of CDX2 marker in moderate differentiated adenocarcinoma of esophagus (40X)

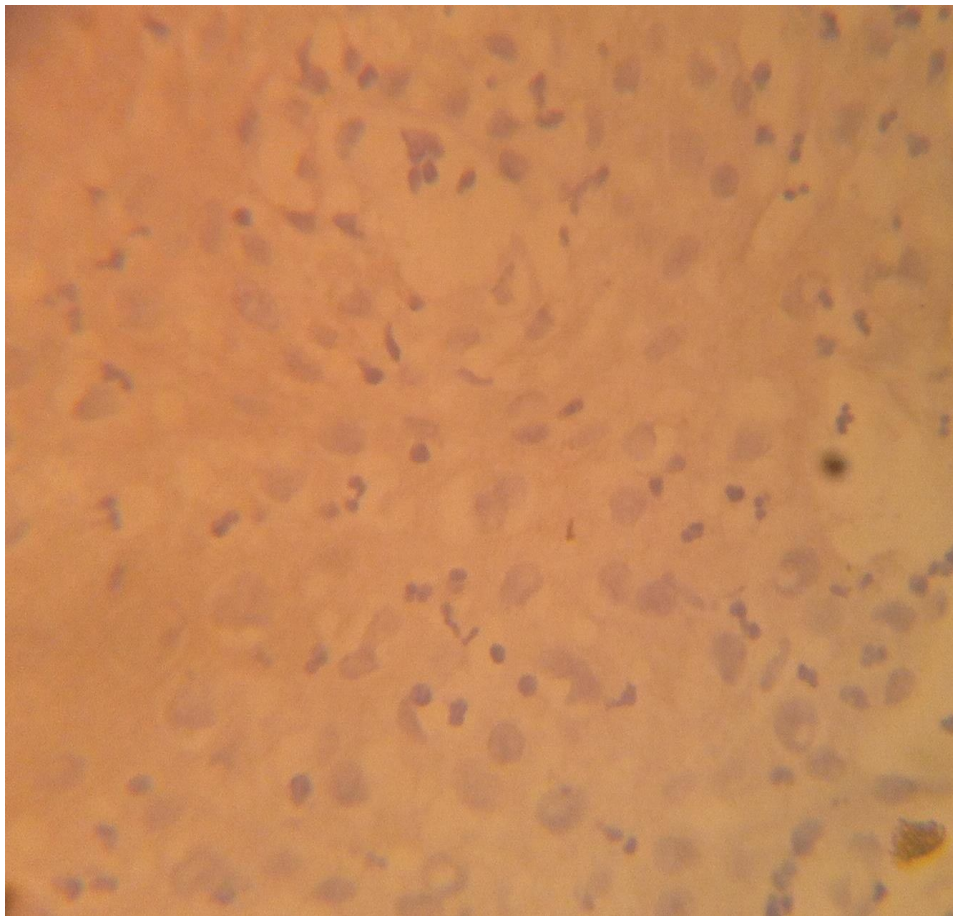


Fig. 4.2: Positive immunohistochemical staining of Human Papilloma Virus in adenocarcinoma of esophagus (40X)

5. Discussion:

Esophageal cancer is one of the most common malignant tumor worldwide, it shows marked geographic variation across the world with exceptionally high rates in certain areas of china (li, 1982; cheng, 1994). A number of environmental factors could be important in the carcinogenesis, such as virus, in the development of esophageal carcinoma either by producing carcinogens or by interacting directly with host cell (Chang,*et al.*, 1994 ; Baehr and Mcdonald, 1994) . In this study esophageal cancer is more common in male than female this result agreed with study carried out in USA found that men are more than 3 times as likely as women to get esophageal cancer (American cancer society, 2011) because the male are more exposed to the risk factors that lead to esophageal cancer.

This study found that the disease is more common in the people who are above 60 years this has been agreed by American cancer society,2011.Reported the chance of getting esophageal cancer is low at younger ages and increase with age, less than 15% of cases are found in people younger than age 55(American cancer society , 2014).

When we comparing the expression of CDX2 in malignant and benign individuals their was no significant difference between malignant and benign, the positive result of detection of CDX2 using immunos-histochemistry, were four (13.3%),and twenty six were negative (86.7%),all positive samples were detected in poorly and moderate differentiated adenocarcinoma ,this result explane that the CDX2 expression occur just in intestinal metaplasia of the esophagus , this has been supported by Reda and his colleagues 2011,who said that CDX2 is not expressed in normal esophageal and gastric epithelial cells but its expression associated with intestinal metaplasia of the esophagus. Negative CDX2 staining was observed in normal squamous

esophageal lining, while strong nuclear staining was seen in all cases of Barrett's intestinal metaplasia, dysplasia, and adenocarcinoma (Reda *et al.*, 2011).

30 samples were tested for detection of human papilloma virus using immunohistochemistry, seven were positive (23.3%), and twenty three were negative (76.7%), the comparison of the expression of human papilloma virus in both malignant and benign samples found that, no significant differences between them, this result is similar to study of Seagusa and others who found HPV is not likely to be involved in oesophageal or gastric tumorigenesis in Japanese patients (Seagusa *et al.*, 1997). Another study in Sweden by Løfdahl and his colleagues they found no evidence of association between HPV 16 or 18 infection and esophageal cancer (Løfdahl *et al.*, 1999). And disagreed with study of Surbhi and his colleagues they found that HPV is associated with 3-fold increase in the risk of OSCC (Surbhi *et al.*, 2013). Seven out of all sample which were positive by immunohistochemistry for human papilloma virus, polymerase chain reaction was carried for them, five of these sample were positive and two were negative for human papilloma virus type 18, the two negative samples may be other types of human papilloma virus or may be false positive result by immunohistochemistry, because the polymerase chain reactions is more sensitive than immunohistochemistry.

6. Conclusions and Recommendations

6.1. Conclusions:

On the basis of this study we concluded that:

- There is no significant relation between human papilloma virus infection and esophageal cancer, and HPV18 is more common one.
- No relation between CDX2 expression and esophageal cancer.

6.2. Recommendations:

On the basis of this study we recommended:

- further study should be with large sample size for detection of CDX2.
- Screening programe should be applied for patients with esophageal tumor for Human Papilloma Virus.

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Appendix 1-Instrument and

material 1-1 instrument:

-rotary microtome

-oven

-coplinjars

-staining racks

-stainless microtome blade

-coated slide

-cover glass

-water bath

-dako pen

-thermal cycler

-workstation

-pipettors -tube

rack

1-2 Materials:

-xylene

-ethyle alcohol

-mayer's haematoxyline

-distilled water

-citrate buffer

-peroxidase blocker

-Anti CDX2 antibodies (primary antibodies) -Anti human papilloma virus antibody (primary antibodies)

-Dextran polymer conjugated secondary antibodies and HRP

-3-3 diaminobenzidinetetrahydrochloridin substrate buffer

-DPX mounting media

-PCR mix 18-59

-buffer

-taq polymerase

-mineral oil

-DNA buffer

-HPV genotype control type18

-DNA extraction kit

-detection agarose kit

2-reagents sheet