# HUMAN PAPILLOMA VIRUS (HPV) IN SQUAMOUS CELL CARCINOMA OF THE OESOPHAGUS.


***A dissertation submitted to the Dr. M.G.R. Medical University, Tamil Nadu; in partial fulfilment of the requirement for the M.S. branch I (General Surgery) examination to be held in April 2013***.

# Certificate

This is to certify that the dissertation entitled

*"HUMAN PAPILLOMA VIRUS (HPV) IN SQUAMOUS CELL CARCINOMA OF THE OESOPHAGUS."*

is a bonafide work done by Dr. Smit Kumar, post graduate resident in Masters of General Surgery 2010 - 2013 at the Christian Medical College, Vellore, towards partial fulfilment for the MS General Surgery-Branch 1 final examination to be held in April 2013.

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**Guide:**

Dr. Inian Samarasam

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## CONTENTS

# ABSTRACT

TITLE OF THE ABSTRACT : **HUMAN PAPILLOMA VIRUS (HPV) IN SQUAMOUS CELL CARCINOMA OF THE OESOPHAGUS.**

DEPARTMENT : GENERAL SURGERY
NAME OF THE CANDIDATE : SMIT KUMAR
DEGREE AND SUBJECT : M.S. (GENERAL SURGERY)
NAME OF THE GUIDE : Dr. INIAN SAMARASAM.

AIMS AND OBJECTIVES: To evaluate the probable association of HPV infection with high risk genotypes 16 and 18 in patients with squamous cell oesophageal carcinoma.

BACKGROUND: Oesophageal carcinomas are the eighth most common cancer worldwide and sixth most common cause of death worldwide from cancer. The etiology is unknown and many factors are involved in the carcinogenesis. The association of high risk HPV types16 and 18 in squamous cell carcinoma of the oesophagus is variable and still controversial in literature. There is limited data from India and no data available from South India. The objective of our study was to determine any association of HPV high risk types 16 and 18 in patient with squamous cell oesophageal cancer.

METHODS: This was a prospective observational case control study done over 2 years. The total number of case participants (Group A ) was 45 and control participants (Group B) were 38. The endoscopy biopsy tissue was collected from both the tumour and adjacent normal healthy mucosa in patients with squamous cell carcinoma of oesophagus. From the control participants biopsy was collected from the normal oesophageal mucosa. The collected samples were coded and sent for analysis for the presence of high risk HPV DNA types 16 and 18 by PCR.

RESULTS: In this study high risk HPV DNA types 16 and 18 were not present in patients with squamous cell carcinoma of oesophagus. There was also absence of HPV in the control participants. However there is need for a larger study to investigate the probable association of HPV in the etiology of squamous cell carcinoma of oesophagus.

# INTRODUCTION

Oesophageal malignancy is the eighth most common cancer worldwide and also sixth most common cause of death worldwide from cancer. (1)The long-term survival rate from the oesophageal cancer is about 19%, which is due to the fact that patients present at advanced stage. (2) (3)

It is also known that the incidence of oesophageal malignancy shows wide variations across the different geographical regions of the world. (4)

Oesophageal malignancy is common in Indian population. (5) A variation in incidence rate is also seen in different parts of our country. (5) It is an important leading site of cancer in both males and females. The average age adjusted incidence rate for cancers of oesophagus in India is 8 per 100,000 population. (1)

The precise etiologic factor for squamous cell carcinoma (SCC) of the oesophagus is not known. (8)

However, it is important to understand the various agents causing cancer to understand its precise mechanism of molecular pathogenesis, identify biomarkers for early diagnosis and better management of this malignancy. (5)

Carcinogenesis being a multistep process, a number of various biological and environmental agents is involved. (6)

Infection with oncogenic HPV types may be an integral step in this multilevel process that leads to oesophageal carcinoma. (6)

The first observation of involvement of Human Papilloma virus (HPV) in the aetiology of squamous cell carcinoma of the oesophagus dates back to 1982. (7) Following this, there is wide variation in the literature regarding HPV detection in patients with SCC oesophagus. .

The available evidence in the literature suggests that the similar mechanisms of carcinogenesis occur in cervical cancer and SCC of the oesophagus. The HPV E6 and E7 oncogenes interfere with normal cell cycle regulation resulting in abnormal proliferation leading to malignancy. (8)(9)

The prevalence of HPV in cases of SCC oesophagus is highly variable across various geographical regions worldwide, being significantly higher in high-incidence regions as compared to low-incidence regions. (10)(11)(12)(13)

This concept was recently substantiated by a large meta-analysis of the data from 1954 – 2012 recently elaborated the concept that oesophageal squamous cell carcinoma might have a different aetiology in low and high incidence geographic regions. (14) The HPV plays a far more important role in the high incidence regions of oesophageal malignancy. (14)

It is possible that different etiologic factors may exist in high risk as opposed to low risk geographical areas. This can explain the difference in detection rates of HPV in oesophageal malignancy from different geographical areas worldwide.

Although India also has high prevalence of oesophageal cancer in the sub continent, the incidence is not as high compared to mainland China, Iran and Traneki region of South Africa, where the prevalence rate has been quoted to about 250 per 100,000 populations. (71). There is very limited data from India which would identify any such association of high risk HPV types with squamous cell carcinoma of the oesophagus.

India being a low prevalence area for oesophageal carcinoma, the role of HPV virus in the aetiology of this cancer is doubtful.

Secondly, there exists variation in the prevalence of this malignancy in different geographic areas within the country. (This malignancy is common in the Southern states, North eastern states and in the Valley of Kashmir).

This study was designed to assess the prevalence of HPV infection (high risk genotypes 16 and 18) in patients with biopsy proven squamous cell oesophageal carcinoma.

The study includes patients from different geographic areas within India to look for any possible association in any of the geographic high prevalence areas in India.

## AIM

*To evaluate the probable association of HPV infection with genotypes 16 and 18 in patients with squamous cell oesophageal carcinoma in Indian patients.*

## OBJECTIVE

*To assess the detection rate of high risk HPV types 16 and 18 in patient with squamous cell oesophageal carcinoma.*

# LITERATURE REVIEW

Oesophageal carcinomas are the eighth most common cancer worldwide, with about 481, 000 new cases (3.8% of the total) estimated in 2008, and sixth most common cause of death from cancer worldwide from cancer with 406,000 deaths (5.4% of the total). (1) This figure includes both the two major types of oesophageal malignancy i.e. squamous cell carcinoma (SCC) and adenocarcinoma. (1)

Of the two common types, the squamous cell type of oesophageal malignancy is the most common type worldwide.(14)(72) It is also the fifth most common cancer in the developing countries.(1) More than 80% of the cases and deaths due to oesophageal carcinoma occur in developing countries.(1)

The long-term survival rate of oesophageal malignancy is 19 %,( 2) which are largely due to the fact that these cancers are detected in an advanced stage. (15) Thus they present a therapeutic challenge to the clinicians. There is reported little difference in outcome between the two histologic types of carcinoma. (16)

The oesophageal cancer male: female ratio is 3 – 4: 1 in low risk countries but in high risk countries or region there exist no gender differences.

The squamous cell carcinogenesis of the oesophagus is complex multistep process and it is hypothesised to have multifactor aetiology and no single agent is identified to cause malignancy. In the past two decades HPV has gained the interest to be the most likely infectious viral agent leading to oesophageal cancer.

**EPIDEMIOLOGY** -

The oesophageal cancer incidence rate varies internationally by nearly 16-fold across the different regions worldwide. The regions reported with highest incidence are Southern and Eastern Africa with regions of Eastern Asia, and regions with lowest of incidence are Western and Middle Africa and Central America. (17)

The area stretching across the Northern Iran through the central Asian republics to North-Central China which is often referred as “oesophageal cancer belt” .This is the region with highest risk for oesophageal carcinoma. The 90 percent of the cancer cases here are of squamous cell type. (18)

In such endemic areas the incidence is as high as 250 cases per 100,000 persons. (19) In contrast, the combined incidence of squamous cell and adenocarcinoma of the oesophagus in the United States is 4 cases per 100,000 persons. The reasons for such a vast geographic discrepancy are unknown.

It is suggested that it is linked to the local arid climate and alkaline soil, as well as ingestion of nitrosamines and inversely to the consumption of riboflavin, nicotinic acid, magnesium and zinc. The highest mortality rate among both male and female occurs in developing countries across Eastern and Southern Africa, and in Eastern Asia.(1)

In North America and Western Europe in contrast, alcohol and tobacco use are the major risk factors for causing squamous cell carcinoma and account for 80 – 90% of cases. (20)(21) A study describes the relative risk of oesophageal cancer by the amount of alcohol and tobacco consumed as of 155: 1 when consuming > 30gm per day of tobacco along with 121gm of alcohol. (22)

## CHANGE IN TREND OF OESOPHAGEAL CANCER IN WEST

In United States, there is report of dramatic rise in incidence of adenocarcinoma of the oesophagus. In 1987, adenocarcinoma was reported in 34% and 12% of oesophageal cancers in Caucasian men and women with 3% and 1% for African American men and women, respectively. In the last 20 years there has been an increase in incidence of adenocarcinoma at a rate of 5% to 10 % per year.(23)(24)

The reasons for such changes in trend is not well known but it has been hypothesised that it is due to increase in risk factors as overweight and obesity in the country's general population. The trend in India has not shown any similar changes. The squamous cell carcinoma of the oesophagus is still the most common histological type of oesophageal carcinoma in our populations.

## THE INDIAN SCENARIO

In India the cancer of oesophagus is one of the most common malignancies.(25) The incidence of the disease varies across the regions in different parts of the country .It is an important leading site of cancer in both males and females.(1)

Of the two most common histological types, squamous cell carcinoma is the more common (90%), as compared with the adenocarcinoma of oesophagus. (26) There is no change in trend of the histologic types for the oesophageal cancer as documented in the US population. (26)

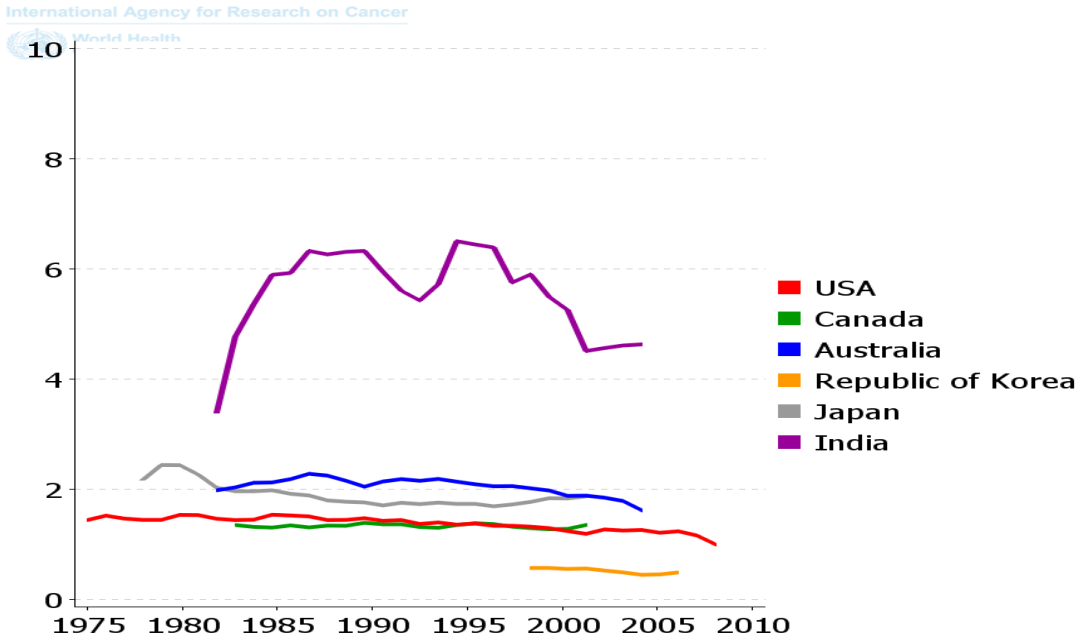
The Cancer of the oesophagus is third leading sites of cancer in Mumbai, Chennai and Bangalore registry, fourth in Bhopal and eighth in Delhi registry. (25)

Average age adjusted incidence rate for cancers of oesophagus is 8 per 100,000 populations. (1)

The estimated mortality for Oesophageal carcinoma for the year 2008 among men in India was 6 per 1, 00,000 population and for women was 4.2 per 1, 00,000 population. (1)

Periodic variation in incidence rate of oesophageal cancer in India for women (age - standardised rate (W) per 100,000 women) is as shown (1) in Figure 1

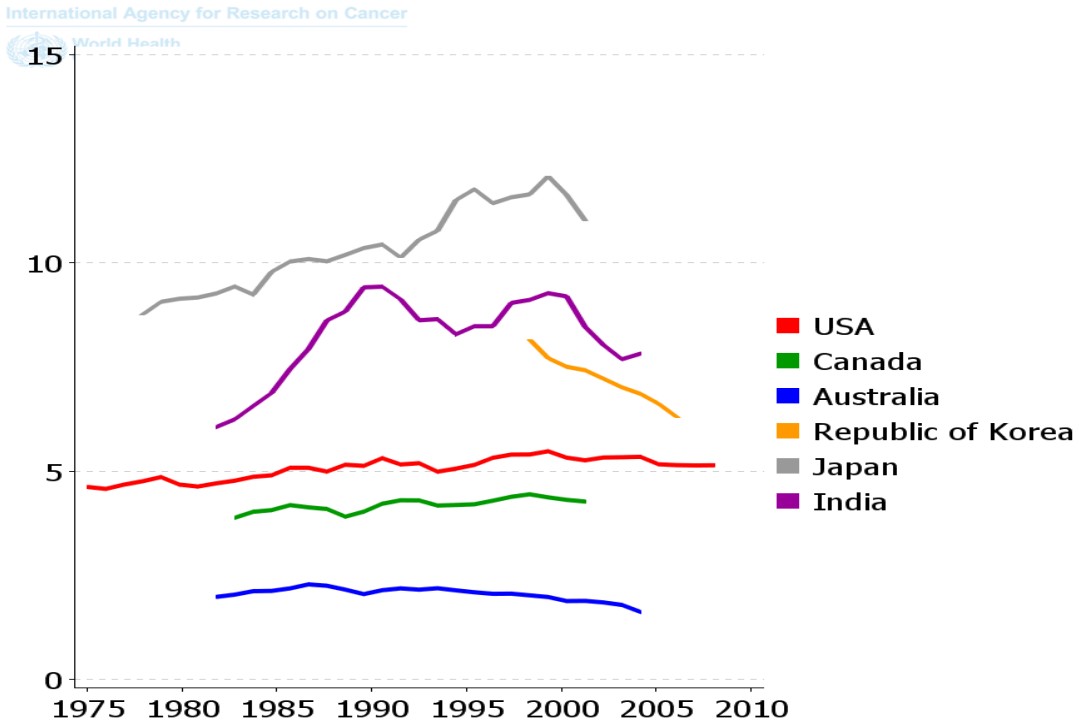
Figure 1 Variation in incidence rate of oesophageal cancer in Women




*(Adapted from* GLOBOCAN 2008 (IARC (1)

Periodic variation in incidence rate of oesophageal cancer in India for men (age-standardised rate (W) per 100,000 men) is as shown: (1) Figure 2

Figure 2 Variation in incidence rate of oesophageal cancer in Women




*(Adapted from* GLOBOCAN 2008 (IARC)*)* (1)

## THE OESOPHAGUS

The oesophagus is a 25 cm long thin, muscular tube connecting the pharynx to the stomach. It extends from the lower border of the cricoid cartilage (at the level of the sixth cervical vertebra) to the cardiac orifice of the stomach at the side of the body of the 11th thoracic vertebra. (27)

The oesophageal mucosa is lined over most of its length from cricopharyngeus muscle at the level of cricoid cartilage superiorly to the gastroesophageal junction inferiorly with stratified keratinized squamous epithelium.

This epithelium gives rise to the squamous cell carcinoma, the most common neoplasm of the oesophagus. These cancers thus most often occur in the cervical and in the upper and middle thoracic oesophagus.

The distal 2 to 3 cm of the oesophagus and the cardia are lined by columnar epithelium, in which adenocarcinoma may occur. (28)

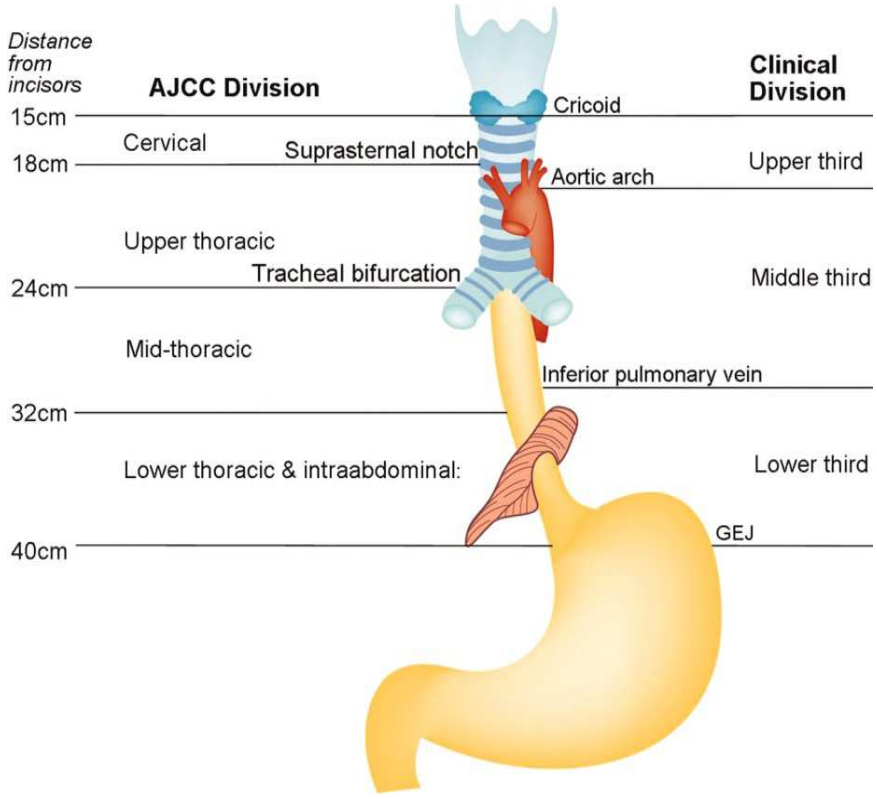
The oesophagus is divided into 3 parts. (Figure 3) They are -

(1) The cervical part which extend from cricopharyngeus to the suprasternal notch.

(2) The thoracic part which extends from suprasternal notch to the diaphragm.

(3) The abdominal part extends from diaphragm to cardia of stomach.

Figure 3: Different parts of Oesophagus




(Figure 3 adapted from Kim T J et al. Radiographics 2009; 29:403-421)

## LYMPHATIC DRAINAGE

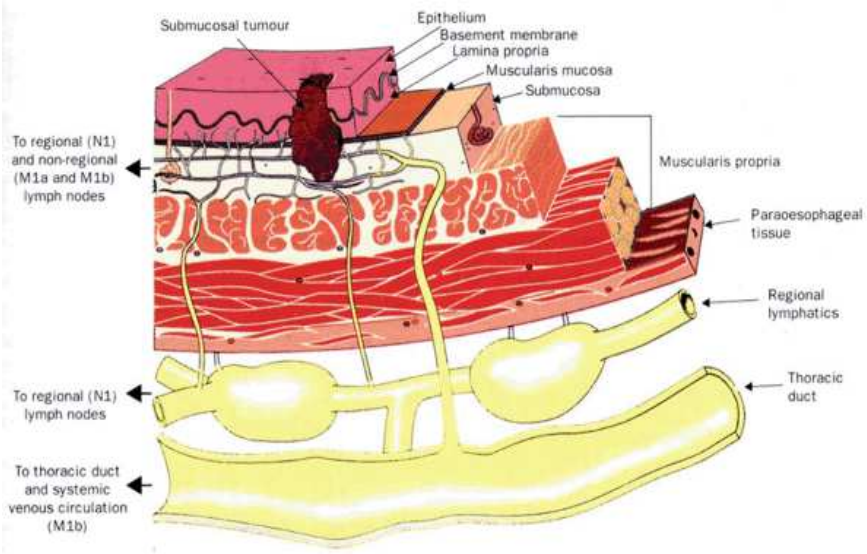
The lymphatic system of oesophagus offers little barrier to the spread of malignancy as they are highly interconnected. Hence, the oesophageal tumour spreads early via lymphatics and has nodal deposits away from the primary lesion. This causes the presence of “skip areas” in between the lesions seen in oesophageal malignancy. (30)

The oesophagus has an extensive, longitudinally continuous, submucosal lymphatic system. The oesophagus has 2 types of lymphatic vessels which are (as depicted in figure 4)

A plexus of large lymphatic vessels is present in the mucous membrane which is continuous above with the mucosal lymphatic vessels of pharynx and below with mucosal lymphatic vessels of gastric mucosa.

The second plexus of finer vessels situated in the muscular coat. Efferent vessels from the cervical part drain into the deep cervical nodes. Lymphatics from the thoracic region drains in the posterior mediastinal nodes and lymphatic from the abdominal part drains in to the left gastric nodes. Lymphatics can also drain directly in to the thoracic duct. (27) (28)

Figure 4 – Lymphatic system of Oesophagus




(Figure 4 Adapted from Rice *T*: Superficial oesophageal carcinoma: is there a need for three-field lymphadenectomy? Lancet 354:793, 1999) (29)

## ETIOLOGIC FACTORS

The squamous cell carcinogenesis of the oesophagus is complex multistep process and it is hypothesised to have multifactor aetiology and no single agent is identified to cause malignancy.

The following are the probable aetiologies' for the squamous carcinoma of the oesophagus-

(a) Hereditary

In regions with high incidence of oesophageal squamous cell carcinoma from China there are reports of familial aggregation. (31) Familial clustering was also reported from Sweden. (32)(33) But the role of hereditary factors in the pathogenesis of oesophageal cancer remains uncertain.

(b) Geographic variations -

The regions of high incidence of oesophageal cancer are found in Southern and Eastern Africa with Eastern Asia and regions with low incidence are Central America with Western and Middle Africa. (1)

(c) Smoking and alcohol

Smoking and drinking regular alcohol are important risk factors for malignancies. High risk of oesophageal squamous cell carcinoma is associated with cigar and pipe smoking. Tobacco and alcohol together may increase the risk of carcinogenesis. (34)(35)

(d) Dietary factors

Several dietary ingredients are associated with oesophageal squamous cell carcinoma such as consuming N-nitroso compounds, chewing of areca nuts or betel quid, drinking hot tea or eating red meat.(36)(37)

Low selenium levels also increases the risk of oesophageal cancer. (38) Zinc deficiency may enhance the carcinogenic actions of nitrosamines. (39)

Drinking hot beverages as hot tea can increase the risk of oesophageal malignancy. A case control study from northern Iran shows a very significant association of drinking very hot tea and risk of squamous cell carcinoma of oesophagus. (40)

(d) Underlying oesophageal disease

The presence of pre-existing oesophageal conditions such as achalasia and corrosive strictures may increase the risk of oesophageal malignancy. (41) (42)

(e) Prior gastrectomy

Patients who had partial gastrectomy in past are at increased risk of developing oesophageal squamous cell cancer. (43)

(f) Atrophic gastritis

Atrophic gastritis is associated with two times increased risk of developing oesophageal cancer. (44)

(g) Tylosis

It is a rare autosomal dominant disease with hyperkeratosis of palms of hands and soles of the feet. It is associated with a high rate of oesophageal cancer..(45)

(h) Upper aerodigestive tract cancer

Possible association is seen between current or past history of SCC of head and neck with synchronous or metachronous squamous cell carcinoma of the oesophagus. (47)(48)

(i) Bisphosphonates

Use of oral Bisphosphonates is reported to cause oesophageal malignancy in post-marketing surveillance. (46)

(j) Human Papilloma Virus

There is a huge variation (0 to 100 percent) in the prevalence of HPV associated with oesophageal malignancy in literature. HPV role is still debatable.

## NATURAL HISTORY AND PATTERN OF SPREAD OF SQUAMOUS CELL CARCINOMA OF THE OESOPHAGUS

Squamous cell carcinoma of oesophagus is characterised by both extensive local growth and affinity towards lymph nodal metastases. (49) As oesophagus has no serosal layer there is very early direct invasion of the contiguous structures.

The growth in the oesophagus can impinge or invade the recurrent laryngeal nerves, carotid arteries and trachea.

If extra oesophageal extension occurs in the mediastinum, trachaeoesophageal or bronchoesophageal fistula can occur.

The growth in the lower third of oesophagus can invade the aorta or pericardium causing mediastinitis, massive haemorrhage or empyema.

The location of lymph node metastasis is influenced by the origin of the primary tumour. Metastatic lymph nodal disease is found in approximately 70%of patients at autopsy.

Akiyama studied the pattern of lymphatic metastases in patient with oesophageal cancer who had had underwent surgical resection.

He found that in upper oesophageal cancer metastasis in neck lymph nodes was 44.1%, upper mediastinum 50 %, middle mediastinum 20.6%, and lower mediastinum 5.9% with upper gastric area 14.7%.

In mid oesophageal carcinoma metastasis in neck lymph nodes was 32.9%, upper mediastinum 38.1 %, middle mediastinum 41%, and lower mediastinum 20.2% with upper gastric area 42.5%.

The involvement of abdominal nodes increases as ones disease involves more distally in oesophagus to the gastroesophageal junction

For T1 lesions incidence of nodal spread is 14% to 21% and for T2 lesions incidence of nodal spread is 38% to 60 %.( 50)

For patient with lower oesophageal and gastroesophageal junction adenocarcinoma, approximately 70% will have nodal metastasis at presentation. The involvement of both mediastinal and abdominal lymph nodes is common. The involvement of abdominal nodes increases as ones disease involves more distally in oesophagus to the gastroesophageal junction.

## CLINICAL PRESENTATION

The region of the primary tumour in the oesophagus may influence the presenting symptoms in patients.

Dysphagia is most commonly seen in more than 90% of patient regardless of location.

Odynophagia is a complaint in 50% of patients.

Weight loss is also common in 40% to 70% of patients and the extent of weight loss is associated with worse prognosis.

Less frequent symptoms of this condition are hoarseness, cough and glossopharyngeal neuralgia.

In advanced lesions the signs and symptoms of tumour invasion into local structures predominates. These symptoms include hematemesis, haemoptysis, melena, dyspnoea and persistent cough secondary to tracheobronchial or bronchoesophageal fistula.

The compression or invasion of the left recurrent laryngeal nerve or phrenic nerve can cause dysphonia or hemi diaphragm palsy.

Other symptoms of Superior Vena Cava syndrome and Horner's syndrome can also occur.

Pleural effusion and exsanguinations due to aortic communication is also seen.

Abdominal and back ache can occur in patient with celiac node involvement by the lower oesophageal growth.

**PROGNOSTIC FACTORS**

Stage of the oesophageal carcinoma at presentation is the most important prognostic factor in determining survival of the patient. The increase in depth of tumour penetration (i.e. T stage), lymph node involvement (i.e. N stage) and absence or presence of metastasis influences the outcome. Usually the patients with distant metastasis are rarely curable. The presence of lymphatic invasion suggests advanced stage and worse long term survival for the patient. (51)

The site of tumour is reported to influence the survival. The patients with lower third experience improved outcome versus patient with lesion in the upper third of the oesophagus. (52)

Tumour size also influences its outcome. According to a study the 2 year survival rate was 19.2% for patient with tumour <5cm in size versus 1.9% for patient with tumour >9cm. (52)

Progression of tumour size is related with unresectability and more chances of distant metastasis.

The histological type of tumour is regarded as an independent prognostic factor in patient undergoing surgery. Siewert et al (53) had analysed 1000 patients with carcinoma oesophagus who had surgical resection done and on follow up found that 5 year survival rate was 47% for patient with adenocarcinoma and 37% for squamous cell carcinoma. (53)

It is also found that women tend to fare better than men with regard to survival. (52)

It is suggested that Race may be a factor influencing survival. A study by Hussey reported higher survival rate in Caucasians than Afro American (52) In contrast to a recent study done in patient who were treated with radiation therapy and chemotherapy there was no statistical difference found between the races. (54)

Age is also been considered to be significant and it is shown that patient older than 65 years age faring less well. (15)

The extent of weight loss and patient's low overall performance status also indicate poor prognosis in the disease. (52)

The presence of deep ulceration of the lesion, sinus tract formation and fistula formation are other poor prognostic factors which also suggest local invasion.

At surgery the importance of obtaining uninvolved resection margins is of significance with regards to the long term outcomes in patient. An Intergroup study of patients who had chemotherapy preceding and following R1,R2 resection and patient not undergoing resection showed a similar outcomes but only patient who had R0 showed a substantial long term disease free survival. (55)

In an observation by patterns of care survey done to evaluate the survival outcome of treatment in patients with squamous cell cancer and adenocarcinoma of the oesophagus between 1996 and 99.

The multivariate analysis showed a considerable improvement in the survival of patients are being treated in centres with >500 new cancer patient per year as compared with centres who have <500. (56)

## TNM staging of 0esophageal squamous cell cancer (SCC) (2010 Edition)

The latest TNM staging system used is the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (UICC) 2010 edition for the oesophageal cancer. As a result of analysis of worldwide data from patient with oesophageal cancer there have been changes between the 2002 and the latest 2010 edition.

The major changes which are present in the 2012 edition are-

(1) There is separate staging group for squamous cell carcinoma and adenocarcinoma of oesophagus.

(2) The tumours present at the oesophagogastric junction and proximal 5 cm of the stomach extending into the GE junction or oesophagus are now staged as oesophageal cancers.

(3) The tumours with an epicentre in the stomach >5 cm from the GE junction, or those within 5 cm of the GE junction without extension into the oesophagus are classified as stomach cancers.

(4) The Tis is redefined as high grade dysplasia (HGD) which includes non invasive neoplasias. The term “carcinoma in situ” is no longer used.

(5) T4 disease is subclassified based upon respectability of invaded adjacent structures/organ.

(6) Nodal status is subclassified as per the number of regional nodes with metastases.

# TNM staging of 0esophageal squamous cell cancer (SCC)

**Table 1 –(Staging of Oesophageal carcinoma) Adapted from** *AJCC Cancer Staging Manual, Seventh Edition (2010))**(57)***

| | |
|---|---|
| **Primary tumour (T)*** | |
| TX | Primary tumour cannot be assessed |
| T0 | No evidence of primary tumour |
| Tis | High-grade dysplasia• |
| T1 | Tumour invades lamina propria, muscularis mucosa, or submucosa |
| T1a | Tumour invades lamina propria or muscularis mucosa |
| T1b | Tumour invades submucosa |
| T2 | Tumour invades muscularis propria |
| T3 | Tumour invades adventitia |
| T4 | Tumour invades adjacent structures |
| T4a | Resectable tumour invading pleura, pericardium, or diaphragm |
| T4b | Unresectable tumour invading other adjacent structures, such as aorta, vertebral body, trachea, etc. |
| **Regional lymph nodes (N)Δ** | |
| NX | Regional lymph node(s) cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in 1-2 regional lymph nodes |
| N2 | Metastasis in 3-6 regional lymph nodes |
| N3 | Metastasis in seven or more regional lymph nodes |
| **Distant metastasis (M)** | |
| M0 | No distant metastasis |
| M1 | Distant metastasis |

| Histologic grade (G) | |
|---|---|
| GX | Grade cannot be assessed - stage grouping as G1 |
| G1 | Well differentiated |
| G2 | Moderately differentiated |
| G3 | Poorly differentiated |
| G4 | Undifferentiated - stage grouping as G3 squamous |

**Anatomic stage/prognostic groups**

**Squamous cell carcinoma◊**

| Stage | T | N | M | Grade | Tumour location § |
|---|---|---|---|---|---|
| 0 | Tis | N0 | M0 | 1, X | Any |
| IA | T1 | N0 | M0 | 1, X | Any |
| IB | T1 | N0 | M0 | 2-3 | Any |
| | T2-3 | N0 | M0 | 1, X | Lower, X |
| IIA | T2-3 | N0 | M0 | 1, X | Upper, middle |
| | T2-3 | N0 | M0 | 2-3 | Lower, X |
| IIB | T2-3 | N0 | M0 | 2-3 | Upper, middle |
| | T1-2 | N1 | M0 | Any | Any |
| IIIA | T1-2 | N2 | M0 | Any | Any |
| | T3 | N1 | M0 | Any | Any |
| | T4a | N0 | M0 | Any | Any |
| IIIB | T3 | N2 | M0 | Any | Any |
| IIIC | T4a | N1-2 | M0 | Any | Any |
| | T4b | Any | M0 | Any | Any |
| | Any | N3 | M0 | Any | Any |
| IV | Any | Any | M1 | Any | Any |

Note: cTNM is the clinical classification,

pTNM is the pathologic classification.

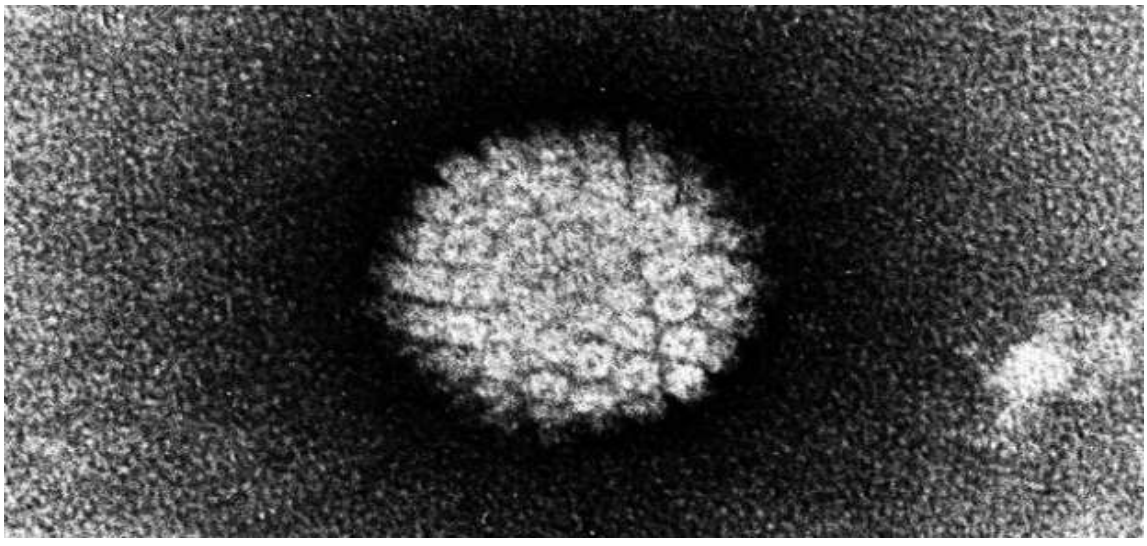
## HUMAN PAPILLOMA VIRUS

Human papilloma viruses (HPV) are small, double stranded, non-enveloped DNA viruses that belong to family Papillomaviridae. They have icosahedral capsid made up of 72 capsomeres (figure 5) the double stranded supercoiled DNA contains about 3000 basepairs (bp).

The DNA encodes for early genes E1 to E8 and late genomes L1 and L2. E6 and E7 early genes are implicated in oesophageal carcinogenesis.

More than 100 different HPV types are described associated with infection of anogenital tract and other sites. HPV infections (HPV 16, HPV 18) are most commonly transmitted by sexual contact. (59)

Figure 5 - Human papilloma virus, under electron microscope



(Figure 5 Adapted from National Institutes of Health)

Papilloma virus does not have oncogenic potential but subsets of papilloma virus are clearly implicated in carcinogenesis in humans. (60)

The evidence to suggest HPV association with human malignant diseases came first from the observations on epidermodysplasia verruciformis. This disease causes susceptibility to HPV types 5 and 8 which produces multiple flat warts. In one third of these patients squamous cell carcinoma of these warty lesions develops. (61)

HPV associated with malignancy of both genital tract and non-genital tract have been divided as –

High Risk types (HPV 16, 18, 31, 35, 39, 45, 51, 52, 56, 59, 66, 68, 69, 73 and 82).

Low-Risk types (HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81)
These are associated with benign lesions such as genital and skin warts.

The different high risk HPV types have tendency to infect a specific sites of the human body according to their “tropism”.

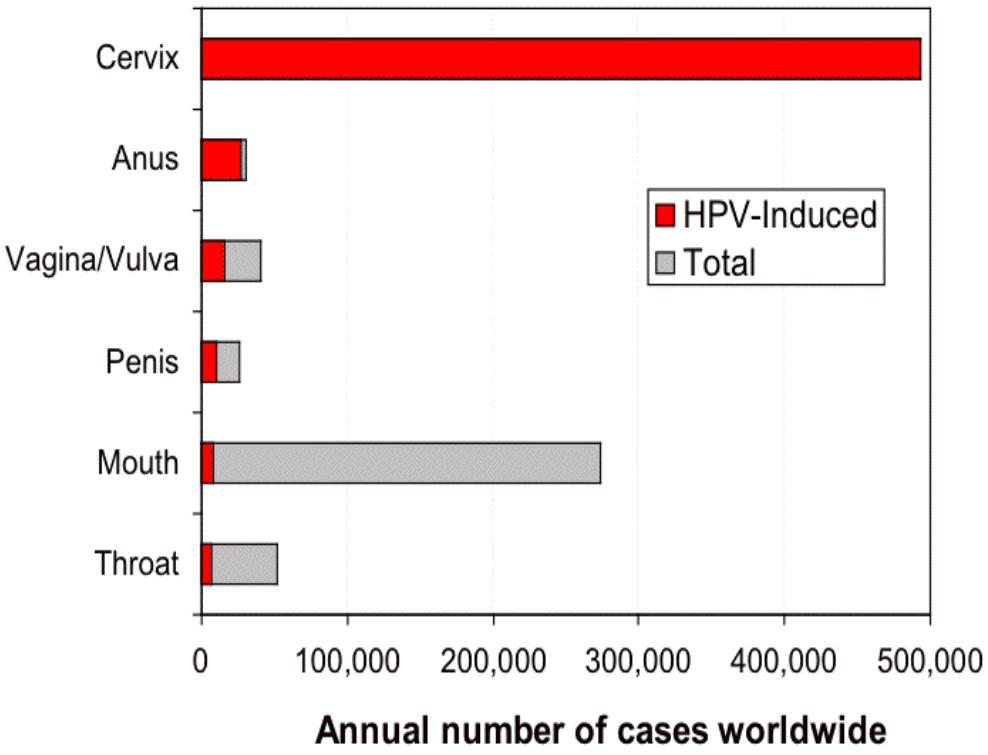
They show 2 types of tropism -

(a) Tropism for epithelial cell: these HPV types have preference towards cutaneous epithelium and cause infections as common warts, plantar warts and butcher's warts in skin.

(b) Tropism for mucous membranes: these HPV types have preference for mucous membrane and they cause infection of penis, scrotum, perineum, perianal region, vaginal introitus, vulva, and cervix.

As per the worldwide data in 2002, HPV is attributed in 5.2% of new cases (about 561,200 cases) involving cancer of cervix, anus, vagina/vulva, penis, mouth and throat. It is also the most commonly associated infectious agent in 84% of new cervical cancer.

Table 2 **-** Worldwide incidence and distribution of cancers attributed to high risk HPV




. (Table 2 Adapted from Global cancer statistics, 2002,) (58)

## MECHANISM OF VIRUS REPLICATION AND ONCOGENICITY

The mode of exposure of oesophageal mucosa to the HPV is not known. There are several theories to suggest the probable HPV transmission.

One theory suggests that increase in practise of oral sex could cause the HPV transmission. The similar mode is also ascribed to the HPV related oropharyngeal cancer.

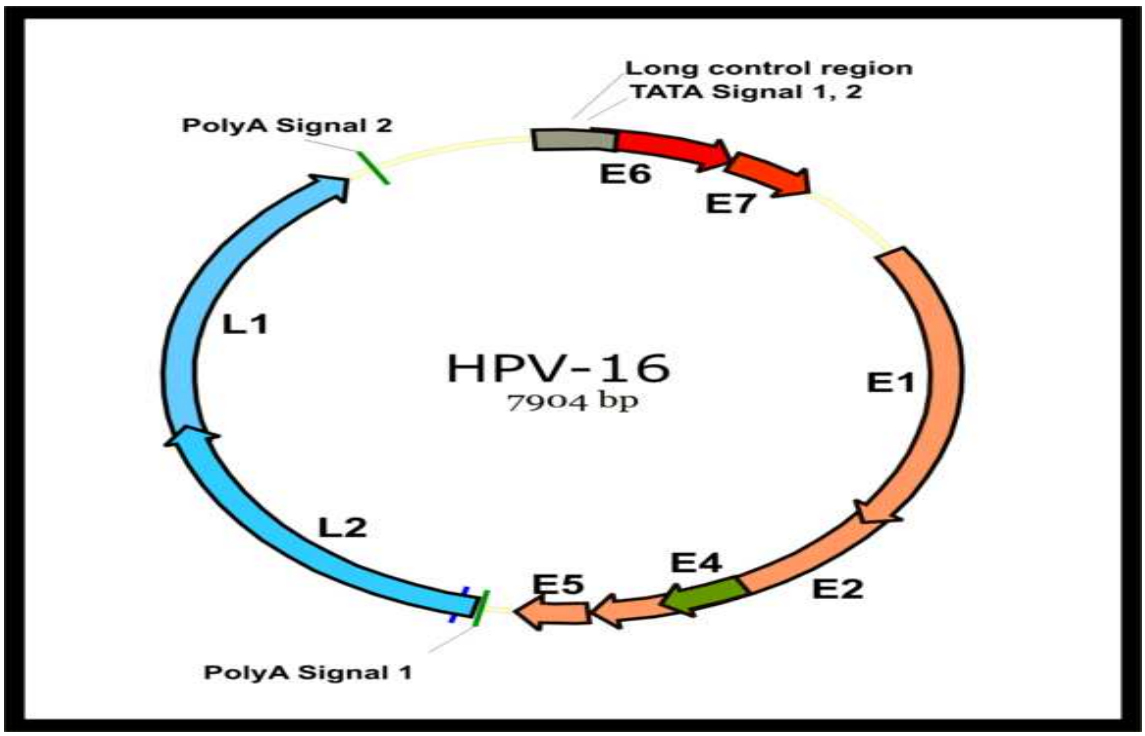
Some report postulate that intrapartum transmission during passage of baby through the HPV infected mothers can lead to the HPV transmission. But the real mode of transmission is not known.

Usually the Papilloma virus gains entry through wounds or abrasions at the site. The virions then bind to heparan and cell surface glycosaminoglycans on human keratinocytes.The entry in to cell is then by clathrin dependent, receptor mediated endocytosis. Following which the disassembly of the viral capsid occurs and viral genome is exposed inside the endosome.

For oncogenesis a part of viral genome is integrated to the host cellular genome. A break at E1 –E2 region during integration lead to interruption of E2 gene. The

deletion of E2 gene leads to loss of control over E 6 and E 7 genes. These HPV early gene products of E6 and E7 formed are implicated in oncogenicity.

Figure 6 THE GENOMIC STRUCTURE OF HPV 16




E6 interaction with p53 accelerates proteolytic degradation of p53, a tumour suppressor gene and reduces its stability. E7 interacts with pRB, retinoblastoma protein, to abolish cell cycle regulation.

pRB is negative regulator of cell cycle and is related with the E2F family of transcription factors which prevent S-phase entry. The binding of E 7 with pRB results with E2F replacement leading to production of proteins required for DNA replication. (60)(62)

The inhibition of p53 and pRB by the viral E6 and E7 results in cell transformation which can further lead to malignancy.

## HPV AND SQUAMOUS CELL OESOPHAGEAL CANCER

The first observations were made by Syrjanen *et al.* in 1982 by examining a series of 60 squamous cell carcinoma of oesophagus. He suggested a probable association of human papillomavirus (HPV) in aetiology of both benign and malignant squamous cell oesophageal lesions. (7)(63)

This morphological similarity found in the gross samples of squamous cell malignancy of oesophagus to the condylomatous lesions related to HPV infections led to further studies. (7)(62)(64)(65)

HPV are known to cause tonsil and oropharyngeal cancers. (62) (66) (67) (68) (69) (72) In view of contiguity of squamous epithelium of oropharynx with oesophageal epithelium the possibility that HPV could be involved in the pathogenesis of oesophageal malignancy could be a possibility.

The evidence of HPV involvement in oesophageal malignancy is supported by various reasons.

(1) Evidence of HPV involvement in benign squamous cell papilloma of oesophagus.

HPV types 16 and 18 are frequently isolated from such papilloma. (62) It is a rare condition and so study with large numbers is not present. There are

about 239 reported cases of oesophageal squamous cell papilloma in literature which have detected HPV in 21.3% (n=51) of cases. (67) (70)

(2) HPV detected in oesophageal precancer sites.

Oesophageal squamous malignancy progresses through the distinct precursor lesions as dysplasia and carcinoma in situ leading to cancer. HPV was detected in such lesions. (71)(70)

(3) Evidence of HPV in animal studies.

The studies on cattle experimentally demonstrated papillomatosis and malignant transformation to carcinoma following infection of bovine papillomavirus type 4 (BPV-4). BPV - 4 DNA is also frequently isolated from naturally or experimentally occurring papillomas in cattles. (73)(74)

It is also found that bracken fern consumed by cattle act as a co factor in BPV induced carcinogenesis. (73)

(4) In vitro studies:

The cultured oesophageal epithelial cells in vitro incubated with HPV showed malignant transformation. The study provides a model to demonstrate the association between HPV and etiopathogenesis of oesophageal malignancy. (75)(76)

(5) Seroepidemiological evidence:

The presence of antibodies to virus like particles(VLP) specific to HPV in patients with squamous cell carcinoma of oesophagus HPV infection as a risk factor for this disease. In Shaanxi Province, China studies have shown 24% of patients and 7% of control were seropositive.

However the role of HPV in squamous cell carcinoma of oesophagus is still controversial. The arguments against the possible association include:

(a) Huge variation in HPV prevalence in the present available literature case series ranging from 0 to 100%.

(b) Inconsistent associations between HPV exposure (as measured by serology) and squamous cell carcinoma of oesophagus.

(c) Contamination of sample during collection, procession and testing can lead to false positive results as HPV is known to cause other common epithelial lesions in humans.

A recent meta analysis of 152 studies which covered a total of 10,234 ESCC cases, analysed by different HPV detection methods in different geographic regions concluded that wide variability in HPV detection rates in ESCC is not due to the HPV detection techniques, but by the geographic origin of the study.(14)

These data substantiate the recently elaborated concept that ESCC might have a different etiology in low-incidence and high-incidence geographic regions, HPV playing an important role only in high incidence areas.

## DIFFERENT METHODS USED IN DETECTION OF HPV IN OESOPHAGEAL CARCINOMAS

Various methods are used to detect HPV in the sample of oesophageal carcinomas. From late 1980 different techniques were used and now most have been replaced by the latest technique.

FILTER IN SITU HYBRIDISATION

This technique was used widely in late 1980. It was routinely used to detect HPV DNA from mucosal samples of the genital tract. The technique had low sensitivity and poor specificity. In early 1990 the technique became obsolete.

This technique had resulted in considerable higher positivity for HPV DNA than other technique because of its high false positivity rate.

IN SITU HYBRIDISATION

It is one of the most frequent techniques used in various studies. Different studies including about 1485 cases of oesophageal squamous cell carcinoma ISH was used. The HPV 16 was most frequently detected serotype. It showed HPV DNA in 22.9% of cases with oesophageal cancer.

POLYMERASE CHAIN REACTIONThis is the latest and most sensitive technique used to detect HPV DNA in oesophageal carcinoma. The overall detection rate of HPV as compared to other technique is low. The higher rate of positive result in other hybridisation technique would be due to the cross hybridisation of probe DNA to the human DNA. The detection rate of PCR in different studies with 1183 cases of

squamous cell carcinoma of oesophagus was 15.6 %. In our study PCR was used to detect the presence of HPV in the sample tissue.

## TECHNIQUE OF POLYMERASE CHAIN REACTION

This technique was first published in 1985 by Kary B. Mullis who received the Nobel Prize in chemistry for inventing this technique.

Today PCR is the basis of modern molecular genetics and molecular biology. It causes fast, highly specific amplification of the DNA fragments. It is guided by two main principles –

1) Complimentarity driven DNA formation of duplex.
2) Template driven DNA synthesis by DNA polymerases.

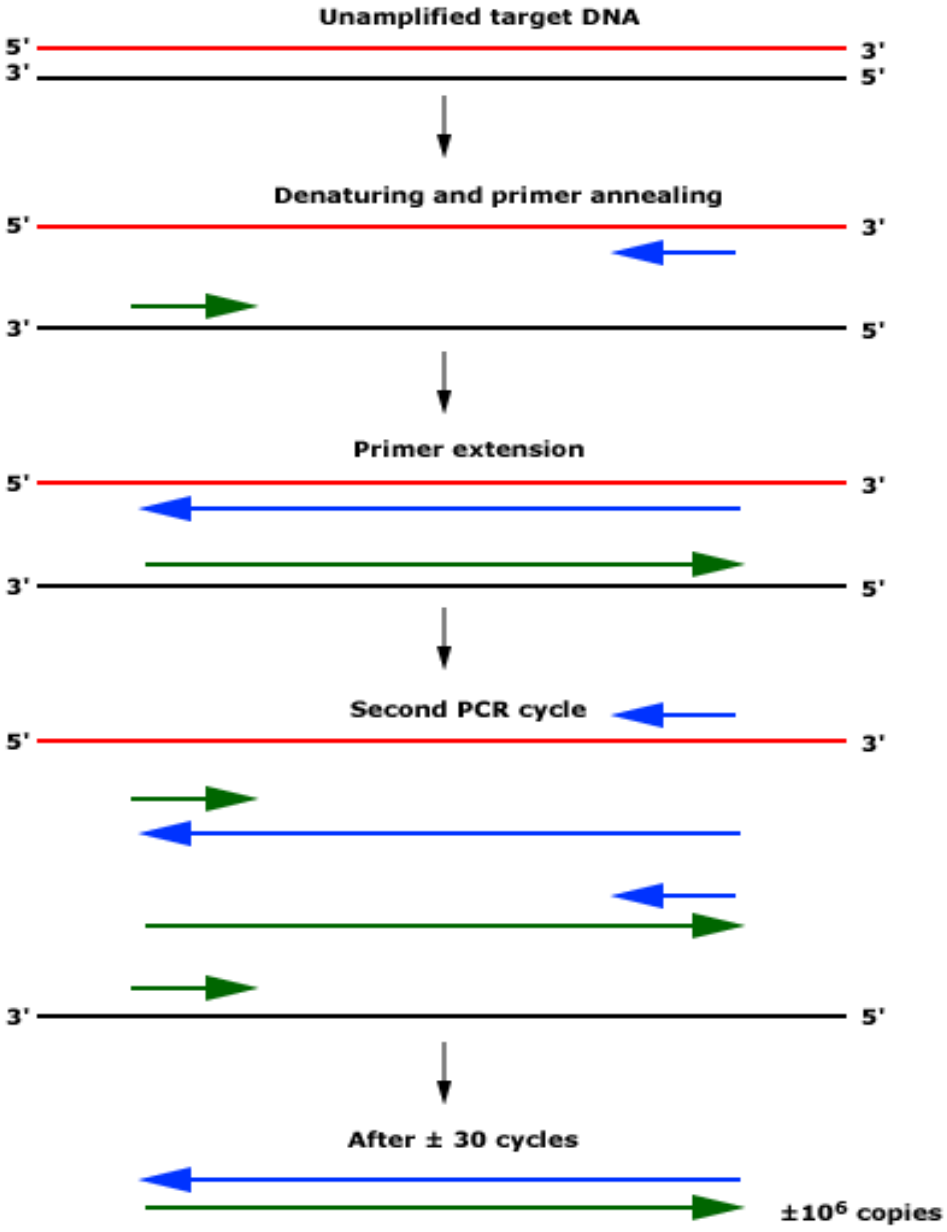
The main process of PCR consists of the following three steps which are repeated for 30 to 40 times. (Figure 8)

STAGE 1 Denaturing: The DNA double strands are denatured to form single strands by heating to 95 degrees.

STAGE 2 Annealing: The mixture is cooled for binding of primer to the single stranded DNA following which the DNA polymerase binds to the 3' end of the primer.

STAGE 3 Elongation: The temperature is raised to 70 – 72 degrees which activates the polymerase to initiate chain elongation. The enzyme polymerase act as catalyst for addition of nucleotides to the primers. The cycle is maintained for 1 minute and then terminated by raising temperature to 95 degrees thus cycling back to stage 1.

Figure 7 Stages of a Polymerase chain reaction




When the process is completed the amplified product in placed for Gel electrophoresis. The negatively charged DNA fragments migrate towards the positive electrode and the speed of the fragment depend upon the size of the DNA. Then

ethidium bromide is added which stain the DNA as fluorescing band in UV light. The gel is photographed for further recording of the same.

## HPV VACCINES AND HPV ASSOSIATED SQUAMOUS CELL CARCINOMA OF THE OESOPHAGUS

Human Papilloma Virus are proven to cause over 70% of cervical cancer. The most common serotype which is associated with cervical cancer is HPV type 16 and 18. There has been a major breakthrough following the introduction of HPV vaccines which target these high risk serotypes.

The vaccines Gardasil is a quadrivalent which also targets HPV serotype 6 and 11 along with the serotype 16 and 18

The vaccine Cervarix is bivalent which specifically targets only the high risk serotype 16 and 18.

The vaccines in a randomised control trial has efficacy rate as high as 90-100% in preventing cervical cancer for women who are HPV sero negative.

These vaccines are intended for prophylactic use and advised for only young girls.

A similar impact can also be expected in regions where there is substantial prove for HPV associated squamous cell carcinoma of the oesophagus.

At present still the etiological significance of HPV is debatable. In view of inconclusive data to support the role of HPV in oesophageal cancer it is not possible to suggest HPV vaccination for prevention of this disease.

However in regions of China where there have been a significant high prevalence of oesophageal cancer even if HPV is associated with small proportion of cases, the vaccines can have significant impact.

# PATIENT SELECTION AND METHODOLOGY

The patients who presented to General Surgery Unit 3 and Department of Gastroenterology with oesophageal carcinoma were included in this study. Informed consent was obtained from the participants who were willing to participate in this study. Patient who were detected to have confirmed squamous cell carcinoma of the oesophagus of the oesophagus were included as **case participants or Group A**. These patients had biopsy of the lesion for routine histopathology and also for the study. A second biopsy of the surrounding normal mucosa not less than 5 cm from the edge of the lesion was also taken for analysis in this study.

The **control group (Group B)** included patients undergoing upper GI endoscopy for dyspeptic symptoms. They were included to study to look at the prevalence of HPV virus in the non malignant population This group had biopsy from the normal mucosa of the middle third of oesophagus for HPV DNA analysis.

The biopsy samples from both the groups which were collected and coded separately and then taken to the Clinical Virology laboratory for further analysis.

## Eligibility criteria

All cases with histopathology proven squamous cell carcinoma of the oesophagus.

## Exclusion criteria

**(i**) Patient who had previous treatment or neoadjuvant therapy for carcinoma oesophagus.

**(ii)** Immunocompromised states (HIV, Cirrhosis of liver, chronic renal failure, uncontrolled diabetes etc.)

### SAMPLE SIZE

HPV infection shows a great variation in geographical distribution worldwide and also in India between different regions. A prospective observational case control study was designed.

Assuming the proportion of HPV positive in case group - 0 .40

Assuming that proportion of HPV positive in control group - 0.10

Power of study (1-β) - 80

α Error % - 5

No. of participants required in “each group “ = 38

TOTAL (case + control) = 76

The following variables were studied in the two groups i.e. Group A and Group B

AGE

GENDER

REGION

RESIDENT OF TOWN /VILLAGE

EDUCATION

SMOKING HABIT

ALCOHOL

DIET

USE OF HOT BEVERAGES AS TEA/COFFEE

OTHER COMORBIDITY

ENDOSCOPY SITE OF LESION

HISTOPATHOLOGICAL GRADE OF CARCINOMA

PRESENCE OF HPV TYPE 16 & 18 AND OTHER TYPES

## STASTISTICAL METHODS:

The categorical variables in this study are described using frequency and percentage. Chi-square test was used to assess any association between these categorical variables and the results will be presented along with odds ratios and their respective confidence intervals. Multivariable logistic regression was performed with candidate variables that were significantly associated with the outcome at 10% significance level and also with those that are clinically relevant. A p-value $< 0.05$ is to be considered statistically significant.

## METHODOLOGY

**Sample collection:**

- The coded biopsy tissue in viral transport medium was collected and transported in an ice container to the Clinical Virology laboratory. (Viral transport medium (VTM) is a balanced isotonic solution at physiological pH. It maintains the virus in the viable state. Generally contains foetal calf serum and wide spectrum antibiotic).

- Once received, the sample was transferred to a 1.5ml eppendorf tube and stored at -80$^0$C until further testing.

- The coded sample was processed through the following steps.

**DNA Extraction protocol:**

DNeasy® Tissue kit: (Qiagen GmbH, Hilden, Germany)

Principle: Column based separation.

- Tissue weighing 25mg and is digested by adding ATL buffer and Proteinase K at 56C.
- Once digested, an equal amount of AL (Lysis buffer) buffer and Ethanol is added.
- The DNA gets precipitated and its washed twice by adding buffers (AW1 and AW2)
- The DNA is then eluted by adding elution buffer.

- The extract (containing the DNA) is stored at -20C until further testing.

**Polymerase Chain Reaction (PCR):**

- A known positive control is used for PCR and beta-globin serves as internal control.
- Primers used: a) PGMY 09/11: Target size: 450 basepair.

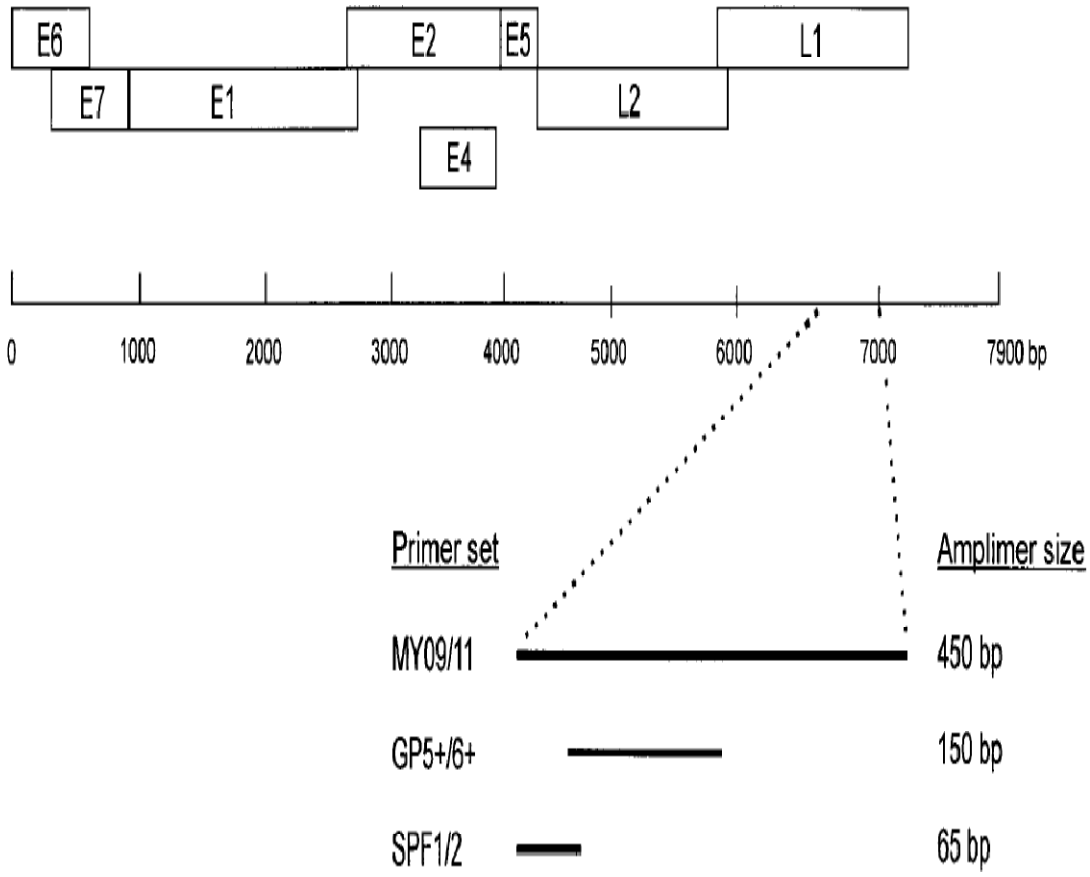
  b) PCO4/GH20 (Beta-globin): 230 basepair
- A sample can be analyzed only if the beta globin is positive.
- Based on the presence or absence of target band (450bp), the sample is interpreted as positive or negative.

The PCR was carried out as a single step non-nested PCR. These universally used primers detect most of the oncogenic HPV types including HPV 16 and 18. Detection of the amplified products was carried out by electrophoresis in an ethidium bromide stained 2.5 % agarose gel. Presence of a 450 base pair HPV DNA specific product and a 230 base pair beta globin specific product indicates a positive result. PGMY09/11 is a set of consensus primers which has higher sensitivity, specificity and reproducibility (Gravitt, P. J. Clin. Microbiol. 38**:**357-361).

The cycling conditions in the PCR are 95$^{o}$C, 10min followed by 40 cycles of 95$^{o}$C, 1min; 55$^{o}$C, 1min and 72$^{o}$C, 10min and a final elongation step of 72$^{o}$C, 10min.

Schematic depiction (figure 8) of different sets of primer utilized for HPV DNA detection studies. Usually the primers targeting L1 gene of HPV viruses are the most often used.

Figure 8 Different sets of primer utilized for HPV DNA detection studies




*(Figure 8 adapted from Kleter B, J Clin Microbiol. 1999 Aug; 37(8):2508-17)*

The thermal cyclers used for PCR are -

a) Veriti TM Thermal Cycler (Applied Biosystem, Foster City, California, USA).

b) GeneAmp® PCR system 9700 (Applied Biosystem, Foster City, California, USA).

**Sequencing of DNA:**

- If sample is positive for HPV, the amplified PCR products will be purified by Millipore filtration and sequenced directly using an ABI Prism Big Dye terminator cycle sequencing ready reaction kit.
- After a post-sequencing clean-up by Millipore filtration, the sequencing reactions will be run on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, CA, USA).
- Finally, the data will be analyzed using Bioedit software version 7.0.5.3 and study sequences compared to the GenBank HPV sequence.

# IMAGE SERIES TO DEPICT THE METHOD OF PROCESSING AND POLYMERASE CHAIN REACTION DONE FOR THE STUDY SAMPLES

## LEGENDS

IMAGE 1 Viral transport media or VTM: It is a balanced isotonic solution at physiological pH. It maintains the virus in the viable state. Generally contains fetal calf serum and antibiotics.

IMAGE 2 Biosafety Cabinet (BSL-B2).

IMAGE 3 Sample transfer to a 1.5ml eppendorf tube.

IMAGE 4 Storage of sample at -80 degrees.

IMAGE 5 Extraction of samples

IMAGE 6 PCR process: Veriti TM Thermal Cycler (Applied Biosystem, Foster City,California, USA).

IMAGE 7 GeneAmp® PCR system 9700 (Applied Biosystem, Foster City, California, USA). Thermal cycler used for PCR.

IMAGE 8 Gel- Doc : apparatus used for the gel electrophoresis of the amplified sample of the PCR process.

IMAGE 9 Sequencer (Applied Biosystem 310 Genetic Analyzer).

Electropherogram picture:

The below picture is just an example of how the readout is from the sequencing machine.

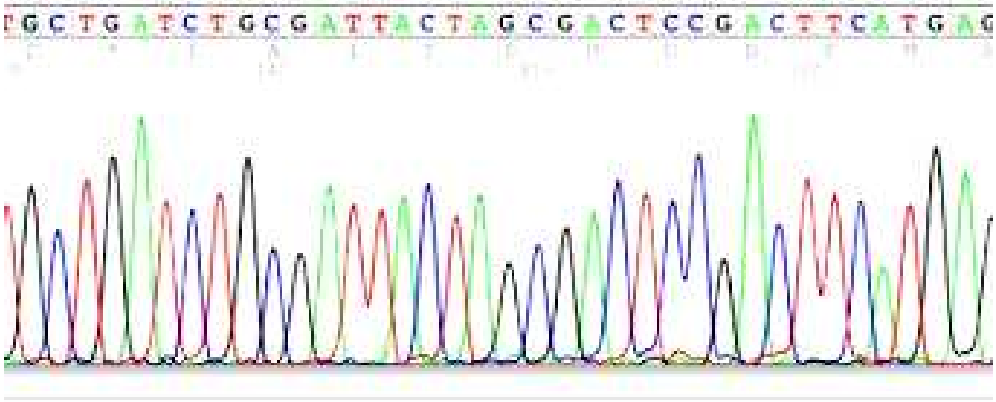
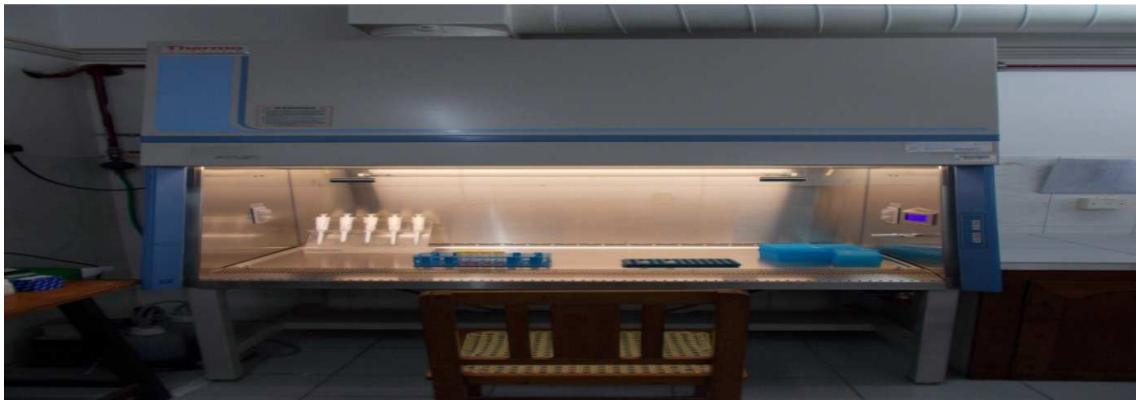
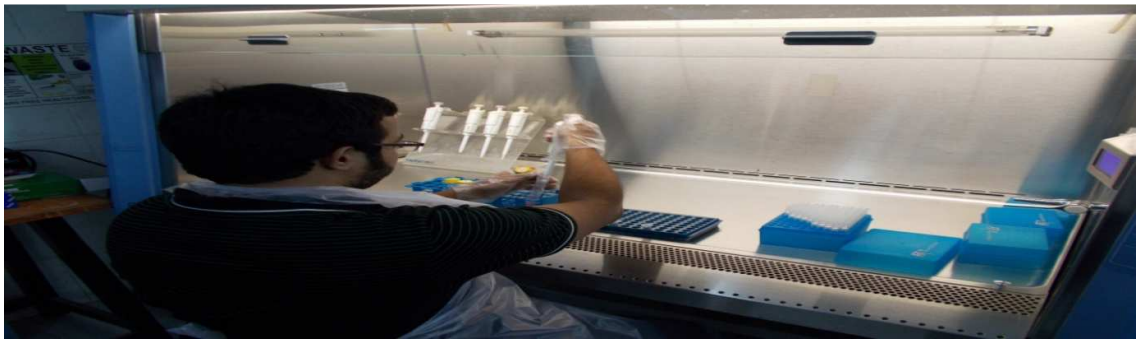


IMAGE 10 Gel Electrophoresis



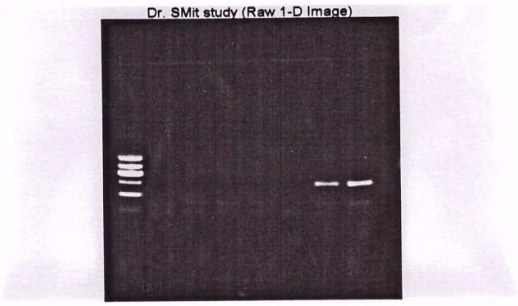



Applied Biosystems
Veriti


GeneAmp
PCR System 9700


310 Genetic Analyzer

## THE ORIGINAL DIGITAL PHOTO OF THE GEL ELECTROPHORESIS OF PRODUCTS OF THE POLYMERASE CHAIN REACTION (PCR) OF THE STUDY SAMPLE




LANE 1 MOLECULAR LADDER CONTAINING BASE PAIR FROM 100 - 1200

LANE 2 STUDY SAMPLE

LANE 3 STUDY SAMPLE

LANE 4 STUDY SAMPLE

LANE 5 STUDY SAMPLE

LANE 6 STUDY SAMPLE

LANE 7 EXTRACTION CONTROL

LANE 8 PCR POSITIVE CONTROL

# RESULTS

The total number of case and control participants at the end of study was as follows.

The total number of case participants with histologically proven squamous cell carcinoma of the oesophagus, who enrolled for the study, was 45. (GROUP A)

The total number of control participants who enrolled for the study was 38. ( GROUP B).

Thus total number of participants who were evaluated was 83.

Figure 9. Distribution of case (Group A) and control (GROUP B) patients in this study.

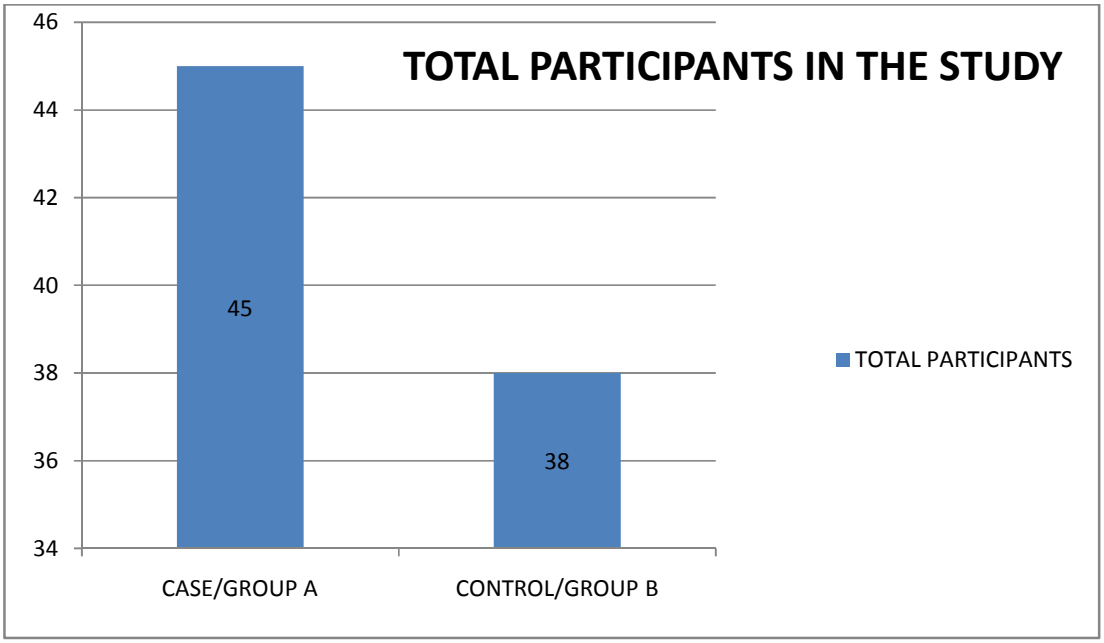
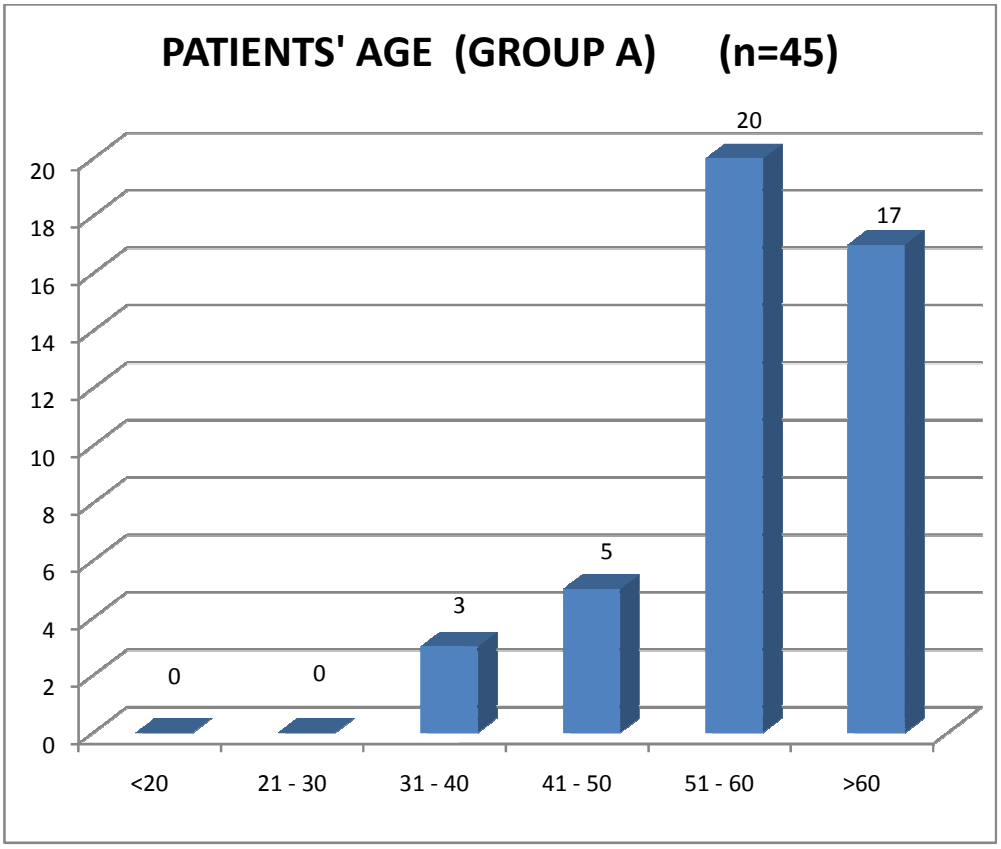
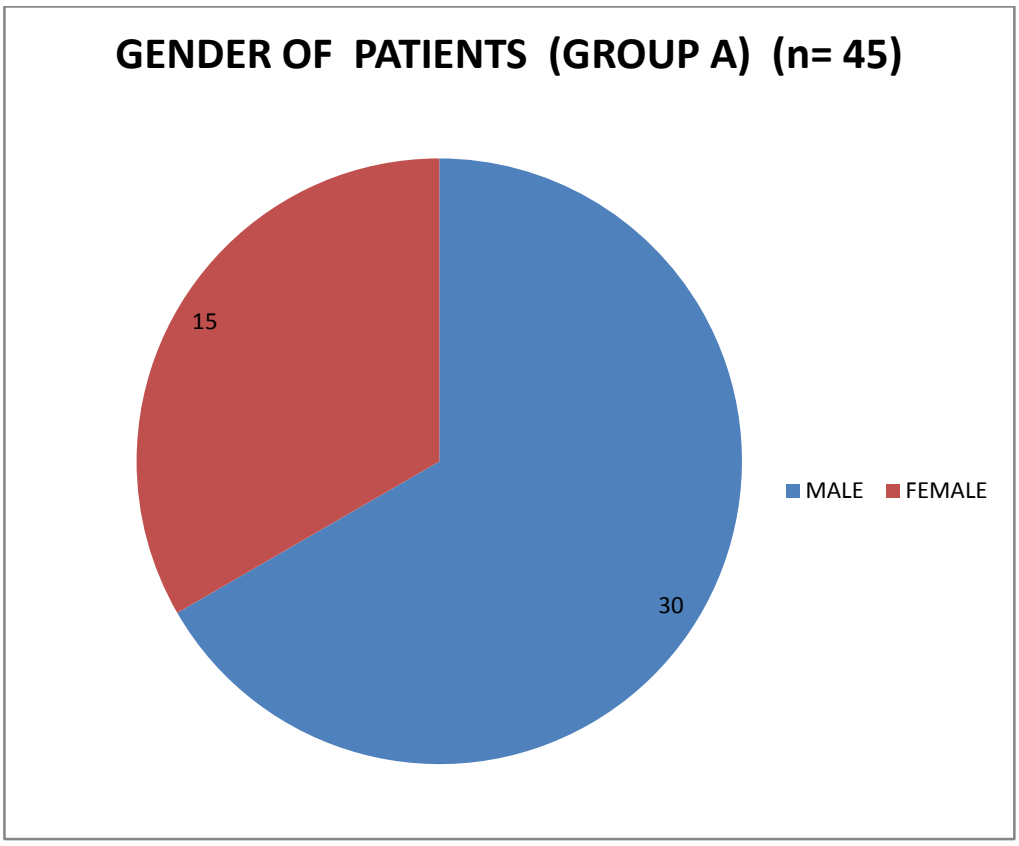


Figure 10: Depicts the distribution of age group of Case /Group A




Most of case participants who had squamous cell carcinoma oesophagus enrolled in our study were more than 50 years of age. This group comprises the most bulk of our study participants with total number of 37. This depicts that carcinoma of oesophagus which we see in our patients at our centre mostly occurs at age after 50 years.

Figure 11. Gender distribution of Case participants / Group A who had squamous cell carcinoma of oesophagus

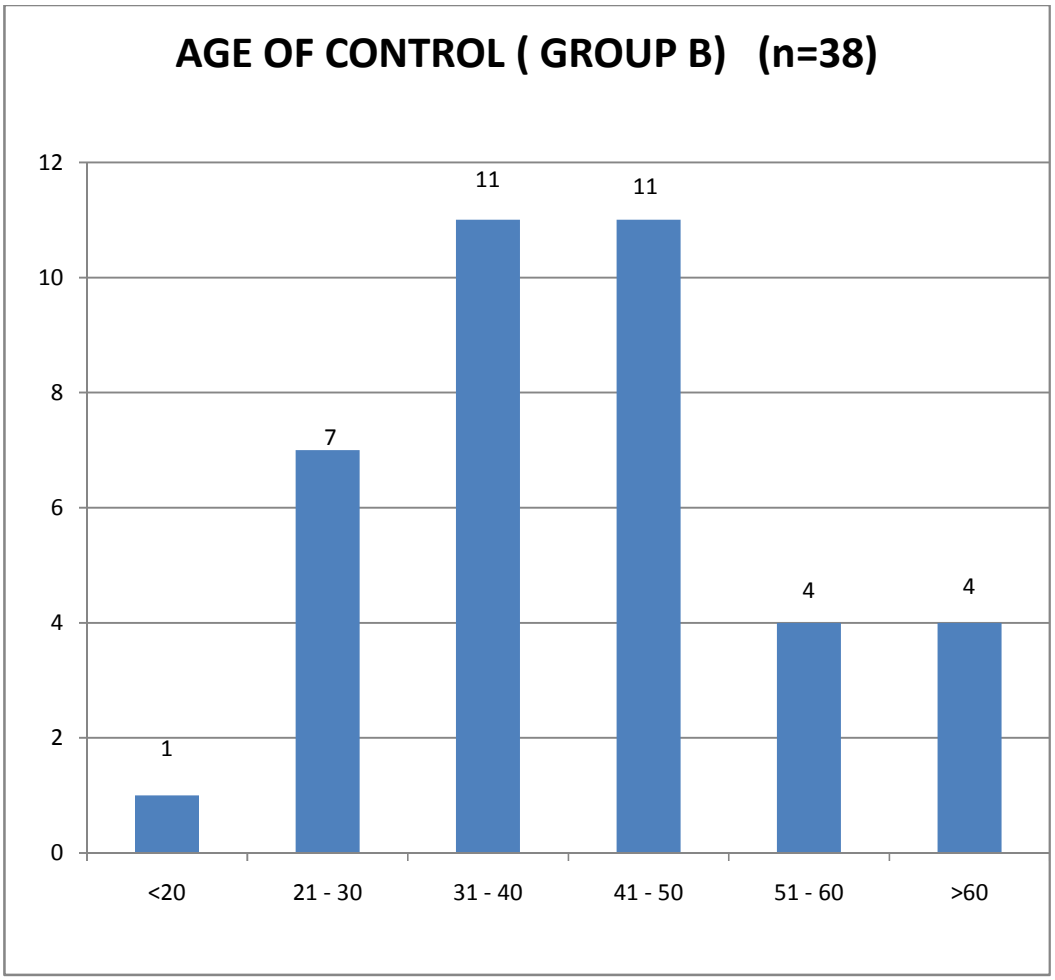



| | |
|---|---|
| MALE | 66.7 % |
| FEMALE | 33.3 % |

The gender profile of our study shows that the squamous cell carcinoma in our participants is present more in male gender i.e. twice as more common .

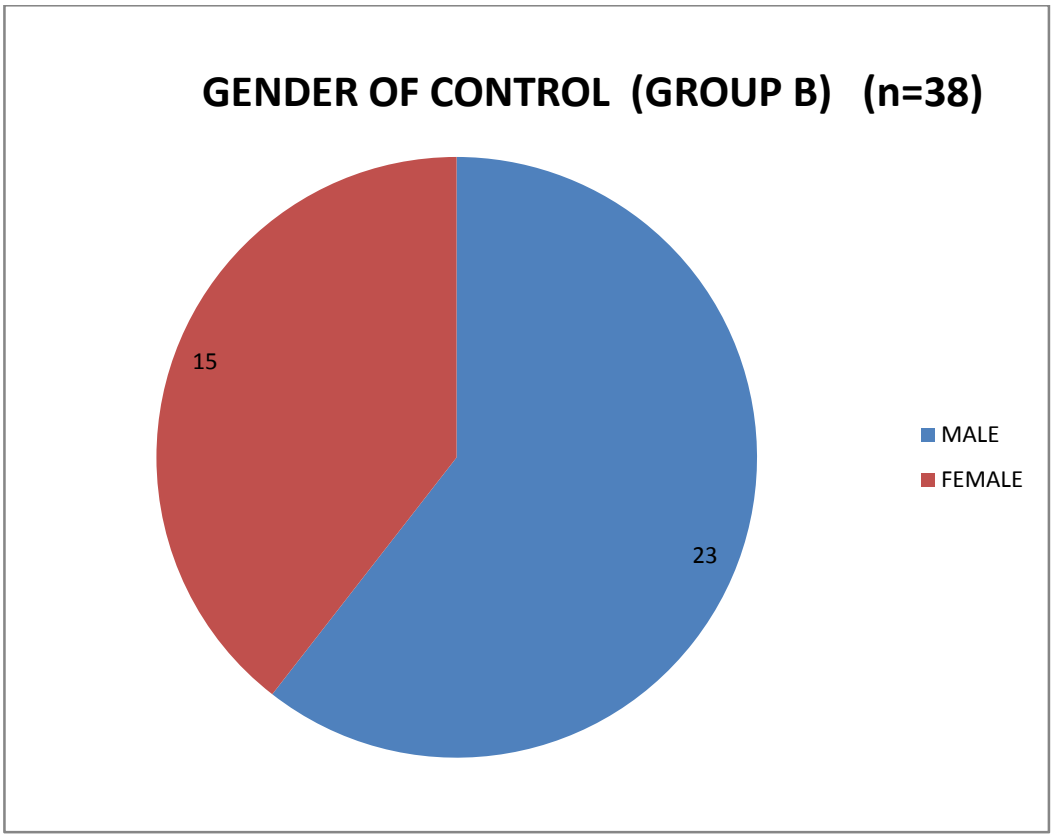
The similar findings are also found in literature which supports the male predominance pattern seen across the world.

Figure 12: Depicts the age distribution for control / group B




The control /group B consist most of the individual in the age group of 21-50 years of age. The control group had patient endoscopy done for dyspeptic symptoms and did not suffer from carcinoma of oesophagus.

Figure 13. Depicts the Gender distribution of GROUP B / Control participants



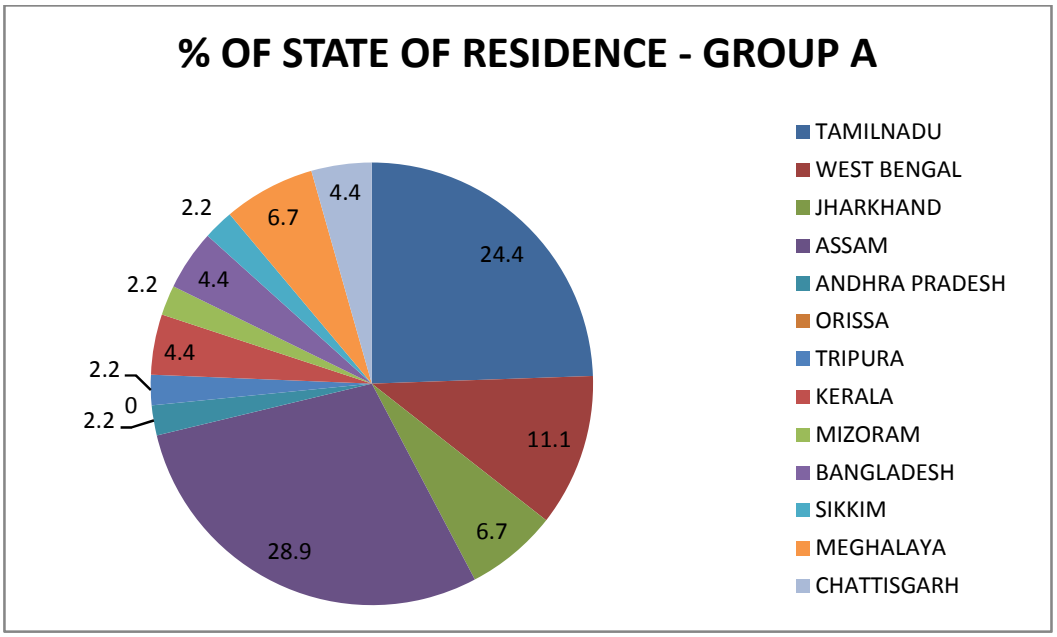

| | |
|---|---|
| MALE | 60.5 % |
| FEMALE | 39.5 % |

The control participants who have enrolled for our study had male predominance with more than half the number comprising of male individual.

Table 3. The distribution table of various state our case participants / Group A belong who come to our centre for treatment.

| STATE | TOTAL NUMBER |
|---|---|
| TAMILNADU | 11 |
| WEST BENGAL | 5 |
| JHARKHAND | 3 |
| ASSAM | 13 |
| ANDHRA PRADESH | 1 |
| ORISSA | 0 |
| TRIPURA | 1 |
| KERALA | 2 |
| MIZORAM | 1 |
| BANGLADESH | 2 |
| SIKKIM | 1 |
| MEGHALAYA | 3 |
| CHATTISGARH | 2 |
| Total | 45 |

Figure 14 Depicting percentage State of residence

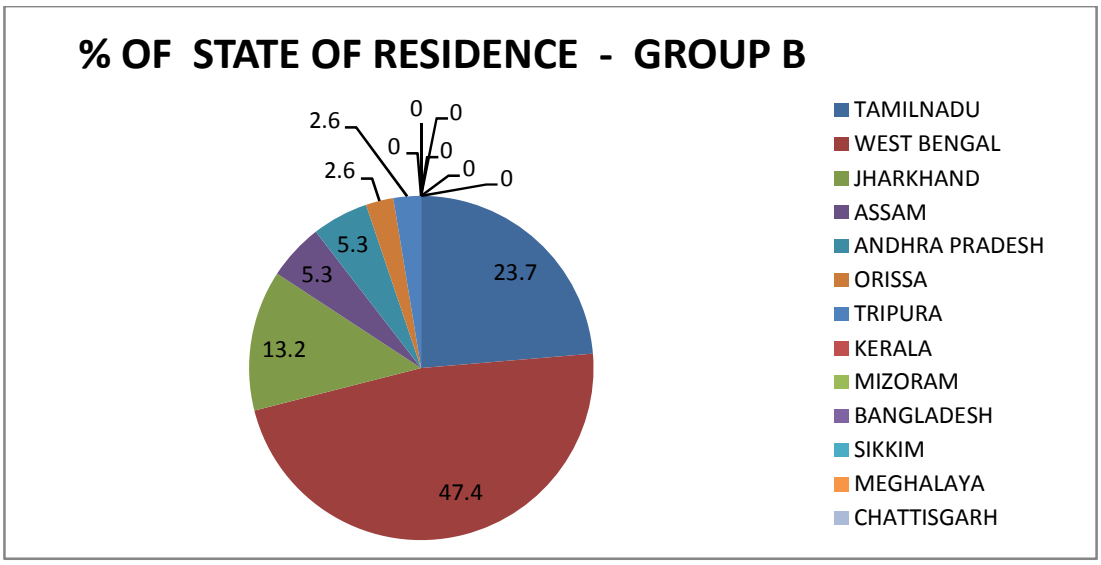



The majority of participants enrolled were from the North east part of India predominantly the state of Assam. The second most common state of origin was Tamil Nadu, because of the geographical location of our centre.

Table 4. State wise distribution of control / Group B population.

| STATE | TOTAL NUMBER OF PATIENT IN CONTROL / GROUP B |
|---|---|
| TAMILNADU | 9 |
| WEST BENGAL | 18 |
| JHARKHAND | 5 |
| ASSAM | 2 |
| ANDHRA PRADESH | 2 |
| ORISSA | 1 |
| TRIPURA | 1 |
| KERALA | 0 |
| MIZORAM | 0 |
| BANGLADESH | 0 |
| SIKKIM | 0 |
| MEGHALAYA | 0 |
| CHATTISGARH | 0 |

Figure 15 Depicting percentage of state of residence.




Most of the patient in control / Group B were from West Bengal.

Figure 16. Educational status of the case / Group A participants who have carcinoma oesophagus.

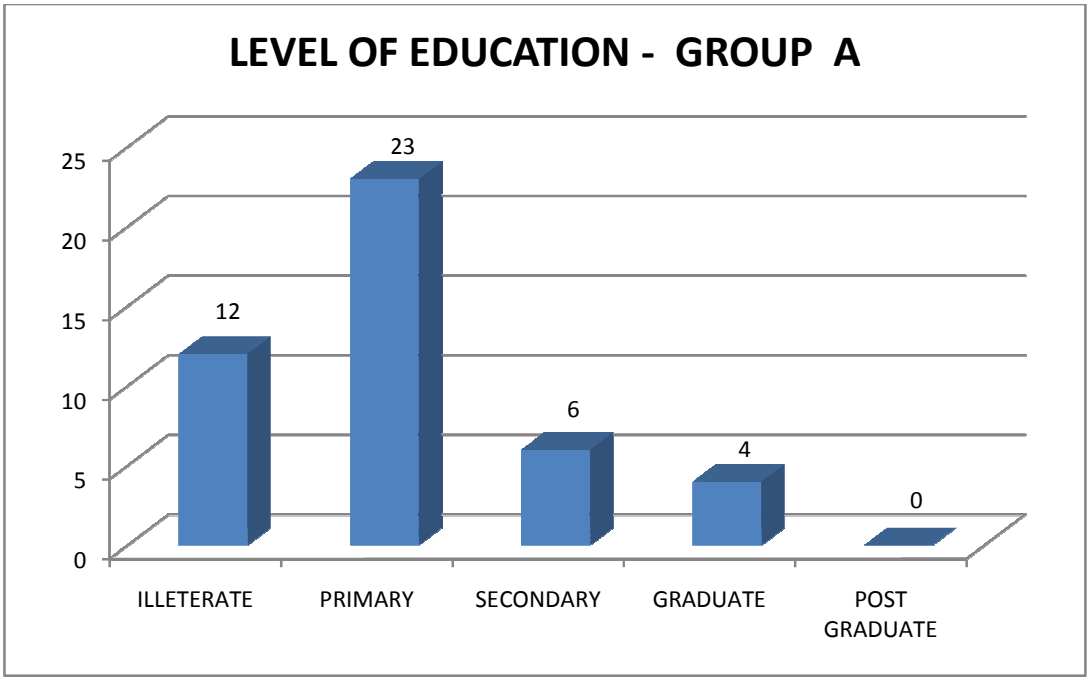



Table 5 **EDUCATIONAL STATUS OF CASES / GROUP A**

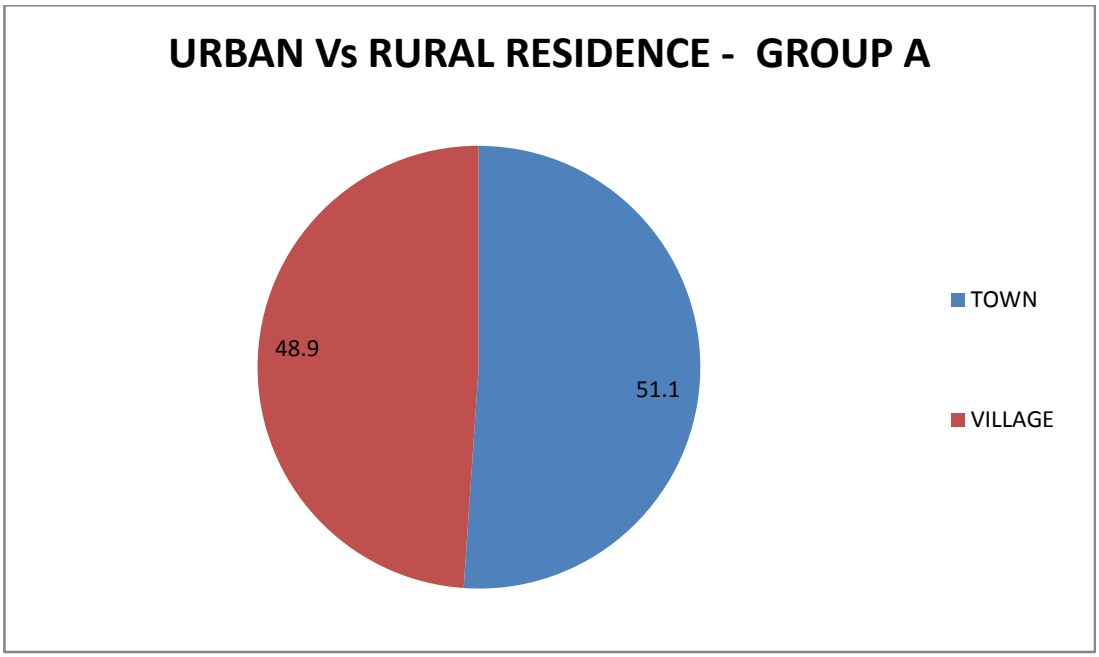
| | % OF CASE / GROUP A |
|---|---|
| ILLETERATE | 26.7 |
| PRIMARY | 51.1 |
| SECONDARY | 13.3 |
| GRADUATE | 8.9 |
| POST GRADUATE | 0 |

Most of the participants who have carcinoma of oesophagus have education level of primary class. The distribution depicts the pattern seen globally as suggested by literature

Table 6. Depicts the place of residence of case participants/ Group A in study

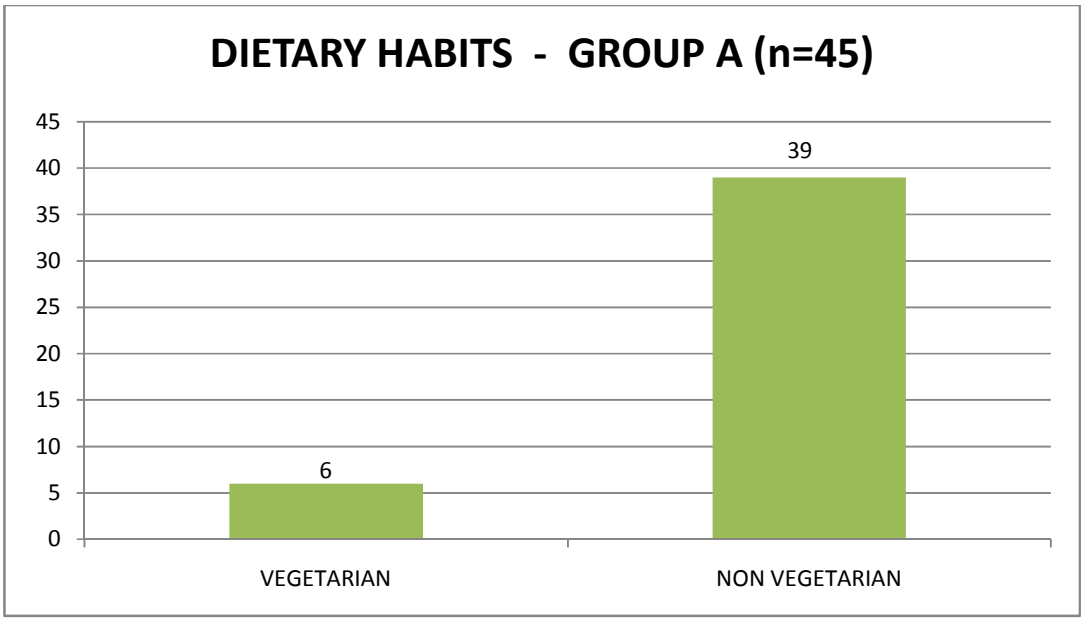
| RESIDENTIAL AREA | NUMBER OF CASE / GROUP A |
|---|---|
| TOWN / URBAN | 23 |
| VILLAGE / RURAL | 22 |

Figure 17 Depicting place of residence of Group A




The case group participants were equally from both town and village. We did not found difference of residence in our study.

Figure 18. Depicts the dietary habit of case participants / Group A




Most participants with SCC of oesophagus had non vegetarian diet status.

Only about 13.3 % of case participants had vegetarian diet.

Table 7. Depicts the smoking habit of Case participants / Group A

| | % OF CASE / GROUP A |
|---|---|
| NON SMOKER | 48.9 |
| SMOKER | 51.1 |

Figure 19 Depicting smoking habits of Group A

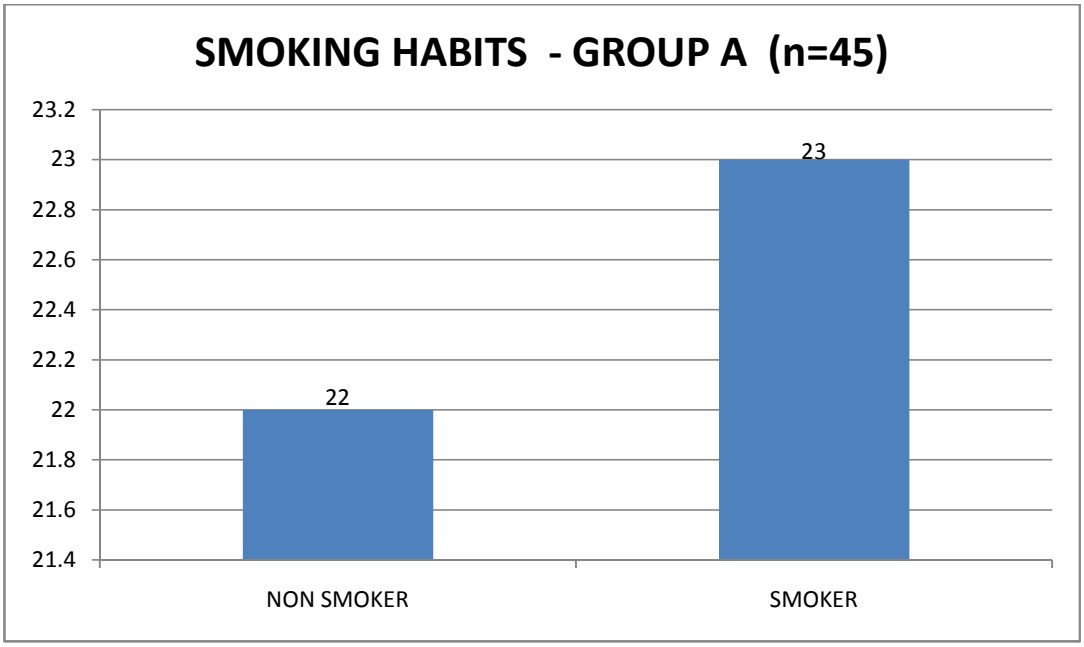
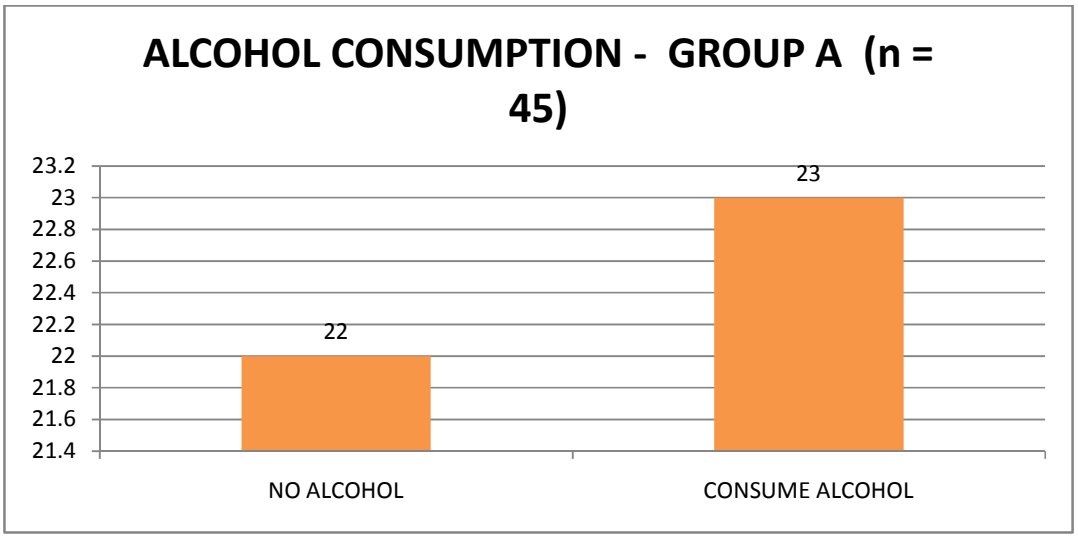



Table 8 Depicting pack years of smokers in Group A

| NUMBER OF PACK YEARS | NUMBER OF SMOKERS IN CASE /GROUP A (n = 23) |
|---|---|
| < 10 | **8** |
| > 10 | 15 |

There was no significant difference in smoking habit in patient who had squamous cell carcinoma of oesophagus. There was presence of similar proportion of smokers and non smoker in the cases as depicted above. Majority of smokers had smoked >10 pack years.

Figure 20. Alcohol consumption pattern of Case participants/ Group A




The proportion of people who consumed alcohol in case / group A was 51.1 %.

Figure 21. Regular consuming hot beverage in Case participants

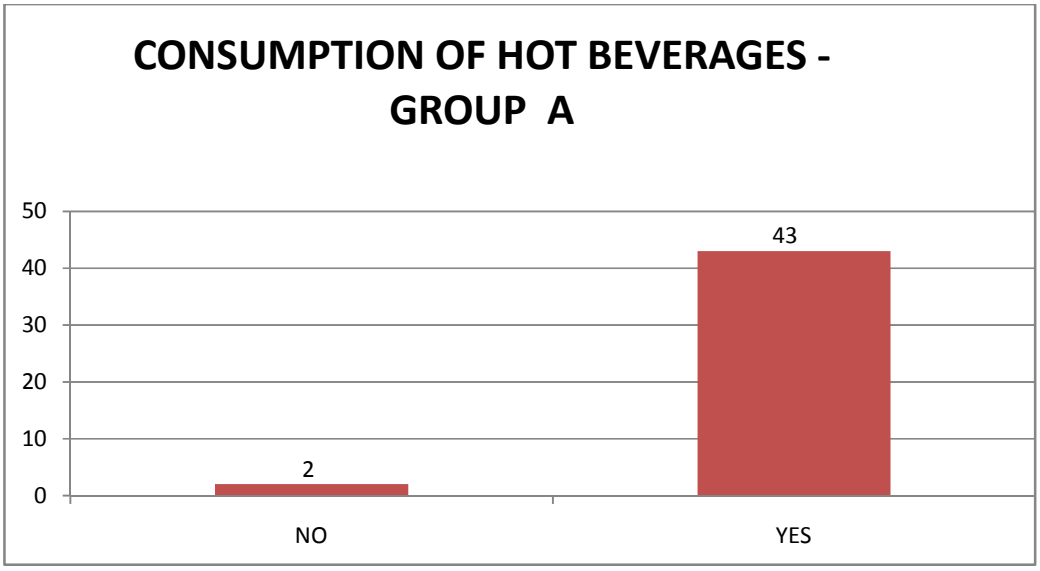
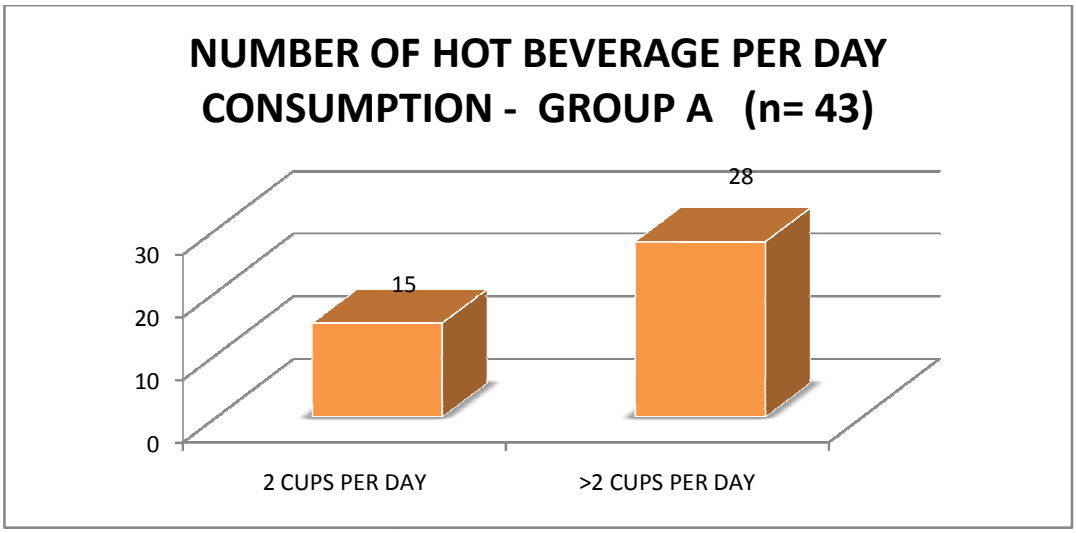



Table 9 Table showing percentage of patient consuming hot beverages in Group A

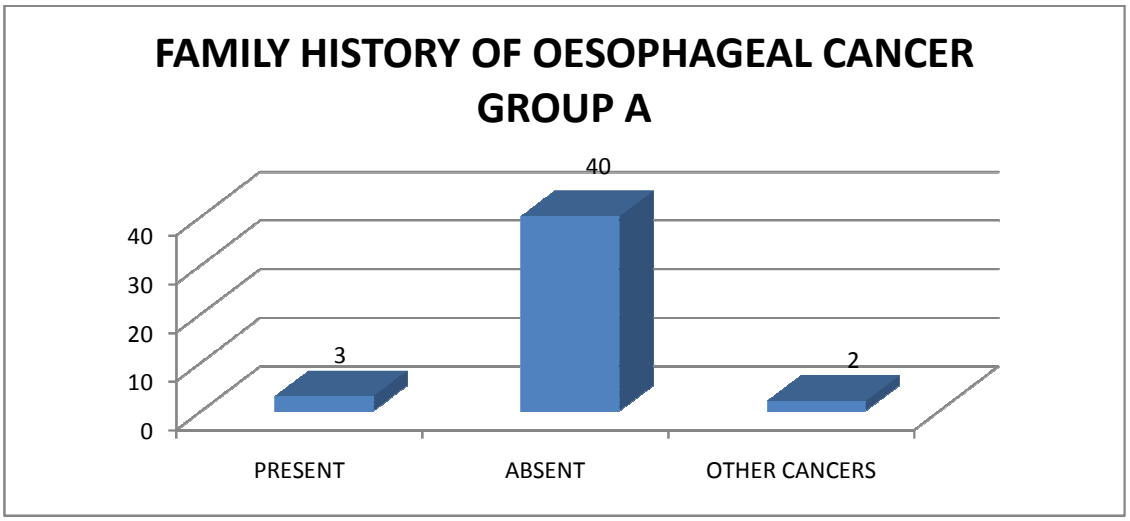
| CONSUMPTION OF HOT BEVERAGES | % OF CASE / GROUP A |
|---|---|
| NO | 4.4 |
| YES | 95.6 |

Figure 22: Depicts number of cups of coffee/tea consumed by Case participants who regularly take such hot beverages.




In case (Group A) who consume hot beverages majority of patient drink more than 2 cups per day.

Figure 23. Depicts the distribution of positive family history in Case/ Group A patients with oesophageal cancer.




The presence of positive family history of carcinoma of oesophagus was seen in 6.7% in case / Group A.

Majority of Case i.e. 88.9% did not have family history of oesophageal carcinoma.

Figure 24. Depicts the predominant symptoms of presentation of case participants with oesophageal carcinoma.

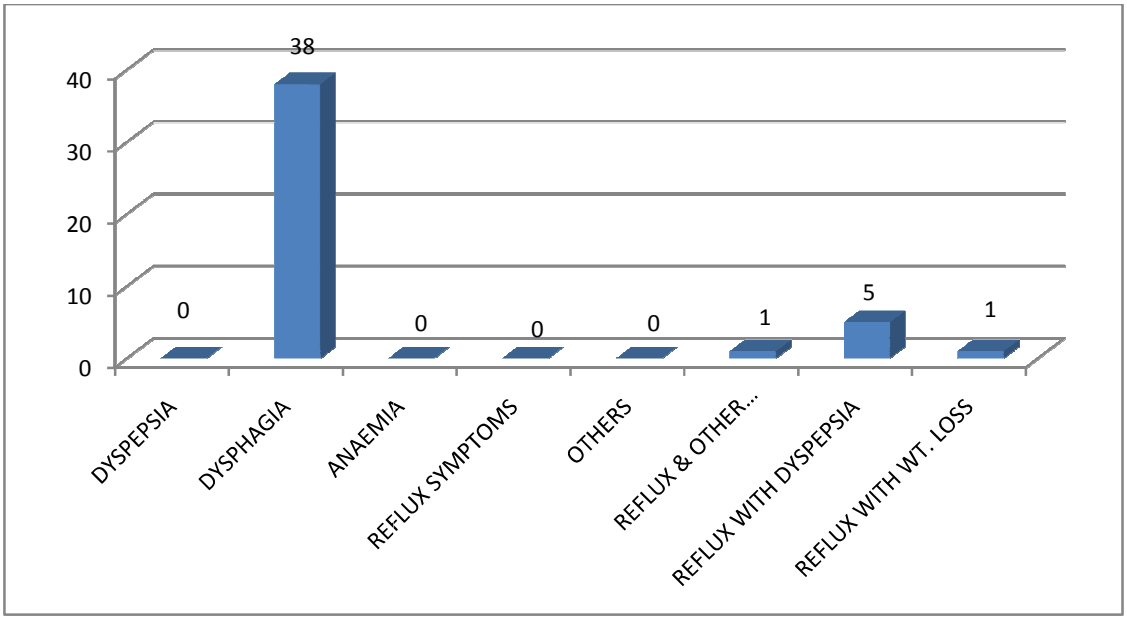



Table 10 showing predominant symptoms of patients in Group A

| PREDOMINANT SYMPTOM AT PRESENTATION | % OF CASE / GROUP A |
|---|---|
| DYSPHAGIA | 84.4 |
| REFUX WITH DYSPEPSIA | 11.1 |
| REFLUX WITH WEIGHT LOSS | 2.2 |
| RELUX WITH OTHER SYMPTOMS | 2.2 |

Majority of our patients in case / Group A who had carcinoma of oesophagus

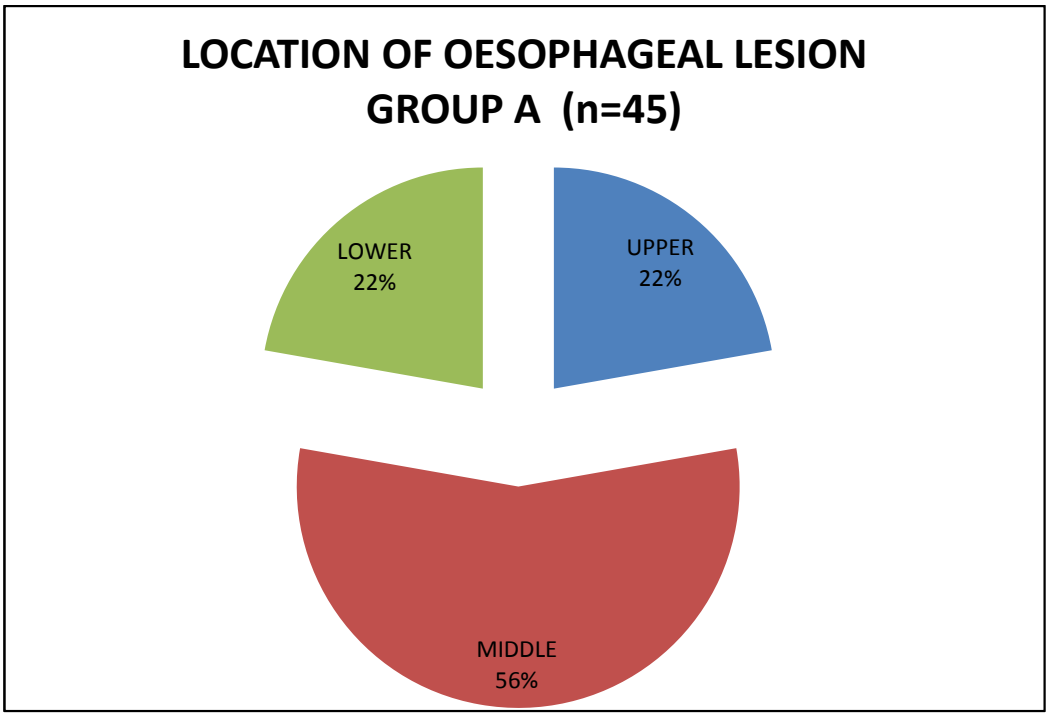
presented with Dysphagia which was progressive in nature.

Dysphagia was seen in 84.4% of cases.

Table 11. Depicts the location of oesophageal lesion in participants with oesophageal carcinoma.

| LOCATION OF OESOPHAGEAL LESION | FREQUENCY (n) |
|---|---|
| UPPER | 10 |
| MIDDLE | 25 |
| LOWER | 10 |

Figure 25 depicting location of oesophageal lesion in Group A



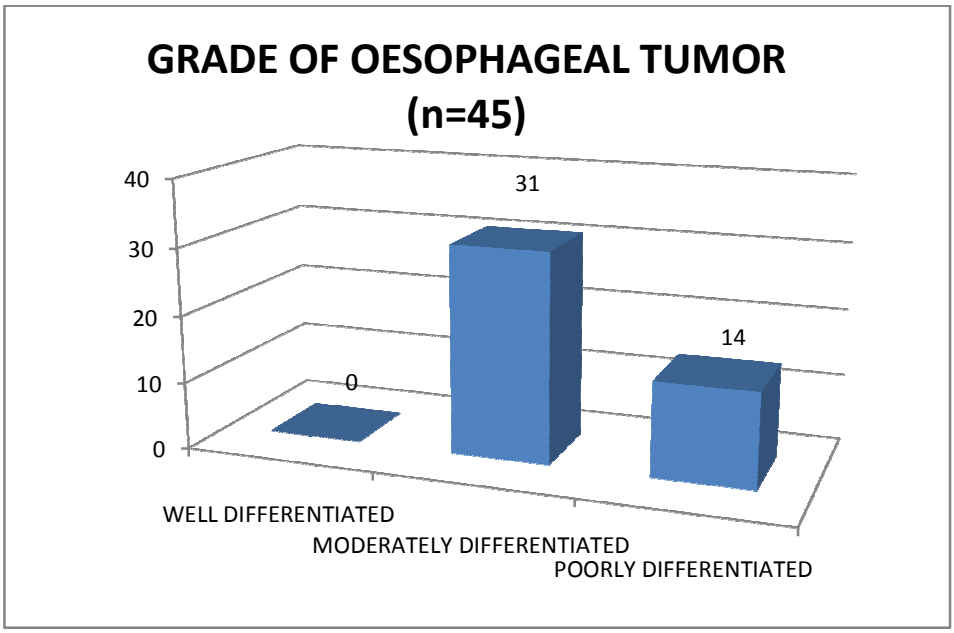

Majority of case participants (i.e. 56%) who had carcinoma of oesophagus had lesion at the middle third of oesophagus.

There was equal distribution in upper and lower part of the oesophagus.

Table 12 Depicts the distribution of the grade of oesophageal tumour lesion in case / Group A

| GRADE OF TUMOUR LESION | % OF CASES / GROUP A |
|---|---|
| WELL DIFFERENTIATED | 0 |
| MODERATELY DIFFERENTIATED | 68.9 |
| POORLY DIFFERENTIATED | 31.1 |

Figure 26 Depicting grade of osephageal tumour

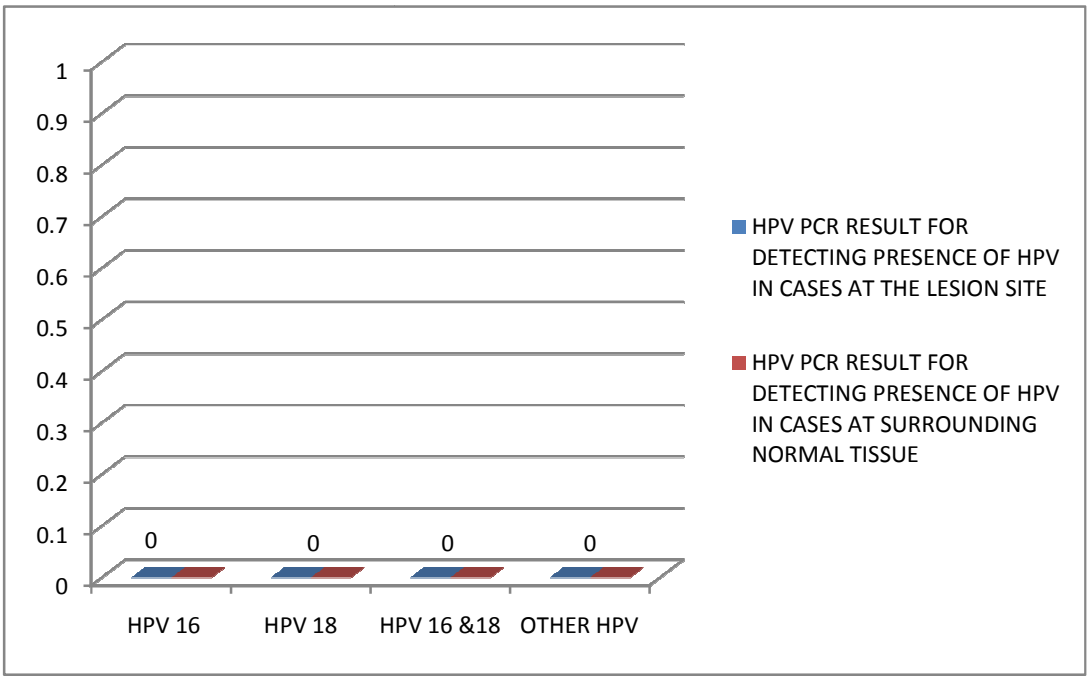



The majority of case / Group A (i.e. 68.9 % of patient) with squamous cell carcinoma of oesophagus had moderately differentiated grade of tumour.

Table 13. Presence of HPV in case participants with SCC of oesophagus

| | |
|---|---|
| Detection of any high risk HPV virus from the tumour lesion in cases | NEGATIVE |
| Detection of high risk HPV type 16 and 18 virus from the tumour lesion | NEGATIVE |
| Detection of any high risk HPV virus from the adjacent normal tissue (CASES) | NEGATIVE |
| Detection of high risk HPV type 16 and 18 virus from the adjacent (CASES | NEGATIVE |
| Detection of any high risk HPV virus from the Control participants | NEGATIVE |
| Detection of high risk HPV type 16 and 18 virus from Controls | NEGATIVE |

Figure 27 Presence of HPV in case participants with SCC of oesophagus



The detection study PCR did not detect any evidence of HPV 16 or HPV 18 in our case participants with histologically proven squamous cell carcinoma of the oesophagus. There was no presence of virus in the tumour tissue and also in the surrounding normal tissue near the lesion.

The PCR also failed to detect any other type of HPV virus present in the tumour tissue and also in the surrounding normal tissue near the lesion.

The PCR also did not show any presence of HPV 16, HPV 18 or any other serotype of HPV in control participants with normal oesophagus.

# DISCUSSION

The first reports suggesting the involvement of human papillomavirus (HPV) in the development of both benign and malignant squamous cell tumours of the oesophagus was in 1982 by Syrjanen *et al*. Since then there are debatable evidences for association of HPV in oesophageal cancer and the role is controversial. The detection rate of HPV in different studies is variable from 0% to 60-70%.

The squamous cell carcinogenesis of the oesophagus is complex multistep process and it is hypothesised to have multifactor aetiology and no single agent is identified to cause malignancy. In the past two decades HPV has gained the interest to be the most likely infectious viral agent leading to oesophageal cancer.

Though till yet the etiological role of HPV in oesophageal cancer is not established and is controversial. It is suggested that infection with HPV may act as co-factor or can synergise the effect of other carcinogens thus leading to carcinogenesis. Therefore it influences the progression rather than the initiation of the disease.

A large Meta analysis of studies done by *K.J Syrjänen* (14) from 152 studies from 1954 – 2012 which included a total of 10,234 squamous oesophageal cancer patients, analysed by different HPV detection methods in different geographic regions.

The results of this analysis conclude that HPV infection plays an important role in the pathogenesis of squamous cell carcinoma of the oesophagus only in high prevalence areas. Therefore HPV infection may not have an important role in the pathogenesis of the malignancy in India, since our country is an area of low prevalence of the malignancy. These findings were in concurrence to an Indian study by *S.Katiyar et.al.* (5), which concluded that HPV may not play important role for oesophageal cancer in India.

In accordance with the evidence in the literature, our study did not show association between the HPV and squamous cell carcinoma of oesophagus.

However a study done in Kashmir by Showket Hussain et al. has found significant proportion of squamous cell carcinoma of oesophagus with HPV infection. (80) The study had 19% HPV positive oesophageal cancer.

It is of interest to note that the valley of Kashmir is a high prevalence area in India and in our study group we did not have patients from this geographical area.

Apart from this, the HPV negativity in this study could be explained by the following reasons.

(1) Low HPV copies present in the tumour lesion in squamous cell carcinoma of oesophagus. The HPV infection of the oesophagus differs from the infection as seen in the cervix.

It is shown in a study by real time PCR that HPV copies in carcinoma oesophagus specimen is less than two orders of magnitude as that found in cervical cancers.

Based on this observation it was tried that a PCR proven series of HPV 16 positive cervical cancer specimens were diluted to 0.1-0.01HPV /cell similar to the average number of copy seen in oesophageal cancers. (78) Then sample were tested under PCR and it was found to be negative for HPV serotype. Thus it was suggested that low HPV copy can cause low detection of high risk HPV in oesophageal cancer.

(2) "Hit and Run" mechanism of carcinogenesis by HPV in oesophagus could be also responsible for absence of any HPV serotype in cancer specimens as suggested by Tone Bjorge et al. (79). They suggested that unlike the cervical carcinoma where there is continued expression of transforming protein of virus E6 and E7 for the growth of tumour in case of oesophageal cancer there is loss of viral DNA as the disease advances. This mechanism is evident in HPV associated Bovine lesions in cattles. (74)

By analogy BPV-4 causes alimentary papillomatosis in cattle which is usually a self limiting disease. But if cattle in fed upon Bracken fern which contains quercetin which is an immunosuppressant and a mutagen it is unable to clear BPV-

Thus the viral protein in presence of the mutagenic agent leads to tumour progression in cattles. The virus though is not found in the tumour lesions.

In our study we found that there was no HPV DNA detected in the adjacent normal tissue around the lesion. This therefore excludes the possibility of chronic presence or colonization of HPV leading to malignancy.

In our study there was also absence of HPV serotypes in the control or Group B with normal oesophagus and also the adjacent normal mucosa from the patient with carcinoma oesophagus. Though we do not have data for the prevalence of HPV in oesophagus from general population and it will requires a larger study to evaluate this.

# CONCLUSIONS

1. This study did not find any high risk HPV serotypes in patients with squamous cell carcinoma of the oesophagus. This is in concurrence with the evidence existing in the literature regarding HPV in low prevalence areas.

2. There exists geographical variation in the incidence of SCC oesophagus in India. However, in this study, HPV infection was not seen in patients from the different geographical areas within India,

3. The study also suggests the absence of high risk serotype HPV in the oesophageal mucosa of the control group as well.

4. There is a need of a larger multicentre study to substantiate the results of this study and to confirm or confidently rule out the association between HPV and oesophageal carcinoma in the Indian population.

# LIMITATIONS

1. The study has a possible selection bias with regards to the geographical area represented by the patients and control groups. For example although Kashmir is a high incidence area in India, this geographical area was not represented in the study.

2. The patient and control groups were not age matched and also differed with regards to the geographical area of origin.

3. The control group included patients with dyspeptic symptoms needing endoscopy. This may be a confounding factor as this group may not necessarily represent the general population.

4. Although the study met the sample size requirement, a larger study is needed to evaluate any significant association.

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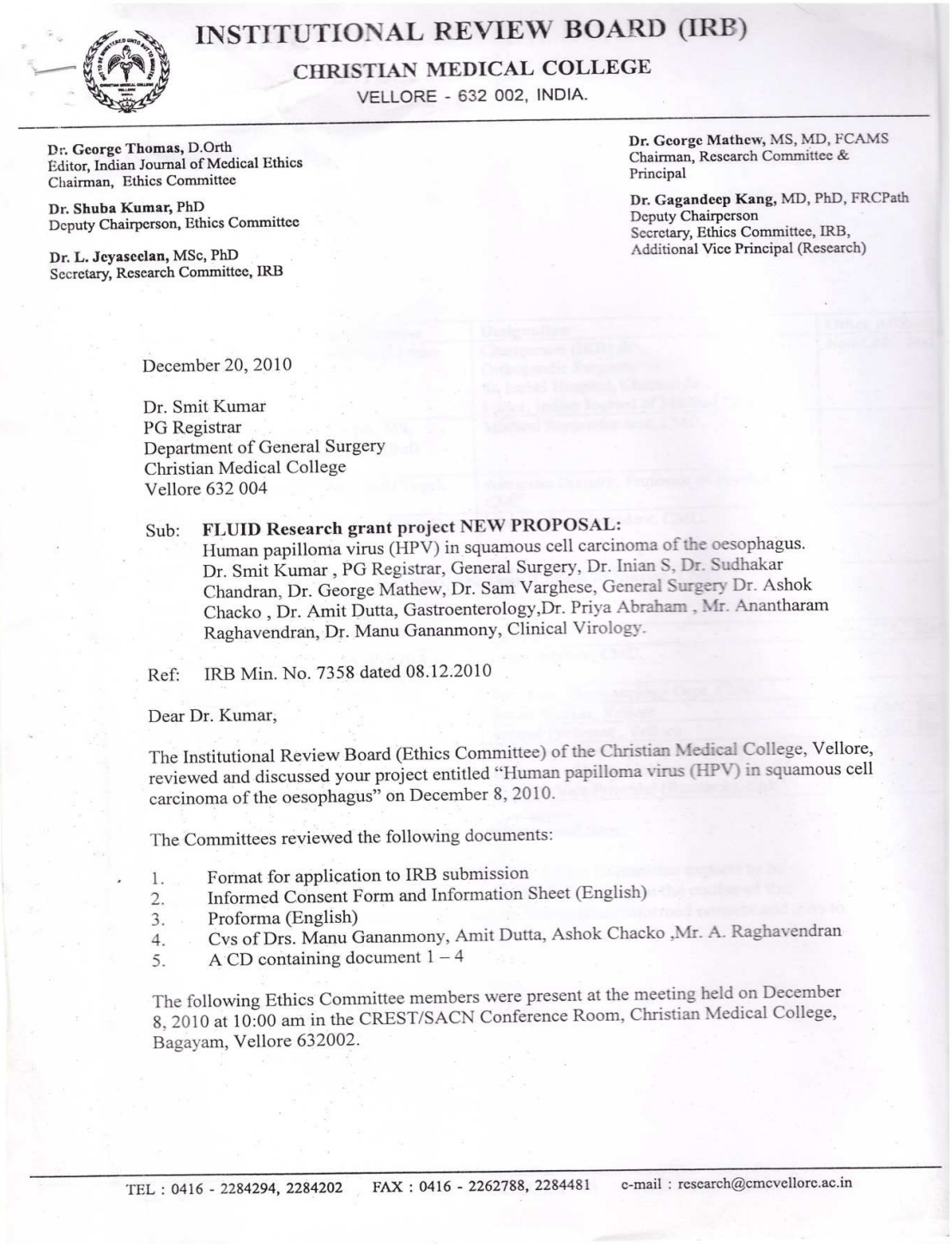
# ANNEXURES



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Secretary, Research Committee, IRB

**Dr. George Mathew,** MS, MD, FCAMS
Chairman, Research Committee &
Principal

**Dr. Gagandeep Kang,** MD, PhD, FRCPath
Deputy Chairperson
Secretary, Ethics Committee, IRB,
Additional Vice Principal (Research)

December 20, 2010

Dr. Smit Kumar
PG Registrar
Department of General Surgery
Christian Medical College
Vellore 632 004

Sub: **FLUID Research grant project NEW PROPOSAL:**
Human papilloma virus (HPV) in squamous cell carcinoma of the oesophagus.
Dr. Smit Kumar , PG Registrar, General Surgery, Dr. Inian S, Dr. Sudhakar Chandran, Dr. George Mathew, Dr. Sam Varghese, General Surgery Dr. Ashok Chacko , Dr. Amit Dutta, Gastroenterology,Dr. Priya Abraham , Mr. Anantharam Raghavendran, Dr. Manu Gananmony, Clinical Virology.

Ref: IRB Min. No. 7358 dated 08.12.2010

Dear Dr. Kumar,

The Institutional Review Board (Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Human papilloma virus (HPV) in squamous cell carcinoma of the oesophagus" on December 8, 2010.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Informed Consent Form and Information Sheet (English)
3. Proforma (English)
4. Cvs of Drs. Manu Gananmony, Amit Dutta, Ashok Chacko ,Mr. A. Raghavendran
5. A CD containing document 1 – 4

The following Ethics Committee members were present at the meeting held on December 8, 2010 at 10:00 am in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 e-mail : research@cmcvellore.ac.in

# INSTITUTIONAL REVIEW BOARD (IRB)

## CHRISTIAN MEDICAL COLLEGE

VELLORE - 632 002, INDIA.



| Name | Qualification | Designation | Other Affiliati |
|---|---|---|---|
| Dr. George Thomas | MBBS, D.Ortho | Chairperson (IRB) &<br>Orthopaedic Surgeon,<br>St. Isabel Hospital, Chennai &<br>Editor, Indian Journal of Medical Ethics | Non-CMC Staf |
| Dr. Prabhakar D Moses (on behalf of Dr. Lionel Gnanaraj) | MBBS, MS,<br>M.Ch. (Urol) | Medical Superintendent, CMC. | |
| Dr. Prathap Tharyan | MD, MRCPsych. | Associate Director, Professor of Psychiatry, CMC | |
| Mrs. Mary Johnson (on behalf of Mrs. Sundari Edwin) | M.Sc. (Nursing) | Nursing Superintendent, CMC. | |
| Mrs. Shirley David (on behalf of Mrs. Rosaline Jayakaran) | M.Sc. (Nursing), RN RM | Dean, College of Nursing, CMC. | |
| Mr. Harikrishnan | BL. | Lawyer | Non-CMC Staf |
| Mr. Samuel Abraham | MA, PGDBA,<br>PGDPM, M.Phil, BL | Legal Advisor, CMC. | |
| Dr. Sujith Chandy | MBBS, MD | Professor, Pharmacology Dept. CMC. | |
| Mrs. S. Pattabiraman | BSc, DSSA | Social Worker, Vellore | Non-CMC Staf |
| Dr. P. Zachariah | MBBS, PhD | Retired Professor , Vellore | Non-CMC Staf |
| Dr. Gagandeep Kang | MD, PhD, FRCPath. | Secretary IRB (EC)& Dy. Chairperson (IRB),<br>Professor of Microbiology<br>& Addl. Vice Principal (Research), CMC. | |

We approve the project to be conducted in its presented form.

The Institutional Ethics Committee / Independent Ethics Committee expects to be informed about the progress of the project, any SAE occurring in the course of the project, any changes in the protocol and patient information/informed consent and asks to be provided a copy of the final report.

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 e-mail : research@cmcvellore.ac.in

# INSTITUTIONAL REVIEW BOARD (IRB)

## CHRISTIAN MEDICAL COLLEGE

VELLORE - 632 002, INDIA.

**Dr. George Thomas,** D.Orth
Editor, Indian Journal of Medical Ethics
Chairman, Ethics Committee

**Dr. Shuba Kumar,** PhD
Deputy Chairperson, Ethics Committee

**Dr. L. Jeyaseelan,** MSc, PhD
Secretary, Research Committee, IRB

**Dr. George Mathew,** MS, MD, FCAMS
Chairman, Research Committee &
Principal

**Dr. Gagandeep Kang,** MD, PhD, FRCPath
Deputy Chairperson
Secretary, Ethics Committee, IRB,
Additional Vice Principal (Research)


A sum of Rs. 80,000/- (Rupees Eighty thousand only) is sanctioned for two year out of which a maximum of Rs. 1,500/- can be spent for stationery, printing, Xeroxing and computer charges (if computers used are within the institution).

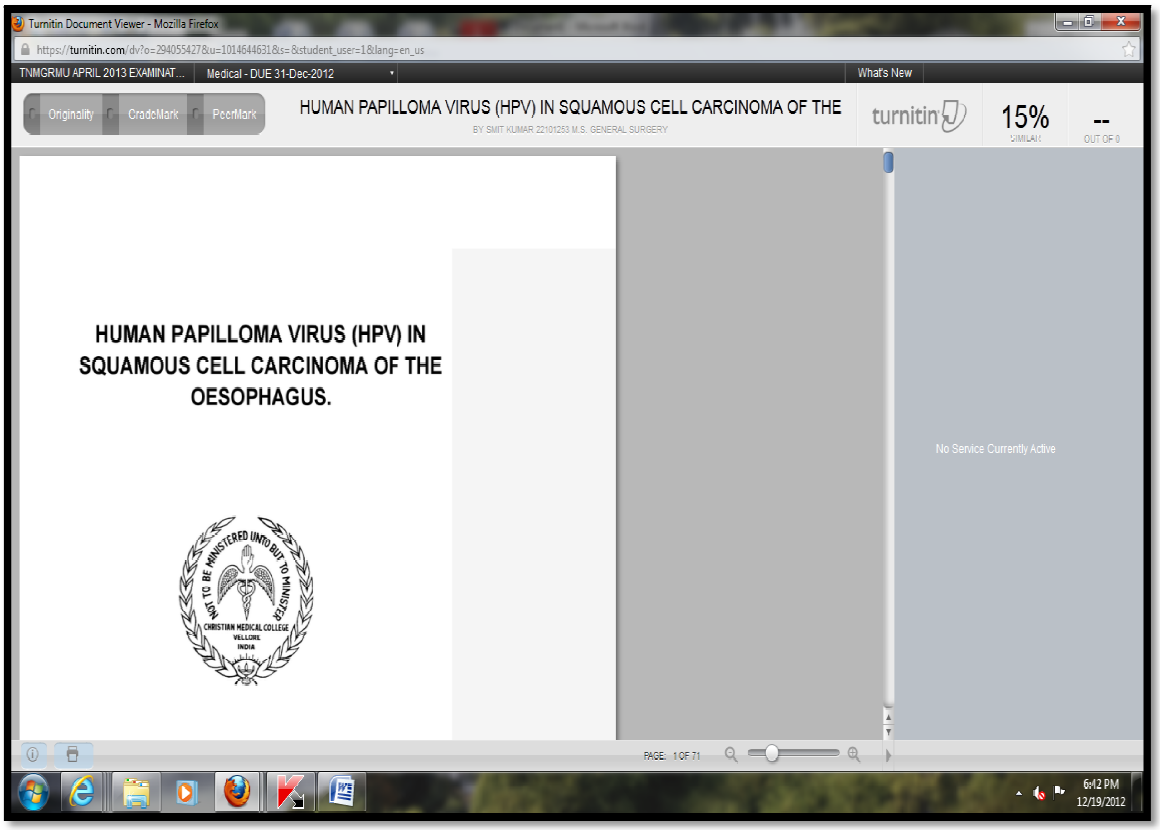
Yours sincerely,

G. Kang

Gagandeep Kang, MD, PhD, FRCPath
Secretary, (IRB)

Secretary
Institutional Review Board
(Ethics Committee)
Christian Medical College
Vellore - 632 002, Tamil Nadu, India


TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 e-mail : research@cmcvellore.ac.in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## INFORMED CONSENT FORM

# STUDY: "HUMAN PAPILLOMA VIRUS (HPV) IN SQUAMOUS CELL CARCINOMA OF THE ESOPHAGUS"

INVESTIGATOR: **Dr. Smit Kumar , PG Registrar,**

Department of General Surgery Unit 3

CHRISTIAN MEDICAL COLLEGE & HOSPITAL, VELLORE

Tel: 0416-2282079

E Mail: drsmitkumar@rediffmail.com

DURATION: 2 years.

## INFORMATION SHEET

Esophageal cancer or cancer of the food pipe is the eighth most common cancer world wide and fifth most common cancer in developing countries. The recent scientific papers suggest that high risk HPV type 16&18 as infectious agent causing oesophageal cancer. In this study we are evaluating if there is any association between high risk Human Papilloma Virus type 16 and 18 and esophageal cancer.

You are thus being requested to participate in this study for evaluation of this viral association with oesophageal cancer.

**If you consent to take part what will you have to do?**

As you are undergoing Endoscopy for evaluation of your symptoms . If you wish to participate in this study then please give consent to take two sets of biopsy **if any growth is found in the esophagus** along with the routine biopsy for histopathology .The biopsies will be taken from the growth and surrounding oesophageal mucosa. The participants will be termed as cases or Group A.

If on Endoscopy there is **no growth** seen then a biopsy of the oesophageal mucosa will be taken for the detection of HPV virus. These participants will be termed as control or Group B.

**Will it cause me pain?**

The Endoscopy is a very safe procedure and biopsies done are very small and superficial, taking only the very top layer of the oesophagus, most patients do not feel any pain and it also does not have any added complications. There is risk of bleeding which is seen in one in thousand cases.

**Can you withdraw from this study?**

Yes, your participation is entirely voluntary and you are free to decide to withdraw from this study. If you withdraw from the study it will not influence your treatment or access to medical care in this hospital.

**Will your personal detail be kept confidential?**

The details collected will be kept confidential and your samples will be coded which will be known only to person involved in the study.

**Will the result of the study informed to you?**

The result of this study will not be informed to you. The test is done later and results will be analysed by scientific method for its significance of importance.

**Will your samples be used for other studies in the future ?**

We seek your permission to use your sample for evaluation in future studies for scientific purposes only .The confidentiality will be maintained as mentioned earlier.

**IF YOU HAVE ANY DOUBTS ABOUT THE PROCEDURE OR STUDY KINDLY CONTACT THE ABOVE INVESTIGATOR.**

**Informed Consent form to participate in a research study**

1. Study Title: **"HUMAN PAPILLOMA VIRUS (HPV) IN SQUAMOUS CELL CARCINOMA OF THE ESOPHAGUS**

2. Subject's Name: _____

Date of Birth / Age:_____
Hospital Number ; _____

I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. I hereby give consent to take biopsies as required for the study and fully understand the risk involved in the procedure.

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s)

I agree to take part in the above study.

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:_____
Date: _____/_____/_____
Signatory's Name: _____

Signature of the Investigator: _____
Date: _____/_____/_____
Study Investigator's Name: _____

Signature of the Witness: _____
Date:_____/_____/_____
Name of the Witness: _____

| ID | GROU | SAMPLE | AGE | SEX | STATE | RESI | EDUC | SMO | PACK_ | ALCOH | DIET | HOT_ | AMOU | FAMILY | DM | HTN | OT | ENDO | TUM | VIR_RE | VIRI | VIRU | REASON FOR SCOPY | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 7030 | 34 | 1 | 2 | 2 | 2 | 2 | 1 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 6 | | |
| 2 | 2 | 7026 | 44 | 1 | 1 | 1 | 3 | 1 | | 2 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 3 | 2 | 7041 | 33 | 2 | 1 | 2 | 3 | 1 | | 1 | 2 | 0 | | 2 | 0 | 0 | 0 | 2 | 0 | 0 | | | 4 | | |
| 4 | 2 | 7006 | 32 | 2 | 2 | 2 | 4 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 5 | 2 | 7013 | 45 | 2 | 3 | 2 | 3 | 1 | | 1 | 1 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 6 | 2 | 7012 | 54 | 2 | 2 | 1 | 4 | 1 | | 1 | 2 | 0 | | 0 | 1 | 1 | 0 | 2 | 0 | 0 | | | 1 | | |
| 7 | 2 | 7005 | 30 | 1 | 1 | 1 | 3 | 1 | | 1 | 1 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 8 | 2 | 7004 | 47 | 1 | 3 | 2 | 3 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 9 | 2 | 7034 | 62 | 1 | 2 | 1 | 3 | 2 | 2 | 1 | 2 | 1 | 2 | 0 | 1 | 1 | 0 | 2 | 0 | 0 | | | 1 | | |
| 10 | 2 | 7033 | 63 | 1 | 2 | 2 | 1 | 2 | 2 | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 11 | 2 | 7032 | 50 | 1 | 1 | 1 | 2 | 2 | 1 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 12 | 2 | 7035 | 29 | 1 | 4 | 2 | 3 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 13 | 2 | 7007 | 43 | 1 | 2 | 2 | 3 | 2 | 1 | 3 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 14 | 2 | 7001 | 24 | 1 | 1 | 2 | 3 | 1 | | 2 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 15 | 2 | 7003 | 50 | 1 | 4 | 2 | 3 | 2 | 1 | 2 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 16 | 2 | 7002 | 42 | 2 | 1 | 2 | 3 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 3 | | |
| 17 | 2 | 7011 | 66 | 2 | 5 | 1 | 3 | 1 | | 1 | 2 | 0 | | 0 | 1 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 18 | 2 | 7008 | 30 | 1 | 2 | 2 | 1 | 2 | 1 | 3 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 19 | 2 | 7009 | 35 | 2 | 2 | 2 | 3 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 20 | 2 | 7010 | 26 | 1 | 1 | 1 | 4 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 3 | | |
| 21 | 2 | 7031 | 50 | 2 | 5 | 2 | 2 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 7 | | |
| 22 | 2 | 7029 | 38 | 1 | 1 | 1 | 3 | 2 | 1 | 1 | 2 | 0 | | 0 | 0 | 1 | 1 | 2 | 0 | 0 | | | 7 | | |
| 23 | 2 | 7028 | 47 | 1 | 2 | 1 | 4 | 1 | | 1 | 2 | 1 | 2 | 2 | 1 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 24 | 2 | 7027 | 37 | 2 | 1 | 2 | 2 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
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| 26 | 2 | 7014 | 42 | 1 | 2 | 1 | 3 | 2 | 1 | 2 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
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| 29 | 2 | 7016 | 35 | 2 | 6 | 1 | 2 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
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| 31 | 2 | 7021 | 58 | 2 | 2 | 1 | 2 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | | | 3 | | |
| 32 | 2 | 7020 | 79 | 1 | 2 | 2 | 2 | 2 | 1 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 33 | 2 | 7019 | 40 | 2 | 3 | 1 | 4 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 34 | 2 | 7018 | 30 | 2 | 2 | 2 | 3 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 35 | 2 | 7017 | 19 | 1 | 2 | 1 | 4 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 5 | | |
| 36 | 2 | 7015 | 38 | 1 | 7 | 1 | 2 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 37 | 2 | 7023 | 37 | 1 | 2 | 2 | 2 | 1 | | 1 | 2 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 38 | 2 | 7080 | 28 | 1 | 2 | 2 | 3 | 2 | 1 | 1 | 1 | 0 | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | | | 1 | | |
| 39 | 1 | 8019 | 62 | 1 | 8 | 1 | 3 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 3 | 2 | | 0 | 0 | 7 | | |
| 40 | 1 | 8020 | 77 | 2 | 9 | 1 | 1 | 2 | 1 | 1 | 2 | 0 | | 2 | 0 | 0 | 0 | 3 | 2 | | 0 | 0 | 2 | | |
| 41 | 1 | 8008 | 60 | 2 | 7 | 1 | 3 | 1 | | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 42 | 1 | 8003 | 57 | 1 | 4 | 2 | 3 | 1 | | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 3 | | 0 | 0 | 8 | | |
| 43 | 1 | 8011 | 56 | 1 | 10 | 1 | 3 | 2 | 2 | 2 | 2 | 0 | | 2 | 0 | 0 | 0 | 2 | 3 | | 0 | 0 | 2 | | |
| 44 | 1 | 8012 | 68 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 2 | 1 | 1 | 0 | 1 | 0 | 0 | 2 | 3 | | 0 | 0 | 2 | | |
| 45 | 1 | 8017 | 65 | 1 | 4 | 2 | 3 | 2 | 1 | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 3 | | 0 | 0 | 2 | | |
| 46 | 1 | 8010 | 58 | 1 | 1 | 2 | 2 | 2 | 2 | 3 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
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| 48 | 1 | 8009 | 65 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 2 | | 0 | 0 | 2 | | |
| 49 | 1 | 8015 | 49 | 1 | 4 | 1 | 3 | 1 | | 2 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 50 | 1 | 8007 | 56 | 1 | 2 | 1 | 2 | 2 | 2 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
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| 52 | 1 | 8023 | 55 | 2 | 4 | 2 | 2 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 53 | 1 | 8025 | 60 | 1 | 5 | 2 | 2 | 2 | 1 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | | 0 | 0 | 2 | | |
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| 56 | 1 | 8046 | 61 | 2 | 3 | 1 | 1 | 1 | | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 3 | | 0 | 0 | 2 | | |
| 57 | 1 | 8055 | 56 | 2 | 4 | 2 | 1 | 1 | | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 3 | | 0 | 0 | 2 | | |
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| 59 | 1 | 8040 | 52 | 1 | 4 | 2 | 2 | 1 | | 3 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 2 | | 0 | 0 | 2 | | |
| 60 | 1 | 8038 | 60 | 1 | 12 | 2 | 2 | 2 | 2 | 3 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 3 | | 0 | 0 | 2 | | |
| 61 | 1 | 8033 | 64 | 1 | 10 | 2 | 2 | 2 | 2 | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 1 | 2 | 3 | | 0 | 0 | 2 | | |
| 62 | 1 | 8035 | 56 | 1 | 2 | 1 | 2 | 2 | 1 | 2 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 3 | | 0 | 0 | 6 | | |
| 63 | 1 | 8070 | 68 | 2 | 13 | 1 | 2 | 1 | | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 64 | 1 | 8071 | 63 | 1 | 12 | 1 | 2 | 1 | | 3 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 65 | 1 | 8036 | 45 | 1 | 12 | 1 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 66 | 1 | 8043 | 75 | 1 | 3 | 2 | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | | 0 | 0 | 2 | | |
| 67 | 1 | 8044 | 32 | 1 | 4 | 1 | 2 | 2 | 1 | 3 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 68 | 1 | 8045 | 35 | 1 | 1 | 2 | 2 | 1 | | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 3 | | 0 | 0 | 2 | | |
| 69 | 1 | 8047 | 60 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 70 | 1 | 8050 | 70 | 1 | 4 | 1 | 1 | 2 | 2 | 2 | 2 | 1 | 2 | 0 | 0 | 1 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
| 71 | 1 | 8069 | 38 | 2 | 1 | 1 | 4 | 1 | | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 3 | | 0 | 0 | 7 | | |
| 72 | 1 | 8042 | 62 | 2 | 2 | 2 | 2 | 1 | | 1 | 2 | 1 | 2 | 0 | 0 | 1 | 0 | 1 | 2 | | 0 | 0 | 2 | | |
| 73 | 1 | 8048 | 59 | 1 | 4 | 1 | 2 | 2 | 2 | 3 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 2 | | 0 | 0 | 7 | | |
| 74 | 1 | 8049 | 42 | 2 | 1 | 1 | 4 | 1 | | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
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| 77 | 1 | 8021 | 69 | 1 | 2 | 1 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | | 0 | 0 | 2 | | |
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| 79 | 1 | 8030 | 61 | 1 | 4 | 2 | 1 | 2 | 2 | 2 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 2 | | 0 | 0 | 2 | | |
| 80 | 1 | 8037 | 67 | 2 | 1 | 1 | 1 | 1 | | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 3 | | 0 | 0 | 2 | | |
| 81 | 1 | 8032 | 43 | 2 | 1 | 1 | 2 | 1 | | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 2 | | 0 | 0 | 7 | | |
| 82 | 1 | 8029 | 53 | 2 | 4 | 2 | 2 | 1 | | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 3 | | 0 | 0 | 2 | | |
| 83 | 1 | 8013 | 54 | 1 | 13 | 1 | 4 | 1 | | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 2 | | 0 | 0 | 2 | | |