بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Detection of CDX2 Tumor Marker and Human Papilloma Virus (HPV) in Esophageal Tumor among Sudanese patients using Immunohistochemistery and Polymerase Chain Reaction

الكشف عن الواسمة الورمية CDX2 وفيروس الورم الحليمي البشري في أورام المريء لدي المرضي السودانيين بإستخدام كيمياء الأنسجة المناعية وتفاعل البلمرة التسلسلي

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الآيـــــــــــــة

قال تعالى:

(وَقُلِ اعْمَلُوْا فَسَيَرَى اللّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ وَسَتُرَدُّونَ إِلَى عَالِمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُم بِمَا كُنتُمْ تَعْمَلُونَ)

صدق الله العظيم

سورة التوبة: الآية 105

Dedications

I dedicate this research to my parents

To my second mama Nadia
To my uncle

To my husband and son

To my brother and sisters

for their support and kindness

To my friends and colleagues

Acknowledgment

This research took me almost a seven month, by that time; I have met with great people whose contribute in many ways came out with this projects. It is a pleasure to convey my gratitude to them all in my humble acknowledgment.

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Abstract

This is a descriptive retrospective study conducted at Ibn Siena hospital and

Sudan University of Science and Technology during the period from April

2014 to October 2014.The study aimed to detecte CDX2 tumor marker and human papilloma virus (HPV) in esophageal tumor among Sudanese patient using immunohistochemical method and Polymerase chain reaction. In this study 30 samples were collected from patients previously diagnosed with esophageal tumor (20of them with esophageal cancer and 10 of them with benign tumor). The p a t i e n t 's age ranging between 8 to 82 years with mean age 37 years, with no association between age group and type of esophageal tumor.

From each block two sections were cut, one for CDX2 detection and other for HPV detection , also 20 micro liter was cut from each block in ependorff tube for detection of HPV type 31 gene using PCR. SPSS version 16 computer programm was used to analyze the data, mean, frequency and chi square were calculated.

Out of thirty patients twenty (66.7%) were males and ten (33.3%) were females, with the ratio 2:1. CDX2 was positive in four samples (13.3%) all of them were malignant, and negative in 26 (86.7%) .

Seven samples (23.3%) were positive for humanpapilloma virus and 23 (76.7) samples were negative by using immunochistochemistery. The detection of human papilloma virus type 31 was done for 30 samples by using PCR two samples (6.7%) were positive, and 28 (93.3%) were negatives. The study concluded that, there is no association between CDX2 expression and HPV infection with the type of esophageal tumor.

المستخلص

أجريت هذه الدراسة الوصفية التراجعية في مستشفي ابن سينا وجامعة السودان للعلوم والتكنولوجيا خلال الفترة من ابريل 2014 إلي أكتوبر 2014 هدفت هذه الدراسة للكشف عن الواسمة الورمية 2CDXوفيروس الورم الحليمي البشري بأورام المريء لدي المرضي السودانيين باستخدام كيمياء الأنسجة المناعية و تفاعل البلمرة التسلسلي تم جمع العينات من 30 مريضا مشخصين مسبقا بأورام المريء(20 عينة مشخصة بسرطان المريء و10 عينات مشخصة بأورام حميدة) تراوحت أعمار المرضي بين 8 إلي 82 سنة و بمتوسط عمر37 سنة مع عدم وجود ارتباط بين العمر ونوع الورم.

من كل قالب تم قطع مقطعين واحد لتحديد2CDX والأخر للكشف عن فيروس الورم الحليمي البشري كما تم قطع 20 ميكرون من كل قالب في أنبوب ابنادرووف للكشف عن نوع فيروس الورم الحليمي البشري 31 الجيني باستخدام تفاعل البلمرة المتسلسل .

تم تحليل البيانات بإستخدام برنامج الحزم الإحصائية للعلوم الاجتماعية وتم حساب المتوسط والتردد واختيار مربع كاي من الثلاثين مريض عشرون(66.7%) كانوا من الذكور وعشرة(33.3%) من الإناث بمعدل 1:2 أعطي الواسمة الورمية2CDX نتيجة ايجابية في أربعة عينات (13.3%) وستة وعشرون عينة كانت سالبة(86.7%) .

عند استخدام تقنية كيمياء مناعة الأنسجة اعطت سبع حالات(33.3%) نتيجة ايجابية لفيروس الورم الحليمي البشري و23 (76.7%) كانت سلبية.

بينما أعطت حالتين نتيجة ايجابية (6.7%) لفيروس الورم الحليمي البشري من النوع 31 باستخدام تفاعل البلمرة المتسلسل و28 (93.3%) نتيجة سلبية.

خلصت الدراسة إلي انه لا يوجد ارتباط بين التعبير النسيجي 2CDX والإصابة بفيروس الورم الحليمي البشري مع نوع ورم المريء.

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CHAPTER ONE

CHAPTER ONE
Introduction

Esophageal cancer (EC) is an important worldwide health problem because of its poor prognosis and a relatively high incidence in some parts of the world. Advances in surgical techniques, chemotherapy and radiotherapy have not substantially modified its prognosis over the last 25 years (Enzinger and Mayer 2003) Esophageal cancer is cancer of the esophagus(WHO.
2014) Symptoms often include trouble swallowing and weight loss other symptoms pain with swallowing, a hoarse voice, enlarged lymph nodes around the clavicle, a dry cough, and possibly coughing upblood or vomiting blood (Ferri and fred .2012).
The large majority of esophageal tumors are accounted for by squamous cell carcinomas (SCCs: 60–70%) or adenocarcinomas (ACs: 20–30%) (Enzinger, *et al,* 2003).
EC is more common in Belgium, China, Iran, Iceland, India, (Stewart, *et al*.

2003). The American Cancer Society estimated that during 2014, approximately 18,170 new esophageal cancer cases diagnosed in the United States and will result in 15,450 deaths (American Cancer Society.2014) In Sudan, According to radiation and iso tope center of Khartoum(RICK)record
,the esophageal cancer is the fourth most common cancer among Sudanese's males, and fifth most common among females (Hamad.2011) .
Barrett's esophagus is considered to be a risk factor for esophageal adenocarcinoma, tobacco50% and alcohol 32% are main risk factors. For squamous cell carcinoma (WHO.2014).

Esophageal cancer classified in to two main subtypes, squamos cell cancer more common in the developing world and adeno carcinoma more common in the developed world (WHO. 2014).
The disease is diagnosed by biopsy done via an endoscope (a fiberoptic camera) (Stahl, *et al.* 2013).Prevention includes stopping smoking and a healthy diet (Ferri, Fred 2012 ,WHO. 2014). Treatment is based on the cancer's stage, its location. Small localized squamous cancers treated with surgery mainly chemotherapy with or without radiation

therapy used. (Stahl, *et al*.2013) Larger tumors growth slowed with chemotherapy and radiation therapy (WHO.2014).

Caudal-related homeobox 2 is a protein that in humans is encoded by the CDX2 gene (German, *et al*. 1995). This protein is a homeobox transcription factor . It is directs early embryogenesis in mice. It is required to form the placenta (Chawengsaksophak , *et al*. 2004) .CDX2 is implicated in the pathogenesis of Barrett's esophagus ,the components from gastro esophageal reflux as bile acids induce the expression of an intestinal differentiation program through up-regulation of CDX2 (Debruyne , *et al* . 2006).

Human papillomavirus (HPV) is a DNA virus from the papillomavirus family that is capable of infecting humans. Like all papillomaviruses, HPVs establish productive infections only in keratinocytes of the skin or mucous membranes. Most HPV infections are subclinical and will cause no physical symptoms (CDC.2008). There are multiple types of HPV, sometimes called "low risk" and "high risk" types. Low risk types cause warts and high risk types can cause lesions or cancer (Schiffman and Castle . 2003).

1.2.Rationale

Esophageal cancers represent the eighth cancers worldwide (Ajani,*et al*.

2011). Tobacco smoking and alcohol consumption was considered causal for esophageal squamous cell carcinoma one possible risk factor for esophageal squamous cell carcinoma is infection with oncogenic human papillomavirus (HPV) types (Freddy Sitas, *et al*.2012) , also the main precursor lesion for the development of esophageal adenocarcinoma is Barrett's metaplasia arises indirectly as a consequence of mutational or environmental modifications in the stromal cells well directly driving trans-differentiation which involve important transcriptional regulators such as CDX2 (Kazumori, *et al*,2009) .In Sudan the main causes of EC is unclear so the diagnosis result is poor, in this study we highlights to one of the risk factor of EC which is HPV31 and the expression of CDX2 in esophageal cancer among Sudanese patient.

1.3. Objectives:

1.3.1. General objective:

To detect CDX2 and HPV31 in esophageal tumors among Sudanese patients.

1.3.2. Specific objectives:

_To detect CDX2 tumor marker and HPV using immunohistochemistry.

_To detect HPV type 31 using PCR

_To correlate between HPV and CDX2 with types of esophageal tumor.

_To correlate between esophageal tumors and patients ag

CHAPTER TWO

Chapter two
Literature Review

2.1. Anatomy, physiology, and histology, of the esophageous:

The esophagus is first components of the human digestive system and human gastrointestinal tract. It I s a fibro muscular tube through which food passes, aided by peristaltic contractions, from the pharynx to the stomach. Is usually 18–25 (cm) long (Edinburgh . 2010). Behind the trachea and heart, passes through the diaphragm to cardia of the stomach. The wall of the esophagus from the lumen outwards consists of mucosa (a stratified squamous epithelium), sub-mucosa (connective tissue), layers of muscle fibers between layers of fibrous tissue, and an outer layer of connective tissue. The esophagus has a mucosa consisting of a tough stratified squamous epithelium without keratin, a smooth lamina propria, and a muscularis mucosa (Kuo.2006).There are two types of glands, with mucous-secreting esophageal glands being found in the submucosa, and cardiac glands located in the lamina propria (Takubo,2007).

2.2. Pathology of theesophagus:

2.2.1. Inflammation of the esophagus:

Its known as esophagitis, Reflux of gastric acids from the stomach, infection, substances ingested for example corrosives, some medications such as bisphosphonates, food allergies, and all lead to esophagitis. Esophagitis cause painful swallowing and is treated by managing the cause of the esophagitis. Esophageal varices engorged blood vessels present within the esophageal walls, lead to partially obstruct the esophagus. as a result of liver diseases such as cirrhosis. Several disorders affect the motility of food as it travels down the esophagus. This c a n c a u s e d i f f i c u l t s w a l l o w i n g , called dysphagia. Achalasia, a failure of the lower esophageal sphincter to relax properly, lead to megaesophagus, and diffuse esophageal spasm . Sclerosis of the esophagus may cause hardening of the walls of the esophagus (Edinburgh. 2010). Congenital malformation is the esophagus fails to develop or there is an abnormal connection between the trachea and esophagus (Shaw-Smith, *et al* 2005).

2.2. 3.Tumors of the esophagus:

Durin various benign esophageal lesions are encountered in the esophagus most are asymptomatic and have no malignant potential, these various benign lesions can originates from different walls layers in the esophagus According to its origen, esophageal tumors can be classified as epithelial tumors as squamous papilloma . non epithelial tumors as leiomyoma, and cyctic tumors as bronchogenic cyst, duplication cyst, lymphangioma, fibroid polyp, lipoma and hemangioma (Hoon,*et al*.2014).

Cancer of the esophagus is two forms: cancer occurs in the squamous cells lining the esophagus it is a carcinoma or occurs in the glands or columnar tissue of the esophagus is an adenocarcinoma for example in Barrett's esophagus, and occurs in the cuboidal cells. Prolonged esophagitis from gastric reflux is one factor in the development of Barrett's esophagus . In this condition, there is metaplasia of the lining of the lower esophagus, which changes from stratified squamous epithelia to simple columnar epithelia. It is one of the main factor of esophageal cancer(Edinburgh. 2010). EC develops in a defined sequence of changes from benign metaplasia (BM), to low- grade dysplasia (LGD), to high-grade dysplasia (HGD), to adenocarcinoma (EC) (Spechler, *et al*.2011).

The common causes of the SCC is smoking tobacco, drinking alcohol, drinking hot drinks, and a poor diet ,the common cause of the ACs is

smoking tobacco, obesity and gastro esophageal reflux disease (Zhang,*et al*.2012). ACs is present in the lower third of the esophagus (David Schottenfeld, *et al*.2006, WHO.2014) . The large majority of esophageal tumors are SCCs or AC, whereas melanomas, leiomyosarcomas, carcinoids and lymphomas are rarely diagnosed (Enzinger and Mayer 2003)**.**

.3. Signs and symptoms of EC:

Difficulty swallowing most common other symptoms painful swallowing (Ferri, 2012). Weight loss, pain behind the sternum or in the epigastrium, husky, raspy, or hoarse-sounding cough, nausea and

vomiting, regurgitation of food, hematemesis, aspiration pneumonia (Enzinger , *et al*. 2003).

2.4. The Risk factor:

Tobacco and alcohol is greatest risk factors (in 90% of all ESCC) 50% of cases tobacco and 32% of cases alcohol (WHO.2014) .tobacco smoking in EAD (Lubin, *et al*. 2012). Other factors are age more common in age over
60 years (Enzinger and Mayer 2003) .esophageal cancer more common in men rather than women. Its more in people close relatives with cancer, Gastroesophageal reflux disease (GERD) exposure to bile acids its resultant Barrett's esophagus (Lagergren, *et al* 1999, Bernstein, *et al*. 2009). Also HPV infection (Syrjänen, 2002). Head and neck cancer, Radiation therapy in the mediastinum for other conditions , strong acid or alkaline (Enzinger and Mayer. 2003). Coeliac disease induces SCC (Green, *et al*.2003). Obesity induces AC (Merry, *et al*.2007). Helicobacter pylori infection (Ye, *et al*.2004, Wong, Fitzgerald. 2005). Chronic gastritis induces reflux (EAC) (Nakajima, Hattori. 2004).

2.5. The epidemiology of EC:

EC is the eighth most common malignant tumor worldwide. In 2010 an estimated 16,640 new cases and 14,500 deaths due to EC occurred in the United States (Ajani, *et al* .2011). It is associated with a 5-year survival rate of 15 to 20%. The lifetime risk of developing this cancer is 0.8% for men and 0.3% for women. The risk increases with age and the mean age at diagnosis is 67 years. Although its incidence is largely variable among different geographical areas, it is endemic in many parts of the world, particularly in Asia, Southern and Eastern Africa (Jemal, *et al* .2011). The area with the highest reported incidence of EC is the so-called Asian esophageal cancer belt, which stretches from eastern Turkey through north- eastern Iran, northern Afghanistan and southern Russia to northern China (Parkin. 2004). Approximately 75% of all ACs are localized in the distal tract of esophagus, whereas SCCs are usually distributed between the middle and lower third (Parkin, 2004. Pohl and Welch. 2005).

2.6. Method of diagnosis of EC:

EC develops in a defined sequence of changes from benign metaplasia (BM), to low-grade dysplasia (LGD), to high-grade dysplasia (HGD), to adenocarcinoma (EC) (Spechler, *et al*. 2011).

2.6.1. Medical history and physical exam:

When symptoms that might be caused by esophageal cancer appear the medical history check for possible risk factors and for abnormal results, to referred to a gastroenterologist for further tests and treatment one of this test is imaging tests (American cancer society .2014).

2.6.2. Imaging tests:

Use x-rays in this test, thick, chalky liquid called barium is swallowed to coat the walls of the esophagus. X-rays of the esophagus are then taken, magnetic fields, sound waves, or radioactive substances to create pictures of the inside body. Imaging tests might be done for a number of reasons both before and after a diagnosis of esophageal cancer; a barium swallow only shows the shape of the inner lining of the esophagus. Computed tomography scan. (CT or CAT) but not usually used to diagnose esophageal cancer, CT used when cancer spread to nearby organs and lymph nodes. also CT-guided needle biopsy, Magnetic resonance imaging (MRI) scan could be done but take a time, another test Positron emission tomography (PET) scan it is a form of radioactive sugar (known as fluorodeoxyglucose or FDG) is injected into the blood. Other imaging test is endoscopy either upper endoscopy this is an important test for diagnosing esophageal cancer, or endoscopic ultrasound as the sometime with upper endoscope. Also bronchoscopey can be used for cancer in the upper part of the esophagus to see if it has spread to the windpipe (trachea) or (bronchi). Thoracoscopy and laparoscopy used to see the lymph nodes and other organs near the esophagus inside the chest (by thoracoscopy) or the abdomen (by laparoscopy) through a hollow lighted tube (American cancer society .2014).

2.6.3. Laboratory testing of biopsy samples:

2.6.3.1. Histopathology examination:

The 4 µm thick paraffin sections cut from each specimen placed on aminopropyltriethoxy-silane-treated slides and routinely stained with hematoxylin and eosin. Specimens examined under light microscopy to confirm the histopathological diagnosis of carcinoma and the tumors graded according to the WHO histological classification criteria.

2.6.3.2. Immunohistochemsitry:

By this process an unlabeled primary antibody binds to the target antigen in the tissue and a labeled secondary antibody that reacts with the primary

antibody is binding to conjugated fluorescent or enzyme reporter (RamosVara and Miller . 2014).

2.6.3.3. PCR technique:

The method relies on thermal cycling to amplify a specific region of a DNA strand (the DNA target) after DNA extracted from tumor tissue biopsy or resection specimens (Cheng, *et al* .1994).

2.6.3.5. Insitu hyperdization (ISH):

It's a type of hybridization that uses a labeled complementary DNA or RNA strand (probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (*in situ*) at elevated temperature, the probe labeled with radioactivity or the other non-radioactive labels that visualized using a fluorescence or brightfield microscope (Gall and Pardue .1969).

2.7. Tumor marker:

Is a biomarker found in the blood, urine, or body tissues that can be elevated in cancer among other tissue types can be produced directly by the tumor or by non-tumor cells a response to the presence of a tumor. Most tumor markers are tumor antigens. (Tumor markers Cancer screening. 2013). Example of esophageal cancer marker Tumor M2-Pyruvate Kinase (Tumor M2-PK)(Kumar, *et al* .2007). Carcinoembryonic Antigen (CEA). Cyfra 21-1 (Tumor markers Cancer screening.2013).

2.7.1. CDX2:

Is a homeobox domain-containing transcription factor that plays an important role in intestinal development by regulating the proliferation and differentiation of intestinal cells (Chawengsaksophak, *et al*.1997, Silberg, *et*

al. 2000, van den Akker, *et al* .2002). CDX2 is expressed within nuclei of epithelial cells of the intestine from the proximal duodenum to the distal rectum, but very limited expression in esophagus and stomach. Therefore CDX2 expression is indicative of intestinal differentiation (Silberg, *et al*.2000). Intestinal metaplasia of the gastric mucosa was demonstrated in transgenic mice engineered to express this transcription factor in gastric epithelial cells (Mutoh, *et al.2002,* Silberg, *et al*.2002). In humans, intestinal metaplasia of the stomach and esophagus is consistently accompanied by CDX2 expression (Mizoshita, *et al*.2001, Satoh, *et al*.2002, Seno, *et al*..2002, Almeida *et al*.2003, Philips, *et al*.2003).

In 2013 Makita, *et al* was found most of the non-neoplastic Barrett's esophageal mucosa showing intestinal-type metaplasia with or without low grade dysplasia was positive for CDX2, but negative for p53. CDX2 expression is restored irrespective of the methylation status of its promoter. In 2003 Phillips *et al* was found that CDX2 protein is a sensitive marker of intestinal metaplasia in the upper gastrointestinal tract and useful in detecting histologically equivocal cases of Barrett's esophagus. CDX2 is present in dysplasia and adenocarcinoma, with some loss of protein primarily in high-grade dysplasia and adenocarcinoma.

In 2006 Tong Liu1 *et al* .was found CDX2 was expressed in most human EAC cell lines, but not in squamous epithelial cell lines. Exposure to acid and/or bile acids may activate CDX2 expression in human esophageal epithelial cells through promoter demethylation, and ectopic CDX2 expression in esophageal squamous epithelial cells contribute to intestinal metaplasia of the esophagus.

2.7.2. HPV:

The role of human papillomavirus (HPV) in the causation of esophageal squamous cell carcinoma is unclear (Freddy Sitas . 2012). The possibility that HPV might play an etiologic role in the development of esophageal squamous cell carcinoma was first proposed in 1982 by Syrjänen et al. based on histological findings that suggested possible associations between HPV and both malignant and benign squamous cell lesions of the esophagus **(**Freddy, *et al* .2012).

In 2007 Ali Eslami Far, *et al* was found there is no correlation between presence and types of HPV with patients' gender and age. The results are consistent with HPV studies conducted in other high-risk areas for ESCC and provided further evidence to support a causal association of HPV infection with ESCC.

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

In 2012 Löfdahl, *et al* was found the prevalence of human papillomavirus (HPV) in esophageal squamous cell carcinoma in relation to anatomical site of the tumor, the tumors containing HPV were not overrepresented in the upper compared to the middle or lower third of the esophagus.

In 2014 Haeri *et al* was found the Human Papilloma Virus and esophageal squamous cell carcinoma was positive for HPV L1 gene.

In 2014 Petrick, *et al* was found the highest HPV prevalence was found in Africa and Asia, not ably among Chinese studies from provinces with high OSCC incidence rates.

In 2012 Yahyapour, *et al* was found 28.3% of upper, 29% of middle and 25.8% of lower third of ESCC samples were positive for HPV DNA.

In 2006 Gao, *et al* was found the high-risk types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, HPV positivity was identified in 13% of subjects without squamous dysplasia, 8% with mild dysplasia, 7% with moderate dysplasia, 16% with severe dysplasia and negative with invasive ESCC."

In 2007 Far *et al* was found there is no correlation between presence and types of HPV with patients' gender and age.

CHAPTER THREE

Chapter three

Materials and Methods

3.1 Study design:

This is retrospective descriptive study aimed to detect CDx2 and HPV by using immunohistochemistry techniques and PCR.

3.2 Study area:

This study was conducted at Ib Sina hospital and Sudan University of Science and Technology, Collage of Medical Laboratory Science.

3.3 Materials:

Formalin fixed paraffin embedded tissue blocks, were obtained from patients previously diagnosed with esophageal tumor were used in this study.

3.4 Study sample:

Thirty paraffin blocks previously diagnosed as esophageal tumor were selected randomly from Ib Sina Hospital, patient , identification data were collected from records include age and sex.

3.5 Sample collection and preparation:

From each paraffin blocks two sections (4µm) were cut , sections were floated into preheated in water path 40C° , and placed in coated slides for immunohistochemistry, one section (20 µm) was cut and placed in Ependorff tube for molecular detection of HPV31.

3.5.1 Immunohistochemical staining procedure:

Rehydration: following deparaffinization in xylene, slides were rehydrated through agraded series of alcohol and placed in DW.

Antigen retrieval: antigen retrieval for CDX2 and HPV was performed target retrieval solution in 95°C for 30 minutes slide was heated in citrate buffer (PH 9.9).

Blocking endogenous peroxidase activity: section were blocked with 3% hydrogen peroxidase for 10 minutes, and then washed in phosphate buffer (PBS) for 2 minutes.

Primary antibody: sections were incubated with 100-200 Ml of primary antibodies (CDX2 and HPV) for 30 minutes at room temperature in a moisture chamber, and then rinsed in phosphate buffer saline for 2 minutes. Primary antibody enhancer: sections were

incubated with 100-200 Ml of primary antibody enhancer for 15 minutes, and then washed in phosphate buffer for 2 minutes.

Secondary antibody: sections were treated with secondary antibody labeled with horse reddish peroxidase for 15 minutes,

DAB chromogen: sections were incubated in diaminobenzidintetrahydrochloride for 3 minutes, and then washed in phosphate buffer for 2 minutes.

Counter stain: sections were counter stained with m a y e r haematoxylin for one minutes, blued in running tap water for 5 minutes and dehydrated in50%,70%,90%,100% ethyl alcohol for each change ,then cleared in xylene for 2 minutes for each, finally mounted in DPX mounting media.

For each run of staining, positive and negative control slides were prepared. The negative control section prepared from the same tissue block, but incubated with PBS instead of primary antibody. While positive control prepared from known positive block and treated with all above steps. Each slide was evaluated with the researcher then the results were confirmed by supervisor.

3.5.2 Polymerase Chain Reaction:

3.5.2.1 DNA extraction:

DNA was extracted from sections according to commercial kits (Sacace Biotechnologies, Italy) according to manufactures instruction.

3.5.2.2 PCR amplification:

DNA was amplified according to commercial kits primer (Sacace Biotechnologies, Italy) then the tube was closed and transferred to the thermalcycler when the temperature was reached 95C, then the program was started. . PCR consist of cycles with each cycle commonly consisting of 2-3 discrete temperature steps, the Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds,then the Annealing step: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C.

3.5.2.3 Analysis of PCR:

The DNA amplifyied by PCR was separated electrophoretically in 3%agarose gel in tris borate EDTA running buffer agarose gel was separated by dissolving 3 gm of agarose in 75ml of TBE buffer ,eththedium bromide was added,4 microliters of the PCR product were loaded into the wells 4 microliters a100bpDNA ladder, was loaded into the first well of the gel. Positive control and samples were loaded with serial number.

3.6 Statistical analysis:

The obtained results and variables arranged in standard master sheet, then analyzed using statistical package for social science (SPSS) program, frequency, mean and Chi square tests were calculated.

3.7 Ethical consideration:

Specimens were taken from Ibn Sena Hospital ethically after ethical clearance. (Appendix's).

CHAPTER FOUR

Chapter Four

Results

In This study 30 paraffin blocks were collected from specimen previously diagnosed as esophageal tumor. 20 (66.7%) of them were males and 10 (33.3%) were females as showed in figure No 4.1.

The patients age ranged between ≥ 60 year were 14 (46.7%) patients and over 60 year were 16 (53.3%) as showed in figure No 4.2.

Twenty (66.7%) samples was diagnosed as malignant and ten (33.3%) as benign as showed in figure No 4.3.

When CDX2 was detected immunohistochemically in30 samples, 4 (13.3%)

Samples were positive and negative in 26 (86.7%) as showed in figure No 4.4.

Human papilloma virus was tested immunohistochemically in 30samples, 7 (23.3%) were positive and negative in 23 (76.7%) as showed in figure No 4.5.

Also human papilloma virus was tested by PCR, in 30 samples, 2 (6.7%) were positive and negative in 28 (93.3%) as showed in figure No 4.6.

The comparison between CDX2 expression and type of tumor the study revealed that: the positive expression of CDX2 in the malignant tumor samples were (4), and no positive result detected in benign tumor .while negative result of CDX2 expression were detect in 16 of malignant samples, and 10 in benign samples as showed in table 4.2. With no significant relation between CDX2 expression and type of tumor (*P*.value 0.129).

The result of HPV PCR and type of tumor the study revealed that: the positive HPV PCR in malignant tumor were (1), and (1) positive result detected in benign tumor. While negative result of HPV were detect in 19 of

malignant samples, and 9 in benign samples as showed in table 4.3. With no significant relation between HPV infection and type of tumor (*P*.value 0.65).

The comparison between the type of tumor and HPV immunohistochemical results the study revealed that: the positive expression of HPV in the malignant tumor samples were (6), and (1)

positive result detected in benign tumor .while negative result of HPV expression were detect in (14) of malignant samples, and (9) in benign samples as showed in table 4.4. With no significant relation between CDX2 expression and type of tumor (*P*.value 0.222).

Relation between the patients age group and type of tumor the study revealed that: the patients age less than or equal 60 year in malignant tumor were 8 and 6 in benign tumor. While the patients age over 60 year in malignant tumor were 12 and 4 in benign tumor as showed in table 4.5, with no significant relation between the patients age group and type of tumor (*P*.value 0.301).

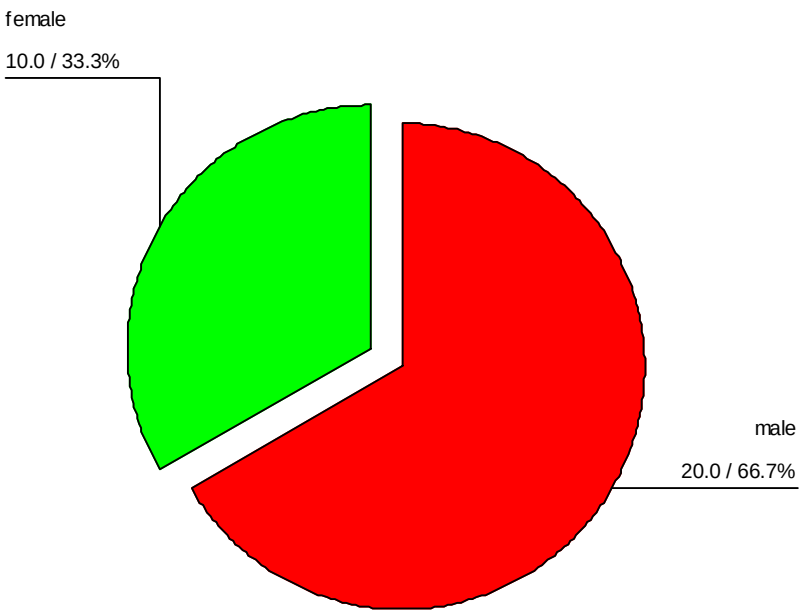


Fig: 4.1. Frequeny of sex among study population

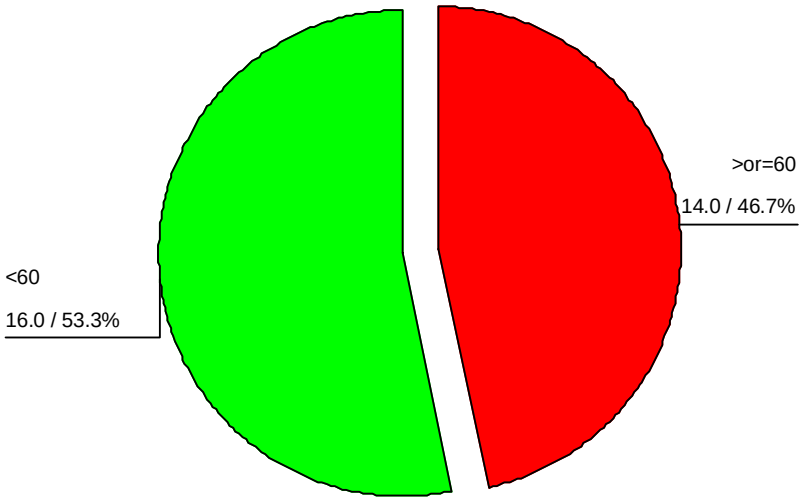


Fig: 4.2. Frequency of age among study population

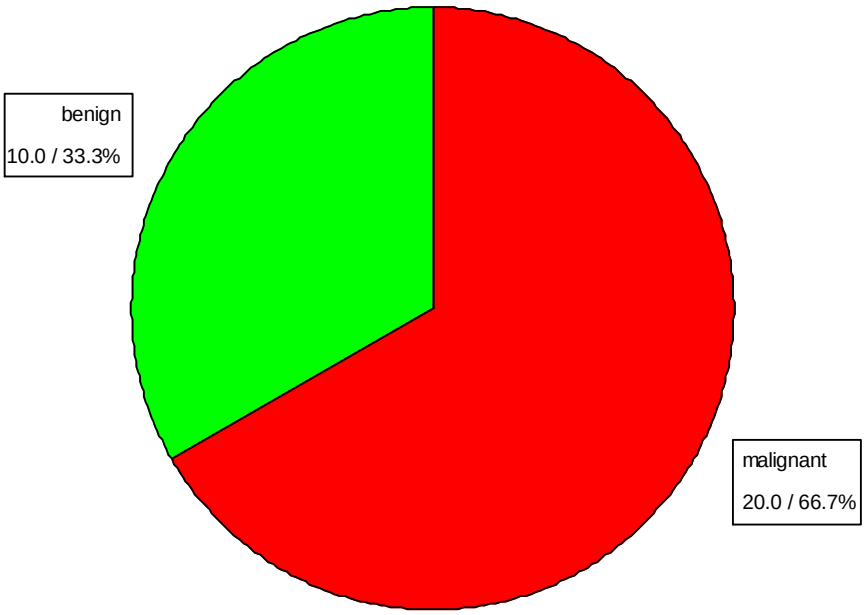


Fig: 4.3.Frequency of histopathology diagnosis.

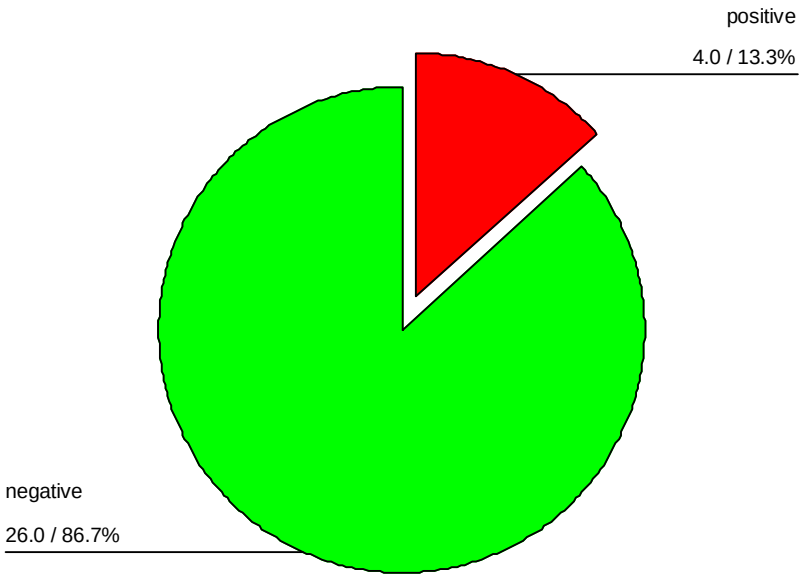


Fig: 4.4. Immunohistochemical result of CDX2

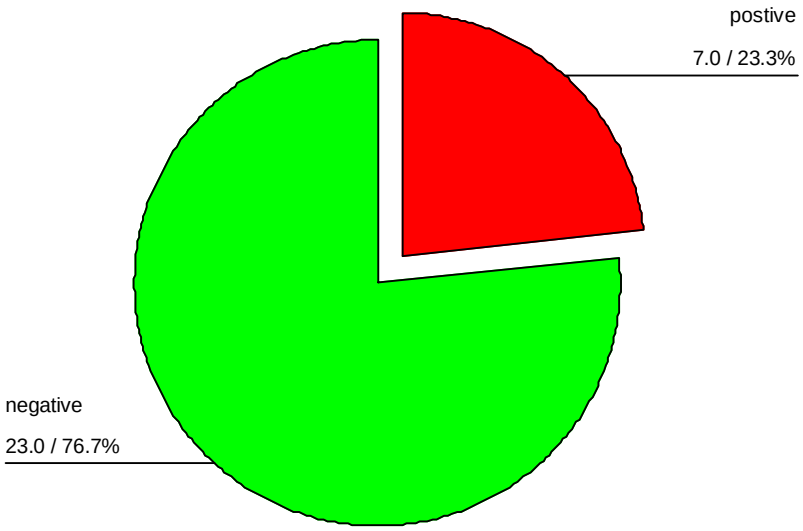


Figure: 4.5. Frequency of immunohistochemical results of HPV.

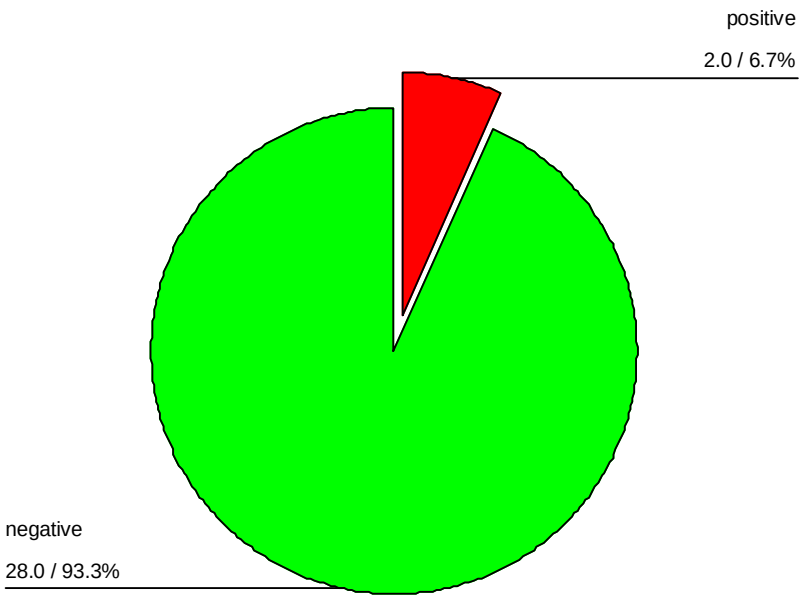


Fig: 4.6.Freguency of PCR results of HPV

Table: 4.1 Relation between type of tumor and CDX2 immunohistochemical results

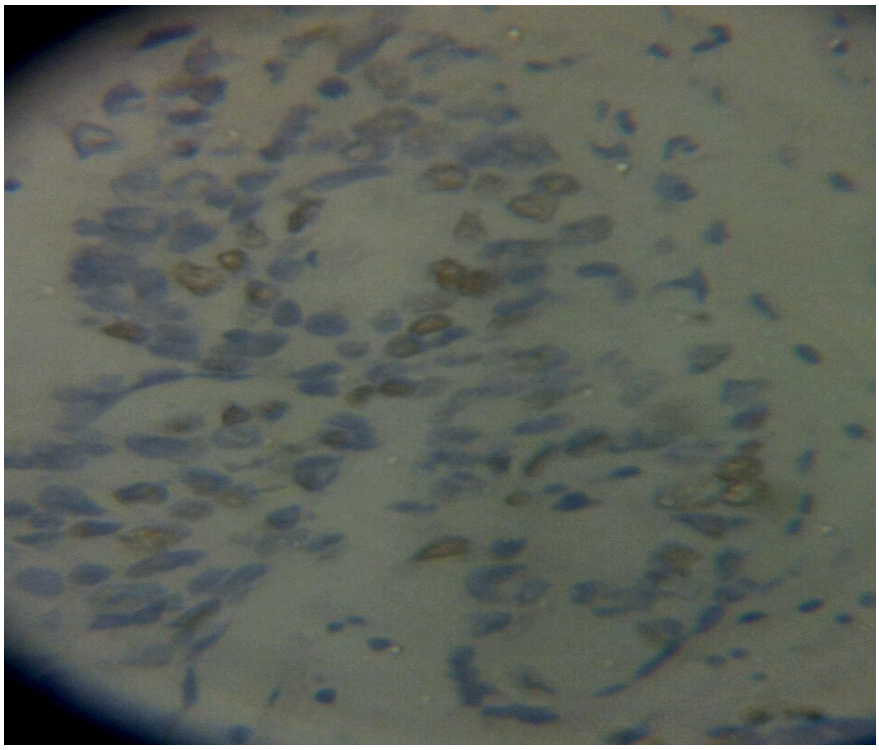
Type of tumor	Immumohistochemical results of CDX2		Total	P.value
	POSITIVE	NEGATIVE		
Malignant	4	16	20	0.129
Benign	0	10	10	
Total	4	26	30	

Table: 4.2. Relation between type of tumor and HPV PCR results

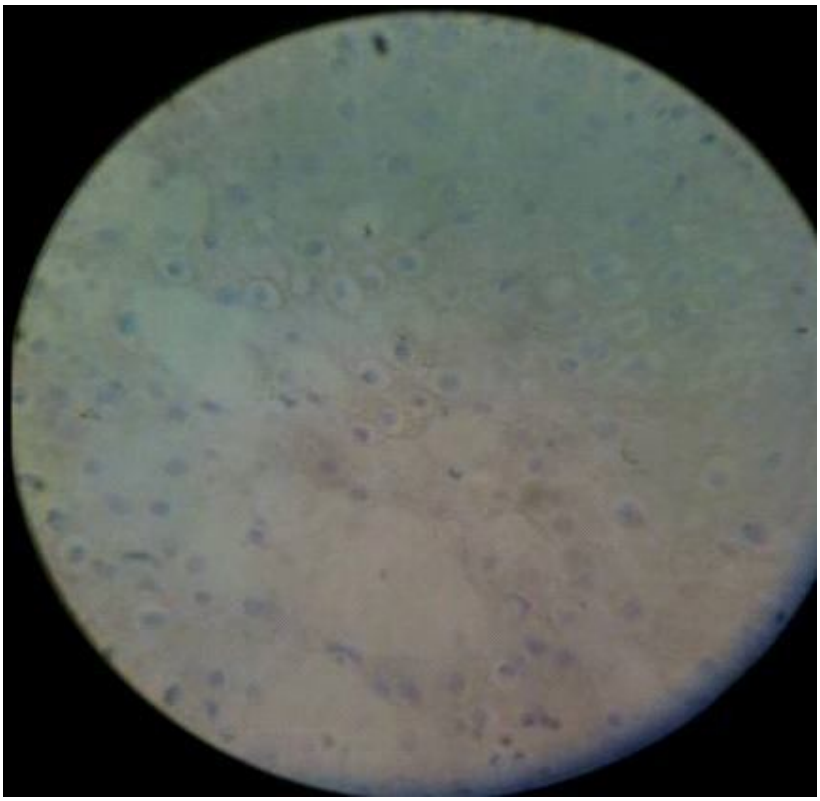
Type of tumor	HPV PCR		Total	P.value
	POSITIVE	NEGATIVE		
Malignant	1	19	20	0.605
Benign	1	9	10	
Total	2	28	30	

Table: 4.3: Relation between type of tumor and immunohistochemical results of HPV

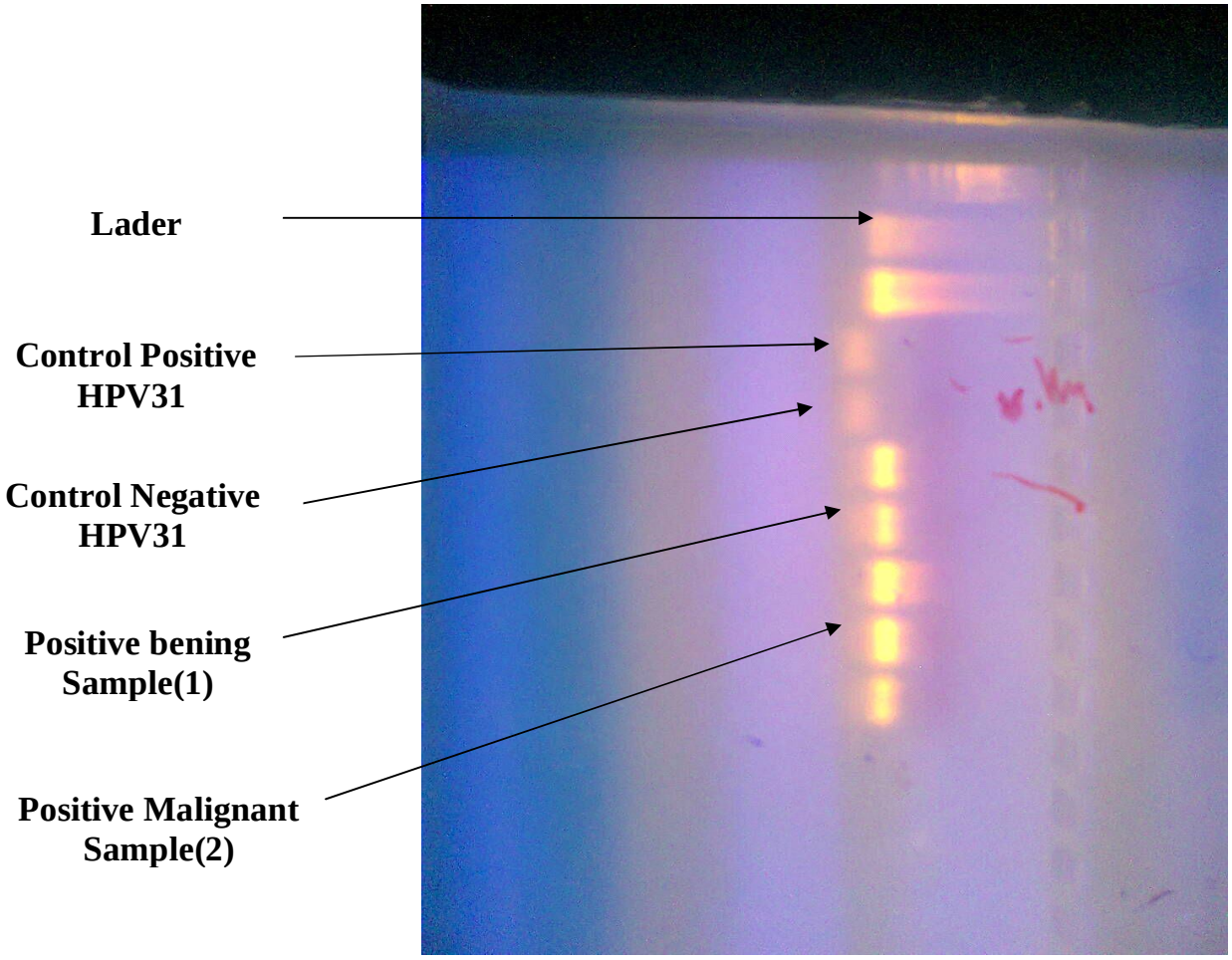
Type of tumor	Immumohistochemical results of HPV		Total	P.value
	POSITIVE	NEGATIVE		
Malignant	6	14	20	0.222
Benign	1	9	10	
Total	7	23	30	



Microphotographic 4.1: Esophageal adenocarcinoma show positive immunohistochemistery of CDX2(40x)



Microphotograph 4.2: Esophageal squamous cell carcinoma show positive immunohistochemistery of HPV (40x)



Microphotograph 4.3: Show positive HPV 31 PCR

(1): no evidence of malignancy.(2).moderately differentiated adenocarcinoma

CHAPTER FIVE

Chapter Five

Discussion

Esophageal cancer risk is strongly related to age and sex (Parkin 2011).

In this study when we compared the type of tumor with age we found the most patients samples are over 60 years has malignant tumor (esophageal cancer) this has been supported by the american cancer society estimated that during 2014 esophageal adenocarcinoma is more common in caucasian men over the age of 60 than it is in African Americans (Kenneth J. Vega ,*et al*.2000, American Cancer Society. 2014). In 2012 Vincenza Conteduca, *et al*, was found the risk increases with age and the mean age at diagnosis is 67 years. Its incidence is variable among different geographical areas. The present study found the disease is more common in men this has been supported by report that in 2012, esophageal cancer is around three times more common in men than women (World Cancer Report 2014).

The American Cancer Society in 2014 estimated that in esophageal cancer approximately the 7th leading cause of cancer death among males. In the United States, SCCs of the esophagus is more common among African American males with a history of heavy smoking or alcohol use (Kenneth J. Vega , *et al*.2000).

This study support our study which the most of the patients is male's patients and diagnosed as SSC. Other study in 2012 by Vincenza Conteduca, *et al* was found the lifetime risk of developing this cancer is 0.8% for men and 0.3% for women.

In this study CDX2 showed there is no significant association between esophageal tumor and it expression. By immunohistochemistry nuclear-localized Cdx2 protein is observed in columnar epithelium of Barrett's esophagus, also low-level of Cdx2 protein localized in the cytoplasm of squamous epithelial cells from Gastroesophageal reflux disease(GERD) patients (Eda , *et al* 2003). Small portions of gastric and esophageal adenocarcinoma heterogeneously express CDX2 (Werling , *et al*.2003).

In contrst to our study in 2003 Phillips, *et al* was found that CDX2 protein is useful in detecting histological equivocal cases of Barrett's

esophagus. It is present in dysplasia and adenocarcinoma, with some loss of protein primarily in high-grade dysplasia and adenocarcinoma

In 2006 Tong Liu1, *et al* was found CDX2 was expressed in most human esophageal adenocarcinoma cell lines, CDX2 expression in esophageal squamous epithelial cells may contribute to intestinal metaplasia of the esophagus.

In 2013 Makita , *et al*. was found most of the non-neoplastic Barrett's esophageal mucosa showing intestinal-type metaplasia with or without low- grade dysplasia was positive for Cdx2.

Our study agree with study in 2004 by Moons, *et al* and Lord , *et al* in 2005 CDX2 expression was detected in normal esophagus where there was no methylation, but was more highly expressed in the adenocarcinomas of the esophagus which also lack methylation of the promoter region. Given that many esophageal adenocarcinomas arise from dysplastic lesions in the background of intestinal metaplasia, CDX2 expression might be a significant contributor to the metaplastic and later neoplastic transformation of the human esophagus to adenocarcinoma .

In this study the immunohistochemical and PCR detections of HPV was show there is no association between HPV infection and esophageal tumor. The present study found that the positive results of HPV31 using PCR were 2 (6.7%).

The first reports suggesting an involvement of (HPV) in the development of both benign and malignant squamous cell tumors of the esophagus date back to 1982 (Syrjänen , 2002).

Our study agree with study in 2008 Koh, *et al* examined HPV types 16, 18, 31, 33, 35, 52b and 58 and type 16-specific primers consensus failed to detect HPV DNA sequences in any of the esophageal samples. In 1998, Poljak, *et al*, They did not found HPV in 121 ESCCs.

(34%) of patients with ESCC from Kochi were positive for HPV DNA type-16 and HPV type-18; in contrast, none were positive for HPV-31 or -33 (Furihata, 1993).

Feng, *et al* in 2013 was found Human papillomavirus was not detected by PCR using multiple consensus primer sets in esophageal adenocarcinomas. Our study results are disagree with HPV studies conducted in other high- risk areas for example this study result disagree with study in 2005 by Bahnassy, *et al* were found HPV was detected in patients with esophageal adenocarcinoma all specimens were positive for HPV.

In 2006 Gao, *et al.* found that the frequency of HPV subtype in tumoural regions as follows: HPV-31, 3%.
In 2007 Far, *et al,* found only HPV-16 in tumor margins also in 2014 Zhang, *et al* was found the distribution of HPV genotypes in esophageal cancer patients from high to low proportion was HPV-16, -58, -18, -33, -31 and -11.

CHAPTER SIX

Chapter Six

Conclusion and Recommendation

6.1. Conclusion:

On the basis of this study we conclude that:

There is no association between HPV infection, CDX2 expression age group and esophageal tumor.

6.2. Recommendation:

On the basis of this study we recommend that:

Further studies with large sample size should be done to detect the CDX2 and HPV in esophageal tumor.

References

References

- Ajani JA, Barthel JS, Bentrem DJ, *et al* (2011). Esophageal and esophago-gastric junction cancers. *J Natl Compr Canc Netw*.9:830–887.
- Ali Eslami Far, Arezoo Aghakhani, Rasool Hamkar, *et al (*2007).Frequency of human papillomavirus infection in oesophageal squamous cell carcinoma in Iranian patients : Arezoo Aghakhani, Clinical Research Dept. *Pasteur Institute of Iran* , Vol. 39, No. 1 , Pages 58-62.
- Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang J, *et al.* (2003) Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. *The Journal of pathology* 199: 36–40.
- American Cancer Society .2014. "Cancer Facts and Figures 2014". Retrieved 28 April 2014.
- Bahnassy, AR Zekri, S Abdallah, AM El-Shehaby, GM Sherif. Human (2005). papillomavirus infection in Egyptian esophageal carcinoma: correlation with p53, p21, mdm2, C-erbB2 and impact on survival *Pathol Int J*.55(2):53-62.
- Bernstein H, Bernstein C, Payne CM, Dvorak K. (2009) Bile acids as endogenous etiologic agents in gastrointestinal cancer. *World J Gastroenterol*. 15(27): 3329–3340.
- Centers for Disease Control and Prevention CDC)(2008)."Genital HPV Infection — CDC Fact Sheet". Retrieved 13 November 2009.
- Chawengsaksophak K, de Graaff W, Rossant J, Deschamps J, Beck F (2004). "Cdx2 is essential for axial elongation in mouse development". *Proc. Natl. Acad. Sci. U.S.A.* 101 (20): 7641–5.
- Cheng, S.; Fockler, C.; Barnes, W. M.; Higuchi, R. (1994). Effective Amplification of Long Targets from Cloned Inserts and Human

Genomic DNA". *Proceedings of the National Academy of Sciences* **91** (12): 5695–5699.

- David Schottenfeld, Joseph F. Fraumeni, Jr. associate editors, Graham A. Colditz, et al . (2006). Cancer epidemiology and prevention (3rd ed). Oxford: Oxford University Press. p. 697.
- Debruyne PR, Witek M, Gong L, Birbe R, Chervoneva I, Jin T, Domon-Cell C, et al . (2006). "Bile acids induce ectopic expression of intestinal guanylyl cyclase C Through nuclear factor-kappaB and Cdx2 in human esophageal cells". *Gastroenterology* 130 (4): 1191–206.
- Eda A, Osawa H, Satoh K, Yanaka I, Kihira K, *et al.* (2003) Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *J Gastroenterol* 38: 14–22.
- Edinburgh. (2010). Churchill Livingstone. Davidson's Principles and Practice of Medicine (21st ed.) .
- Enzinger PC and Mayer RJ. 2003. Esophageal cancer. *N Engl J Med*.349.2241–2252.
- Far, A Aghakhani, R Hamkar, A Ramezani, HF Pishbigar, S Mirmomen, MR Roshan, et al .2007. Frequency of human papillomavirus infection in oesophageal squamous cell carcinoma in Iranian patients. *Scand J Infect*. 39(1):58-62.
- Feng, J Zheng, X Du, Y Tan, H Yang, H Zhang, Z Zhang. (2013) .Human papillomavirus detected by PCR using multiple consensus primer sets in esophageal adenocarcinomas in Chinese patients. *J Med Virol*.85(6):1053-1057.
- Ferri Fred (2012). Ferri's clinical advisor (1st Ed). pp. 389–391.
- Freddy Sitas, Sam Egger, Margaret I. Urban, Philip R. Taylor, Christian C. et al (2012), Associations Between Esophageal Squamous Cell Carcinoma and Human Papillomavirus Serological Markers, *J Natl Cancer Inst*;104(2):147-158.

- Gall, JG; Pardue, ML (1969). "Formation and detection of RNA-DNA hybrid molecules in cytological preparations . *Proceedings of the National Academy of Sciences of the United States of America*. **63** (2)378–83.
- German MS, Wang J, Fernald AA, Espinosa R, Le Beau MM, Bell GI (1995). "Localization of the genes encoding two transcription factors, LMX1 and CDX3, regulating insulin gene expression to human chromosomes 1 and 13*".J Genomics* 24 (2): 403–4.
- GF Gao, MJ Roth, WQ Wei, *et al*. (2006). A cross-sectional study in a high-risk region of China. *Int J Cancer* 15.119(6):1354-1359.
- Green PH, Fleischauer AT, Bhagat G, Goyal R, Jabri B, Neugut AI (2003). "Risk of malignancy in patients with celiac disease". *Am. J. Med*. 115 (3): 191–5.
- Haeri, O Mardani, F Asadi-Amoli, R Shahsiah . (2014). Human papilloma virus and esophageal squamous cell carcinoma, Cao – *ScientificWorldJournal*. 52(3):197-200.
- Hamad, H.M.A.(2011).Cancer inititatives in sudan .Annals oncology,17.
- HE Löfdahl, J Du, A Näsman, E Andersson, *et al. (*2012). Prevalence of human papillomavirus (HPV) in oesophageal squamous cell carcinoma in relation to anatomical site of the tumour . Yahyapour - *Asian Pac J* .7(10): e46538.
- Hoon Jai Chun, Suk-Kyun Yang, Eun Young Park, (2014). Clinical

  Gastrointestinal Endoscopy, Springer Science & Business Media, p:47
- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. (2011) Global cancer statistics. *CA Cancer J Clin*. 61:69–90.
- Petrick, AB Wyss, AM Butler, *et al*, (2014). Prevalence of human papillomavirus among oesophageal squamous cell carcinoma cases: systematic review and meta-analysis, *Br J Cancer* 29; 110(9):2369-2377.
- JS Koh, SS Lee, HJ Baek, YI Kim, (2008). Association of high-risk human papillomavirus with esophageal squamous cell

carcinomas among Koreans, as determined by polymerase chain reaction. Esophagus;21(2):114-117

- K J Syrjänen .(2002). *J Clin Pathol*; 55(10).
- Kazumori H, Ishihara S and Kinoshita Y. (2009). Roles of caudal- related homeobox gene Cdx2 in oesophageal epithelial cells in Barrett's epithelium development. *J Gut*. 58:620–628.
- Kenneth J. Vega, M.D., M. Mazen JamaM.D.l (2000). ."Changing pattern of esophageal cancer incidence in New Mexico". Changing pattern of esophageal cancer incidence in New Mexico. *The American Journal of Gastroenterology*.
- Kumar, Yogesh; Tapuria, Niteen; Kirmani, Naveed; Davidson, Brian R. (2007). "Tumour M2-pyruvate kinase: A gastrointestinal cancer marker". *European Journal of Gastroenterology & Hepatology* 19 (3):265.
- Kuo, Daniela; Urma (2006). "Esophagus - anatomy and development". *GI Motility online*.
- Lagergren J, Bergström R, Lindgren A, Nyrén O (1999). "Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma". *N. Engl. J. Med*. 340 (11): 825–31.
- Lord RV, Brabender J, Wickramasinghe K, DeMeester SR, Holscher A, Schneider PM, Danenberg PV, et al .(2005). Increased CDX2 and decreased PITX1 homeobox gene expression in Barrett's esophagus and Barrett's-associated adenocarcinoma. *Surgery.J ;* 138*:* 924–31.
- Lubin JH, Cook MB, Pandeya N, Vaughan TL, Abnet CC, Giffen C, Webb PM, et al . (2012) The importance of exposure rate on odds ratios by cigarette smoking and alcohol consumption for esophageal adenocarcinoma and squamous cell carcinoma in the Barrett's Esophagus and Esophageal Adenocarcinoma Consortium. *Cancer Epidemiol.J*. 36(3): 306–316.
- M Furihata, Y Ohtsuki, S Ogoshi, A Takahashi, T Tamiya, T Ogata. (1993). Prognostic significance of human papillomavirus genomes (type-16, -18) and aberrant expression of p53 protein in human *esophageal cancerInt J Cancer*. 8.54(2):226-230.

- Makita K, Kitazawa R, Semba S, *et al*. (2013). Cdx2 expression and its promoter methylation during metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus. *World J Gastroenterol*. 19(4):536-41

- Merry AH, Schouten LJ, Goldbohm RA, van den Brandt PA (2007). "Body Mass Index, height and risk of adenocarcinoma of the oesophagus and gastric cardia: a prospective cohort study". *Gut .J*.56 (11): 1503–11.

- Mizoshita T, Inada K, Tsukamoto T, Kodera Y, Yamamura Y, *et al*. (2001) Expression of Cdx1 and Cdx2 mRNAs and relevance of this expression to differentiation in human gastrointestinal mucosa–with special emphasis on participation in intestinal metaplasia of the human stomach. Gastric cancer : official *journal of the International Gastric* Cancer Association and the Japanese Gastric Cancer Association 4: 185–191.

- Moons LM, Bax DA, Kuipers EJ, Van Dekken H, Haringsma J, Van Vliet AH, Siersema PD, Kusters JG. (2004). The homeodomain protein CDX2 is an early marker of Barrett's oesophagus. *J Clin Pathol*; 57*:* 1063–8.

- Mutoh H, Hakamata Y, Sato K, Eda A, Yanaka I, *et al*. (2002) Conversion of gastric mucosa to intestinal metaplasia in Cdx2- expressing transgenic mice. Biochemical and biophysical research communications 294: 470–479.

- Nakajima S, Hattori T (2004). "Oesophageal adenocarcinoma or gastric cancer with or without eradication of Helicobacter pylori infection in chronic atrophic gastritis patients: a hypothetical opinion from a systematic review". *Aliment. Pharmacol*. 20 Suppl 1: 54–61.

- Parkin DM, Boyd L, Walker LC(2011). The fraction of cancer attributable to lifestyle and environmental factors in the UKin 2010. *Br J Cancer* ;105(S2):S77-S81.

- Parkin DM (2004) International variation. *S J Oncogene* 23:6329–6340.
- Phillips RW, Frierson HF Jr, Moskaluk CA (2003) Cdx2 as a marker of epithelial intestinal differentiation in the esophagus. *The American journal of surgical pathology* 27: 1442–1447.
- Phillips, Roy W. MD; Frierson, , Henry F. Jr MD. (2003) Moskaluk, Christopher A. MD, PhD Cdx2 as a Marker of Epithelial Intestinal Differentiation in the Esophagus *American Journal of Surgical Pathology* - Volume 27 - Issue 11 - pp 1442-1447
- Pohl H and Welch HG.(2005)The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. *J Natl Cancer Inst*. 97:142–146.
- QY Zhang, DH Zhang, ZY Shen, LY Xu, EM Li, WW Au (2014) .Infection and integration of human papillomavirus in esophageal carcinoma. *Int J Hyg Environ Health*.(2):156-161.
- Ramos-Vara, JA; Miller MA (2014). "When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry-the red, brown, and blue technique.". *Veterinary Pathology* **51** (1): 42–87.
- Satoh K, Mutoh H, Eda A, Yanaka I, Osawa H, *et al.* (2002) Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of Helicobacter pylori. Helicobacter 7: 192–198.
- Schiffman M, Castle PE (2003). "Human papillomavirus: epidemiology and public health". *Arch Pathol Lab Med* **127** (8): 930-

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- Seno H, Oshima M, Taniguchi MA, Usami K, Ishikawa TO, *et al*. (2002) CDX2 expression in the stomach with intestinal metaplasia and intestinal-type cancer: Prognostic implications. International journal of oncology 21: 769–774.
- Shaw-Smith, C (18 November 2005). "esophageal atresia, tracheo- esophageal fistula, and the vacterl association: review of genetics and epidemiology". Journal of Medical Genetics 43 (7)
- Silber gDG, Swain GP, Suh ER, Traber PG (2000) Cdx1 and cdx2 expression during intestinal development. Gastroenterology 119: 961–971.
- Silberg DG, Sullivan J, Kang E, Swain GP, Moffett J, *et al*. (2002) Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. Gastroenterology 122: 689–696.
- Silberg DG, Swain GP, Suh ER, Traber PG (2000) Cdx1 and cdx2 expression during intestinal development. Gastroenterology 119: 961–971.
- Spechler SJ, Sharma P, Souza R, Inadomi JM, Shaheen NJ. 2011.American Gastroenterological Association medical position statement on the management of Barrett's esophagus. Gastroenterology;140:1084-1091.
- Stahl, M; Mariette, C; Haustermans, K; Cervantes, A; Arnold, D; (2013). ESMO Guidelines Working, Group "Oesophageal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up." Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 24 Suppl 6: vi51–6.
- Stewart, Bernard W. Kleihues, P. (2003). *World cancer report*. Lyon: IARC Press.
- Syrjänen KJ (2002). "HPV infections and oesophageal cancer". *J. Clin.Pathol.* 55 (10): 721–8.
- Takubo, Kaiyo (2007). Springer Verlag. Pathology of the esophagus an atlas and textbook (2nd ed). Tokyo: p. 28.
- Tong Liu, Xinyan Zhang, Chi-Kwong So, *et al* .2006 .Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2

transfection on morphology and gene expression of human esophageal epithelial cells by Oxford University Press.

- Tumor markers Cancer screening. Retrieved December 28, 2013.
- van den Akker E, Forlani S, Chawengsaksophak K, de Graaff W, Beck F, *et al*. (2002) Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. Development 129: 2181–2193.
- Vincenza Conteduca, Domenico Sansonno, Giuseppe Ingravallo, *et al* .2012 .Barrett's esophagus and esophageal cancer: An overview Published online on Thursday, May 17, Pages: 414-424 .
- W Shield, Thomas. LoCicero, Joseph. B. Ponn, Ronald. (2005). *Less Common Malignant Tumors of the Esophagus*. Lippincott Williams & Wilkins. pp. 2325–2340.
- Werling RW, Yaziji H, Bacchi CE, Gown AM (2003) CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. The American journal of surgical pathology 27: 303–310.
- Wong A, Fitzgerald RC (2005). "Epidemiologic risk factors for Barrett's esophagus and associated adenocarcinoma". Clin. Gastroenterol. Hepatol. 3 (1): 1–10.
- World Cancer Report 2014. World Health Organization. 2014. pp. Chapter 5.3.
- Y Yahyapour, M Shamsi-Shahrabadi, M Mahmoudi, *et al*. 2012.Evaluation of human papilloma virus infection in patients with esophageal squamous cell carcinoma from the caspian sea area, north of iran.. Asian Pac J Cancer Prev;13(4):1261-1266.
- Ye W, Held M, Lagergren J, *et al*. (2004). "Helicobacter pylori infection and gastric atrophy: risk of adenocarcinoma and squamous- cell carcinoma of the esophagus and adenocarcinoma of the gastric cardia". *J. Natl. Cancer Inst.* 96 (5): 388–96.
- Zhang, HZ; Jin, GF; Shen, HB (Jun 2012). "Epidemiologic differences in esophageal cancer between Asian and Western populations. Chinese journal of cancer 31 (6): 281–6.

Appendices'

Appendix

1. Measurement and material:

1.1 Insturument:

- Rotary microtome.
- Oven.
- Coplinjars.
- Staining racks.
- Stainless microtome blade.
- Coated slide.
- Cover glass.
- Water path.
- Dako pen.
- Thermal cycler.
- Workstation.
- Pipettorstube rack.

1-2 Materials:

- Xylene.
- Ethyle alcohol.
- Mayers haematoxylene.
- Distilled water.
- Citrate buffer.
- Perioxidase blocker.
- Anti CDX2 antibodies (primary antibodies).
- Anti human papilloma visus 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 type 31.
- DNA extraction kite.
- Detection agarose kite.
- 2-Reagents sheet.

1.3. Master sheet

Number of sample	age	Sex	Diagnosis	Stages	CDX2 IHC	HPV IHC	HPV 31by PCR
1725/13	60yrs	F	Squamous cell carcinoma,large keratinizing cell type	M	-ve	+ve	-ve
1295/13	50yrs	M	Scc	M	-ve	+ve	-ve
1343/13	23yrs	M	Esophageal endoscopy biopsy(Kaposi sarcoma)	M	-ve	-ve	-ve
1049/12	60yrs	M	Scc	M	-ve	-ve	-ve
422/14	40yrs	M	Scc	M	+ve	-ve	-ve
1095/13	70yrs	F	Scc	M	-ve	-ve	-ve
7/14	70yrs	F	Scc	M	-ve	+ve	-ve
451/14	52yrs	M	Scc	M	-ve	-ve	-ve
378/14	72yrs	M	Gastroesophageal junction poorly differentiated adenocarcinoma	M	-ve	-ve	-ve
17/14	61yrs	M	Scc	M	-ve	-ve	-ve

1679/13	82yrs	M	Moderately differentiated adenocarcinoma	M	+ve	+ve	+ve
151/11	77yrs	M	Scc large cell keratinizing type	M	-ve	-ve	-ve
417/14	64yrs	F	Scc	M	-ve	+ve	-ve
1684/13	75yrs	M	Poorly differentiated adenocarcinoma	M	+ve	+ve	-ve
183/12	21yrs	M	Scc large cell keratinizing type	M	-ve	-ve	-ve
183/11	75yrs	F	Well differentiated invasive (keratinizing)scc	M	-ve	-ve	-ve

1303/14	98yrs	M	Moderate differentiated invasive scc	M	-ve	-ve	-ve
1264/14	60yrs	F	Moderate differentiated invasive (keratinizing)scc	M	-ve	-ve	-ve
1100/12	70yrs	M	Scc large cell non keratinizing type	M	-ve	-ve	-ve
1202/13	75yrs	F	Moderately differentiated adenocarcinoma	M	+ve	-ve	-ve
216/14	8yrs	M	Esophagitis	B	-ve	-ve	-ve
2128/13	30yrs	M	No evidence of malignancy	B	-ve	-ve	+ve
251/14	80yrs	M	Gastroesophageal junction chronic inflammation	B	-ve	-ve	-ve
1110/13	75yrs	M	Gastroesophageal junction(inflamatous)gastric polyp	B	-ve	-ve	-ve
112/14	43yrs	F	Mild chronic inflammation	B	-ve	+ve	-ve
1307/14	50yrs	F	Esophagitis	B	-ve	-ve	-ve

1819/13	63yrs	M	No significant pathogenic,no atypia	B	-ve	-ve	-ve
300/14	68yrs	M	Esophagitis gastroesophageal junction	B	-ve	-ve	-ve
423/14	60yrs	M	Gastroesophageal junction	B	-ve	-ve	-ve
1592/12	39yrs	F	No evidence of malignancy	B	-ve	-ve	-ve