PREVALENCE OF HUMAN PAPILLOMA VIRUS IN LARYNGEAL CANCERS

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF **M.S BRANCH –IV (OTORHINOLARYNGOLOGY)** EXAMINATION OF THE TAMILNADU DR.MGR. MEDICAL UNIVERSITY TO BE HELD IN **APRIL 2014**

CERTIFICATE

This is to certify that the dissertation entitled **'PREVALENCE OF HUMAN PAPILLOMA VIRUS IN LARYNGEAL CANCERS'** is a bonafide original work of **Dr Philip George,** submitted in partial fulfillment of the rules and regulations for the MS Branch IV, Otorhinolaryngology examination of The Tamil Nadu Dr. M.G.R Medical University to be held in April 2014.

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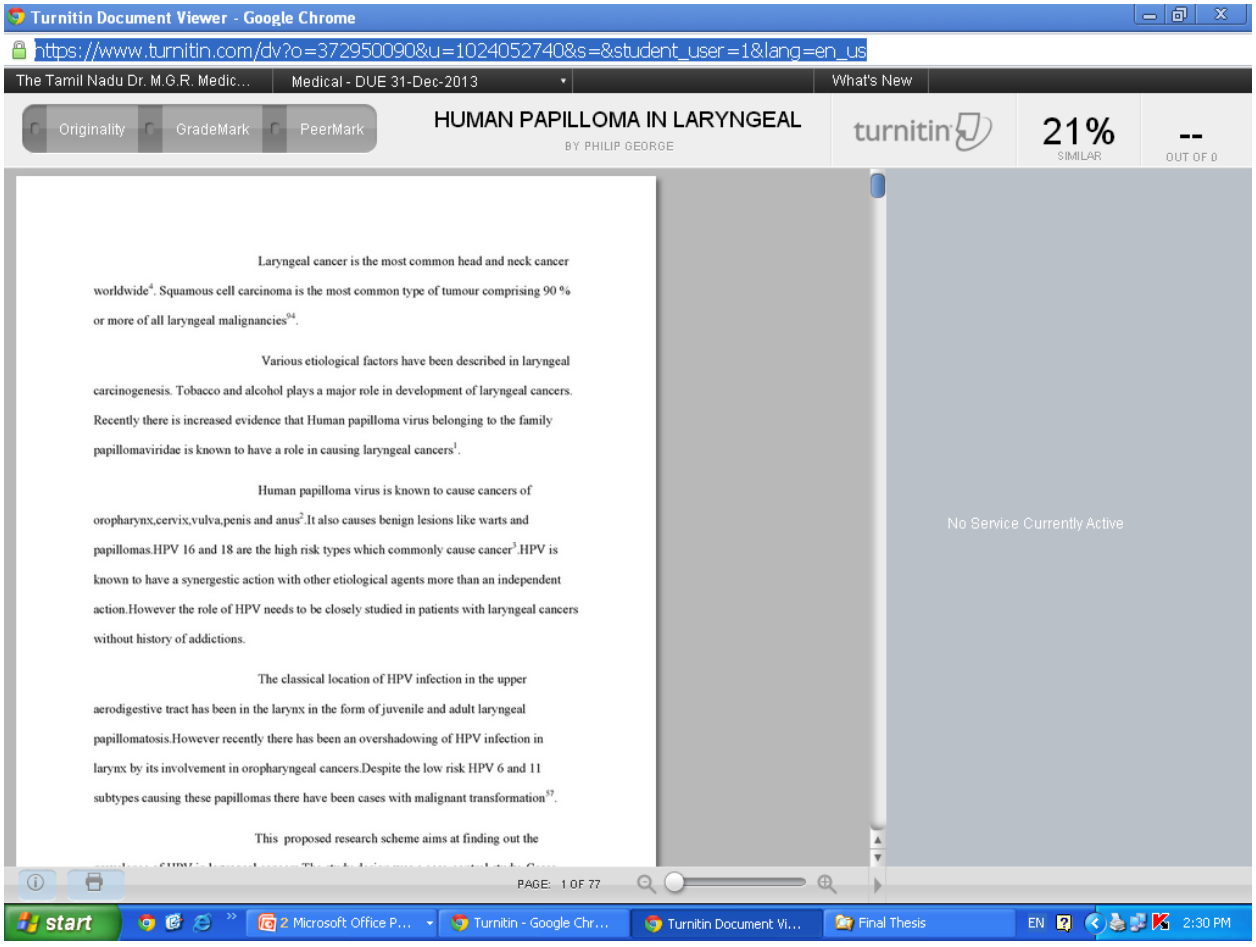
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Laryngeal cancer is the most common head and neck cancer worldwide[4]. Squamous cell carcinoma is the most common type of tumour comprising 90 % or more of all laryngeal malignancies[94].
Various etiological factors have been described in laryngeal carcinogenesis. Tobacco and alcohol plays a major role in development of laryngeal cancers. Recently there is increased evidence that Human papilloma virus belonging to the family papillomaviridae is known to have a role in causing laryngeal cancers[1].
Human papilloma virus is known to cause cancers of oropharynx,cervix,vulva,penis and anus[2].It also causes benign lesions like warts and papillomas.HPV 16 and 18 are the high risk types which commonly cause cancer[3].HPV is known to have a synergestic action with other etiological agents more than an independent action.However the role of HPV needs to be closely studied in patients with laryngeal cancers without history of addictions.
The classical location of HPV infection in the upper aerodigestive tract has been in the larynx in the form of juvenile and adult laryngeal papillomatosis.However recently there has been an overshadowing of HPV infection in larynx by its involvement in oropharyngeal cancers.Despite the low risk HPV 6 and 11 subtypes causing these papillomas there have been cases with malignant transformation[57].
This proposed research scheme aims at finding out the
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ABSTRACT

TITLE OF THE ABSTRACT : PREVALENCE OF HUMAN PAPILLOMA VIRUS IN LARYNGEAL CANCERS

DEPARTMENT : OTORHINOLARYNGOLOGY

NAME OF THE CANDIDATE : PHILIP GEORGE

DEGREE AND SUBJECT : MS ENT

NAME OF THE GUIDE : Dr. RAJIV MICHAEL

OBJECTIVES : To find out the prevalence of Human Papilloma virus in laryngeal cancers in the ENT Department of a tertiary level teaching hospital

METHODS : The study was conducted in the ENT outpatient department and in the ENT operating room at Christian Medical College,Vellore between November 2011 and July 2013. Study included 30 cases of laryngeal cancers and 30 controls which were benign lesions of vocal cords such as polyps,cysts or nodules.Cases and controls were evaluated in the ENT outpatient department and explained about the study in detail and were given an information sheet for clarifications. Informed valid consent was taken.Tissue was taken for the study when patient was posted for a direct laryngoscopic/microlaryngoscopic biopsy of the lesion.

Tissue is taken for histopathological examination as well as HPV study.The tissue obtained is transferred to a VTM tube,i.e viral transport

medium tube and is taken to the virology lab in an ice container. Viral transport medium is a balanced isotonic solution at physiological pH. It maintains the virus in the viable state.It contains fetal calf serum and antibiotics.

Once received in lab, the samples were transferred from the VTM tube to a 1.5ml eppendorf tube and stored at -80^{0}C until further testing in -80 degree freezer.The next step is DNA extraction using DNA extraction kit, DNeasy® Tissue kit: (Qiagen GmbH, Hilden, Germany). The extracted DNA undergoes Polymerase chain reaction.A known positive control was used for PCR and beta-globin serves as internal control.

If sample was positive for HPV,sequencing is done to identify the genotype.The amplified PCR products were purified by Millipore filtration and sequenced directly using an ABI Prism Big Dye terminator cycle sequencing ready reaction kit. Finally, the data was analyzed using Bioedit software version 7.0.5.3 and study sequences compared to the GenBank HPV sequences. WHO recommended CHUV assay had to be used in case of 2 samples which were not able to be sequenced using routine methods

From the previous studies in the literature the average prevalence of HPV in laryngeal carcinoma is 25%.

$n=2pq(Z\alpha + Z\beta)^2/(P1-Po)^2$

By applying the formula n=73

Hence,in each arm 73 cases should be studied.

But the average number of microlaryngoscopy for cases of laryngeal cancers in CMC is 3-4 per month. Hence the aim was to study 30 cases and 30 controls.

RESULTS : Out of the 30 cases of laryngeal squamous cell carcinoma cases,4 were positive for HPV whereas there were no positive HPV cases in the control group. One was HPV 16 type and another one HPV 11 type. Two other positive cases were

not able to be sequenced probably due to low viral load. The results of HPV in laryngeal cancers were statistically insignificant with a p value of 0.052.Our study showed that both smoking and tobacco chewing had 5 times increased risk of acquiring laryngeal cancers.

CONCLUSION : As there were 4 HPV positive cases in the cancer group whereas there were none in the control group,there is more trend towards HPV positivity in the cancer group.Further studies are essential to prove the confirmatory role of HPV in laryngeal cancers HPV subtyping needs to be done for all juvenile and adult-onset laryngeal papillomatosis in view of chances of malignant transformation.These patients need to be followed up regularly. HPV testing should be made mandatory in the workup of laryngeal cancer patients especially in young patients without any co-existing risk habits such as smoking ,tobacco chewing and alcohol consumption.

Key words:Human papilloma virus,laryngeal ancer

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I would like to thank the Fluid Research Committee, CMC Hospital for granting me permission for conducting this study.

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CONTENTS

INTRODUCTION

Laryngeal cancer is the most common head and neck cancer worldwide[4]. Squamous cell carcinoma is the most common type of tumour comprising 90 % or more of all laryngeal malignancies[94].

Various etiological factors have been described in laryngeal carcinogenesis. Tobacco and alcohol plays a major role in development of laryngeal cancers. Recently there is increased evidence that Human papilloma virus belonging to the family papillomaviridae is known to have a role in causing laryngeal cancers[1].

Human papilloma virus is known to cause cancers of oropharynx, cervix, vulva, penis and anus[2].It also causes benign lesions like warts and papillomas.HPV 16 and 18 are the high risk types which commonly cause cancer[3].HPV is known to have a synergestic action with other etiological agents more than an independent action.However the role of HPV needs to be closely studied in patients with laryngeal cancers without history of addictions.

The classical location of HPV infection in the upper aerodigestive tract has been in the larynx in the form of juvenile and adult laryngeal papillomatosis.However recently there has been an overshadowing of HPV infection in larynx by its involvement in oropharyngeal cancers.Despite the low risk HPV 6 and 11 subtypes causing these papillomas there have been cases with malignant transformation[57].

This proposed research scheme aims at finding out the prevalence of HPV in laryngeal cancers.The study design was a case-control study. Cases will be laryngeal cancer

patients and controls will be patients with vocal cord polyps or other benign conditions of vocal cords like nodules or cysts.

As per the western literature the incidence of HPV subtypes in laryngeal carcinoma may be identified in upto 40% of cases[94]. There is paucity of literature on the correlation between HPV and laryngeal cancers in an Indian population[4].Hence this study aims at finding the prevalence of Human papilloma virus in laryngeal cancers in India through a case control study.

AIMS & OBJECTIVES

- **Aim :** To understand the role of HPV in the etiopathogenesis and prognosis of laryngeal cancers

- **Objective :** To find out the prevalence of Human Papilloma virus in laryngeal cancers in the ENT Department of a tertiary level teaching hospital

REVIEW OF LITERATURE

The role of Human papilloma virus in head and neck cancers is being studied in detail in recent years .The role of high risk types of human papilloma virus in cervical carcinogenesis is well established. Recent studies have revealed the pivotal role of HPV in causing oropharyngeal cancers[5].Further studies were aimed at detecting the role of HPV in other head and neck sites mainly the larynx.

Cancer of the larynx is the second most common malignancy of the upper aerodigestive tract (UADT)[101].This accounts for approximately 25% of all head and neck malignancies. Squamous cell carcinoma is the most common histological type accounting for 90%[6] of cases. In India, laryngeal carcinoma constitutes 2.63% of all body cancers, ten times more common in males than females (4.79% vs 0.47%) with an incidence of 3.29 new cases in males and 0.42 new cases in females for one lakh population[100]. There seems to be a tendency for the laryngeal cancer to be mainly a disease of middle aged men with a peak incidence in the seventh decade. Women are affected in a comparatively younger age with a peak incidence at less than sixty years[7].

Although a large variety of malignancies are reported in the larynx, 85-95 percentage of all laryngeal malignancies are squamous cell carcinoma (SCC), arising from the epithelial lining of the larynx[94]. First reports on HPV types in spontaneously arising laryngeal cancers(HPV 16 & 30) appeared in 1986(Kahn et al,1986;Scheurlen et al,1986)[8]

Since then many studies have been looking into the role of Human papilloma virus in the aetiology of laryngeal cancers.

EMBRYOLOGY OF LARYNX

The development of the larynx starts during the fourth week of embryonic development.The tracheobronchial diverticulum appears just below the hypobranchial eminence in the ventral wall of the primitive pharynx . An oesophagotracheal septum is formed from the edges of the groove which fuses caudally,leaving a slit-like aperture cranially into the pharynx. The resulting tube is lined with endoderm from which the epithelial lining of the entire respiratory tract develops. The cranial end of the tube forms the larynx and trachea and the caudal end the bronchi and lungs.

The larynx is subdivided into the supraglottis,the glottis and the subglottis. The buccopharyngeal primordium gives rise to the supraglottic larynx which develops from the third and fourth branchial arches. The glottis and subglottis are derived from the tracheobronchial primordium from the sixth branchial arch and are formed by the union of lateral furrows that develop on each side of the tracheobronchial primordium

Arytenoid swellings appear on both sides of the traheobronchial diverticulum.Aryepiglottic folds are formed from the arytenoids swellings. The hypobranchial eminence becomes the epiglottis. The glottis forms just above the level of the primitive aperture. The thyroid cartilage develops from the cartilages of the fourth pharyngeal arch and the cricoid cartilage and the cartilages of the trachea develop from the sixth arch during the sixth week with the trachea increasing rapidly in length from the fifth week onwards. The mesoderm of each pharyngeal arch differentiates into cartilage, muscle and vascular structures of that arch.

The supraglottis is supplied by the superior laryngeal arteries, and its lymphatics drain into deep cervical chain nodes at levels II and III. The glottis and subglottis are supplied

by the inferior laryngeal arteries, and similarly, lymphatic drainage from these two regions follows these arteries to drain into prelaryngeal and pretracheal nodes (Level VI), before reaching the deep cervical chain nodes in level IV[9].There is an increased chance of bilateral lymphatic metastases from supraglottic carcinoma because the supraglottis is formed without a midline union and its lymphatics drain bilaterally[10].

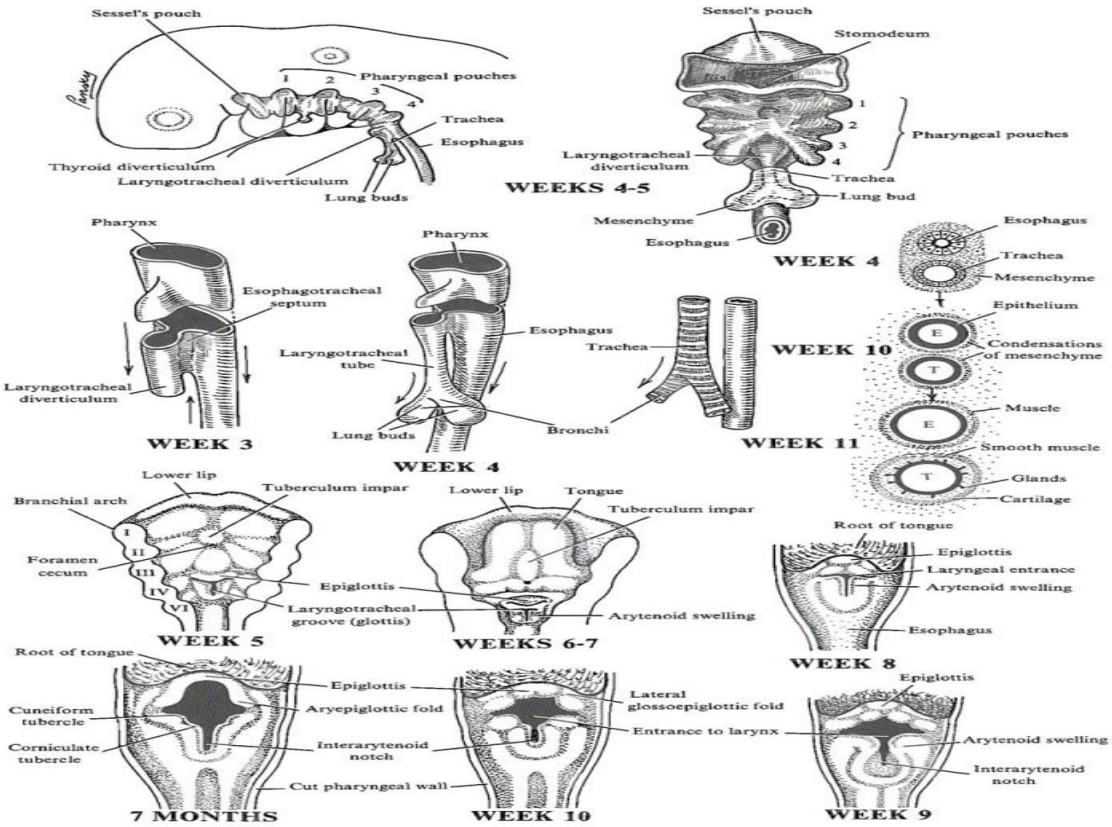


Fig.1 EMBRYOLOGY OF LARYNX(Adopted from Review of MEDICAL EMBRYOLOGY Book by BEN PANSKY, Ph.D, M.D.)

ANATOMY OF THE LARYNX

The larynx extends from the laryngeal inlet to the inferior border of the cricoid cartilage. Anatomically the larynx is divided into the supraglottis,glottis and subglottis by the false and true cords. The supraglottis consists of superiorly the epiglottis and aryepiglottic folds as they sweep down to the arytenoids. Its lower border is formed by the ventricular bands (false cords) which form the upper border of the glottis. The inferior surface of the glottis is a horizontal plane 1cm inferior to the inferior limit of the supraglottis[92].The sub glottis becomes the trachea at the lower border of the cricoid.The framework of the larynx consists of the hyoid bone, paired and unpaired cartilages connected by ligaments,membranes and intrinsic and extrinsic muscles to give it stability. It is lined with a mucous membrane that is continuous above with the pharynx and below with that of the trachea.

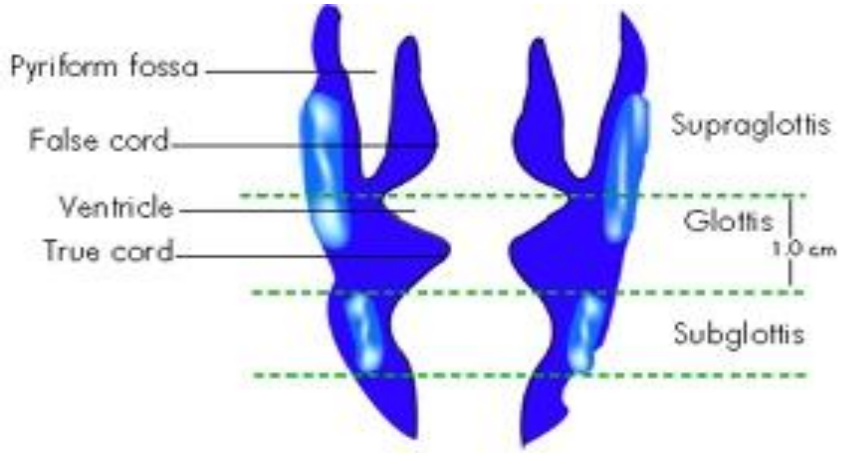


Fig.2 Parts of larynx

Thyroid,cricoid and epiglottis are the unpaired cartilages and corniculate,cuneiform and arytenoids are the paired cartilages(Fig.4).The Thyroid cartilage has two alae which meet anteriorly forming an angle. Vocal folds are attached to the middle of thyroid cartilage.The cricoid cartilage is the only cartilage forming a complete ring and has an expanded lamina posteriorly and a narrow arch anteriorly..The epiglottis is an elastic cartilage forming the anterior wall of the laryngeal inlet. It is divided into suprahyoid and infrahyoid epiglottis by the hyoepiglottic ligament.The arytenoid cartilages are paired cartilages and posses a base,muscular process and a vocal process which gives attatchment to the voal cord.The corniculate cartilage articulates with the apex of arytenoid cartilage.The cuneiform cartilages are situated in the ayepiglottic folds and give support to it.

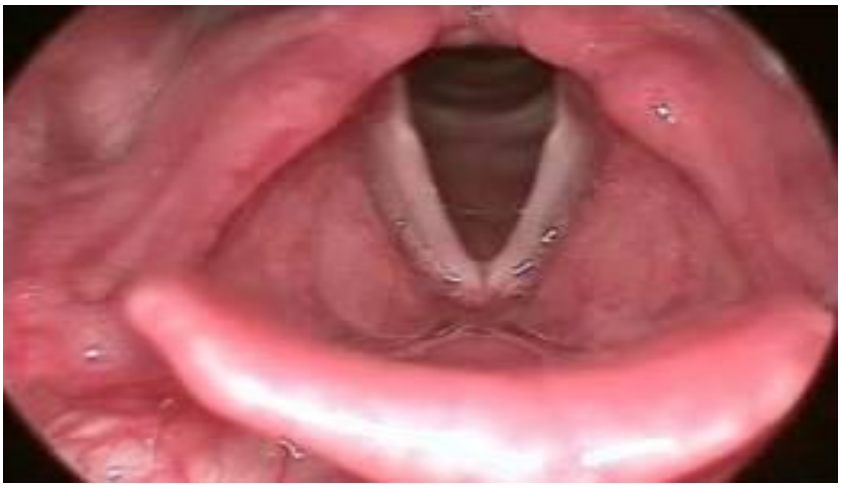


Fig.3 Normal larynx

The muscles of the larynx are divided into extrinsic and intrinsic muscles.Extrinsic muscles attatch the larynx to the neighbouring structures and maintain the position of larynx in the neck.Extrinsic muscles are divided into a suprahyoid and an infrahyoid groups.

The suprahyoid group consists of mylohyoid, geniohyoid, stylohyoid,digastrics ,stylopharyngeus, palatopharyngeus and salpingopharyngeus muscles.The infrahyoid group consists thyrohyoid,sternothyroid and sternohyoid muscles. The intrinsic muscles are all paired and move the cartilages in the larynx and regulate the mechanical properties of the larynx.They comprise the posterior cricoarytenoid, lateral cricoarytenoid,transverse arytenoids,oblique arytenoids, thyroarytenoid, cricohyroid, aryepiglotticus and thyroepiglottius muscles .

The motor and sensory nerves of the larynx are derived from the vagus by way of its superior and recurrent laryngeal nerves. The arterial supply of the larynx is derived from laryngeal branches of the superior and inferior thyroid arteries and

the cricothyroid branch of the superior thyroid artery.The lymphatic drainage of the larynx is divided into upper and lower drainage groups by the vocal folds. The larynx above the vocal folds is drained by vessels that accompany the superior laryngeal vein and pierce the thyrohyoid membrane emptying into the upper deep cervical lymph nodes. The larynx below the vocal folds drains to the lower deep cervical chain often through the prelaryngeal and pretracheal nodes.

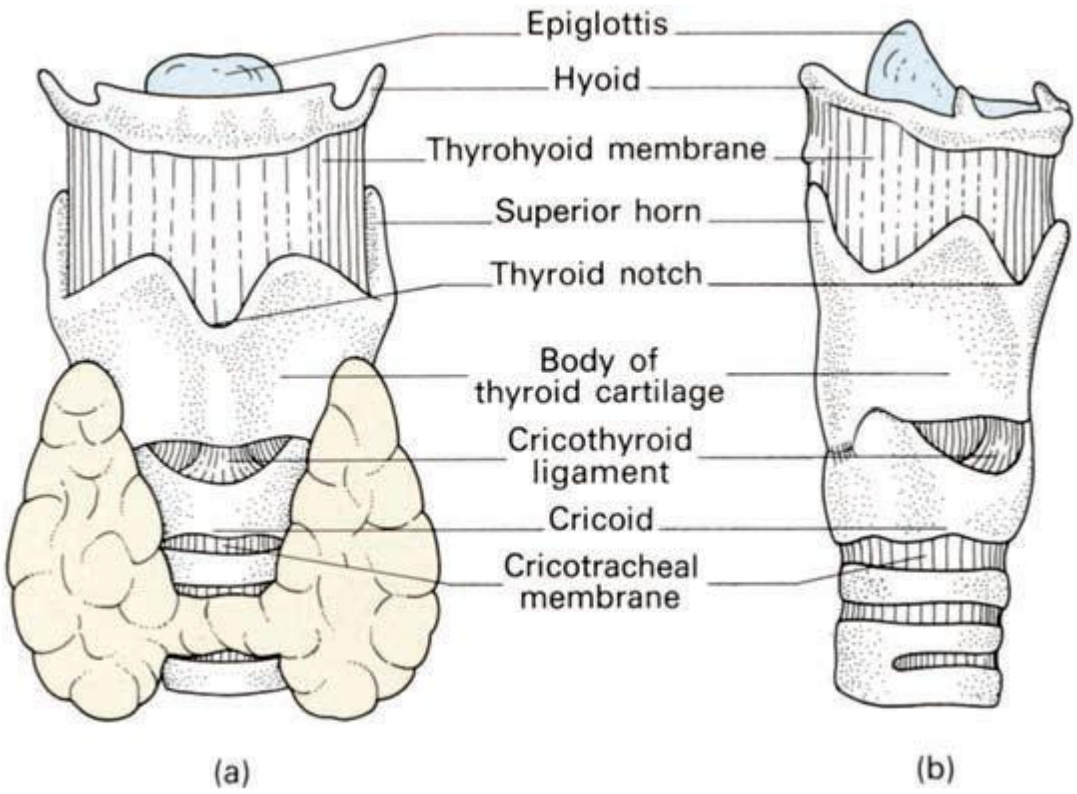


Fig.4 External framework of larynx(*adopted from the book Clinical Anatomy by Harold Ellis*)

Histology of larynx

The mucous membrane lining of the larynx is closely attached over the posterior surface of the epiglottis, the cartilages and over the vocal ligament. Elsewhere, it is loosely attached and prone to oedema. Most of the larynx is lined by the respiratory type epithelium that is pseudo stratified ciliated columnar epithelium. The upper half of the posterior surface of the epiglottis, the upper part of the aryepiglottic fold, the posterior glottis and the vocal folds are covered with nonkeratinizing stratified squamous epithelium.Mucous glands are freely distributed throughout the mucous membranes and are particularly numerous on the posterior surface of the epiglottis where they form

indentations into the cartilage and in the margins of the lower part of the aryepiglottic folds and in the saccules. The vocal folds do not possess any glands

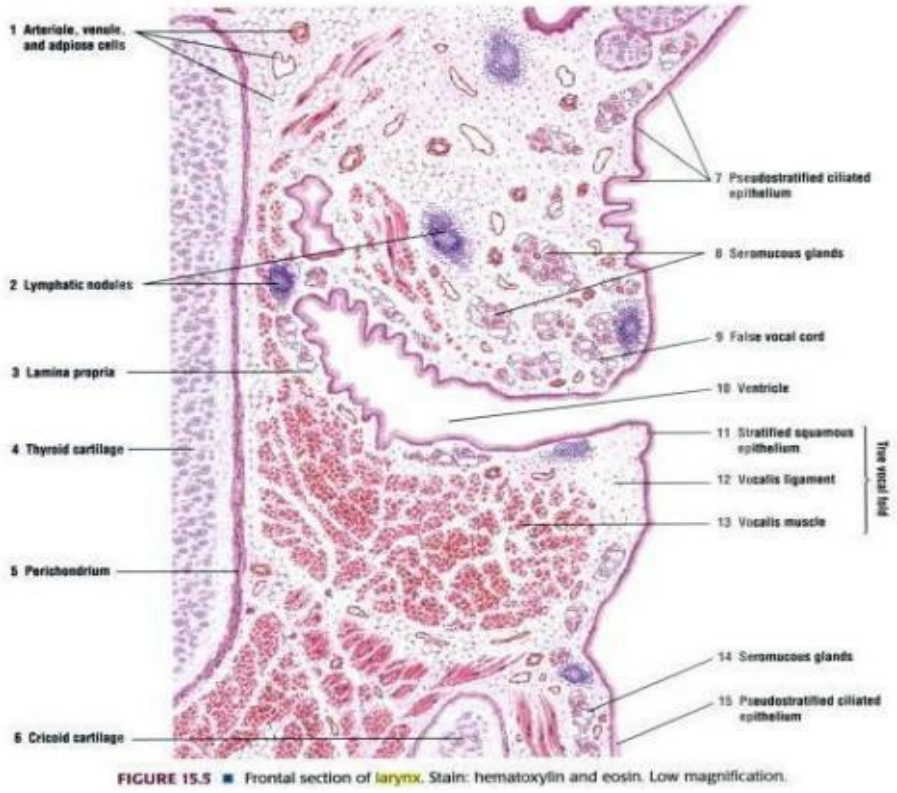


FIGURE 15.5 ■ Frontal section of larynx. Stain: hematoxylin and eosin. Low magnification.

Fig.5 Histology of larynx

Vocal fold polyps

A true vocal polyp is a benign swelling of greater than 3 mm that arises from the free edge of the vocal fold . Vocal fold polyps are caused by inflammation caused by stress or irritation[11].It is usually single, but can occasionally be seen on bilateral vocal cords. It is claimed that polyps are the most common structural abnormality that cause hoarseness and they affect men more than women. They are most frequently seen in smokers and between the ages of 30 and 50 years[12].Voice misuse is an important etiological factor for polyp formation.Certain patients may develop polyp after yelling or shouting usually

when the vocal folds are already inflamed due to laryngitis and gastroesophageal reflux. There will be disruption to the vascular basement membrane, proliferation of capillaries, thrombosis, minute haemorrhage and exudation of fibrin.[15] Vocal fold polyps are typically caused by acute and chronic trauma to the microvasculature of the superficial lamina propria [14] . Hyperfunctional glottal sound production causes shearing stresses which lead to bleeding into the SLP and malformed neo-vascularized masses.Some polyps have a haemorrhagic appearance whereas others are more gelatinous and grey.Speech therapy may provide the patient ease of symptoms,but is unlikely to result in resolution of the polyp.It is advisable to remove the polyps under general anaesthetia.[15] The aim is to restore the smooth edge of the vocal cord allowing them to close fully and vibrate normally.

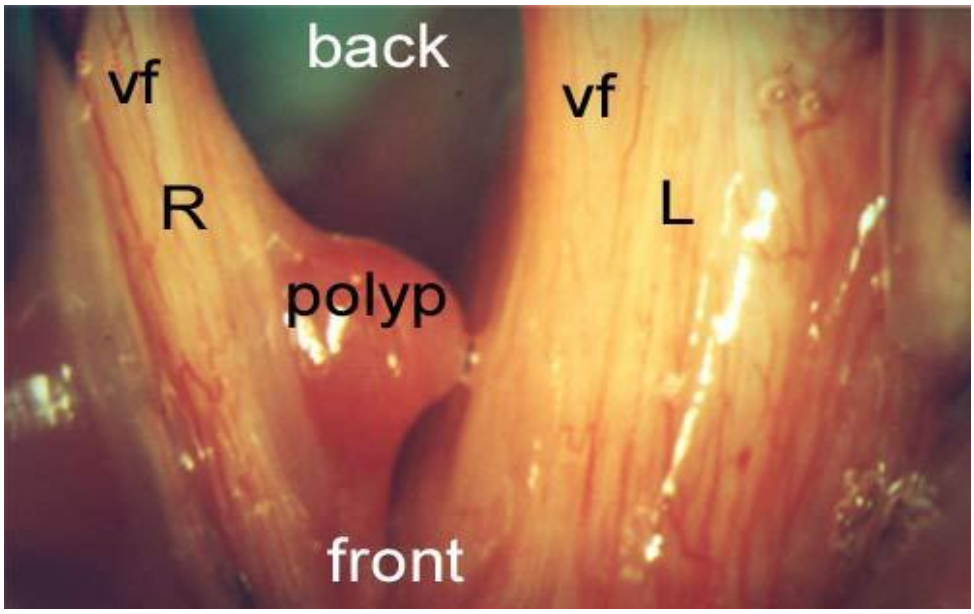


Fig.6 Right vocal cord polyp

Vocal fold nodules

Vocal nodules are bilateral small swellings (less than 3mm in diameter) that develop on the free edge of the vocal fold at the mid membranous portion. Vocal cord nodules have various synonyms.The various synonyms are teachers' nodules, laryngeal nodules, , parsons' nodes laryngeal nodes, corditis nodosa, singers' nodes or screamers' nodes[16].Voice abuse is the commonest etiology of the lesions.Singers and teachers are more prone for this condition.Nodules are formed due to vascular disorders secondary to overstrain[17].Speech therapy is the first line of treatment.Surgical techniques include microsurgical methods and laser excision[18].

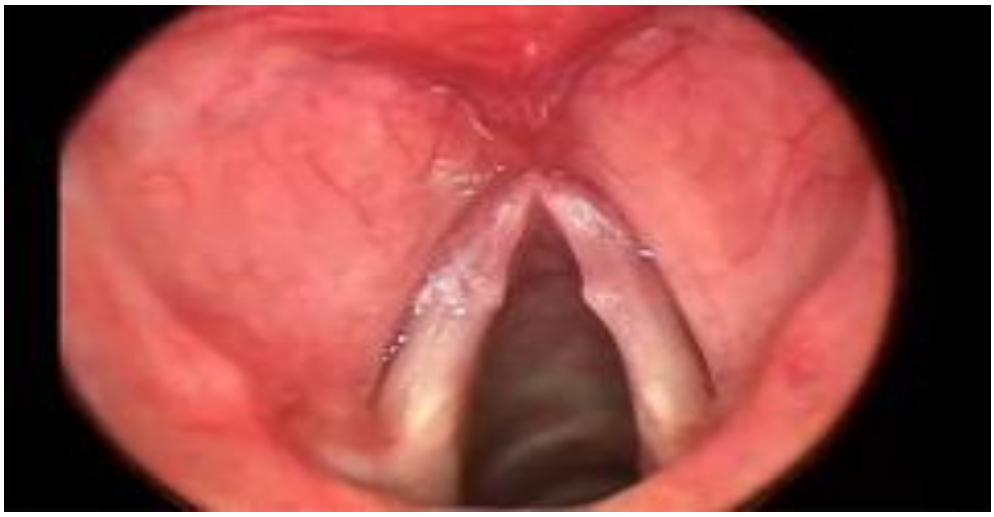


Fig.7 Bilateral vocal cord nodules

Vocal fold cysts

Vocal fold cysts (VFC) may be within the sub epithelium or in the ligament. It is usually unilateral. Significant reduction in vibratory function of the mucosa is noted in stroboscopy. Vocal fold cysts do not resolve with voice therapy. Microlaryngoscopic surgery is needed and it is confirmed intra operatively by the presence of an encapsulated

lesion.

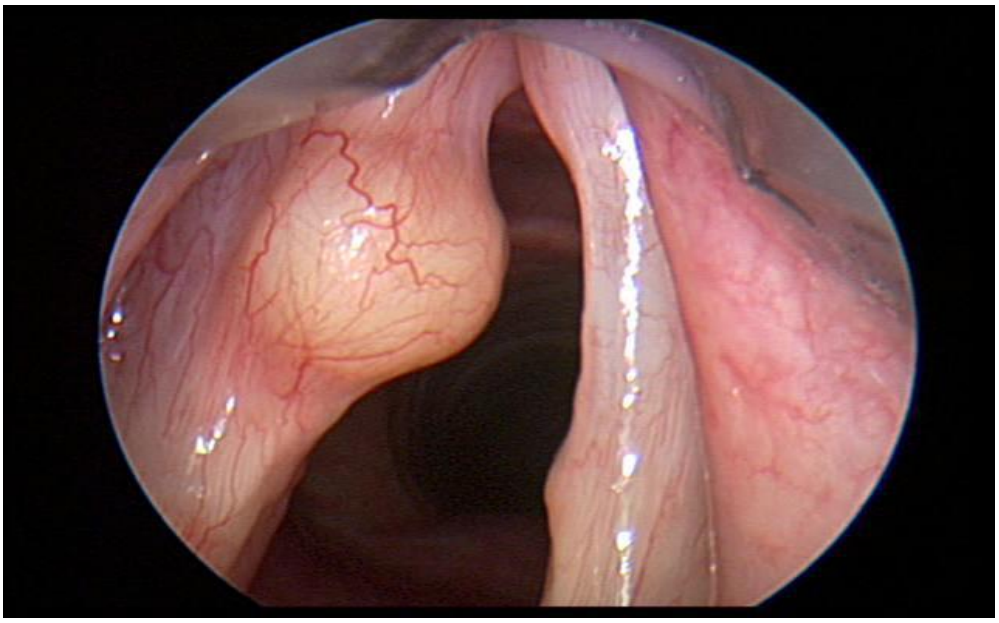


Fig.8 Right Vocal cord cyst

ETIOLOGY OF CARCINOMA LARYNX

The most common head and neck cancer is laryngeal carcinoma .It is well known that the development of laryngeal cancer is influenced by environmental and life style factors like tobacco use and alcohol consumption[19].Other factors which lead to development of laryngeal cancers are exposure to toxins,human papilloma viruses,exposure to radiation,dietary factors and laryngopharyngeal reflux[20].Human papilloma virus acts as a co-adjuvant in the formation of laryngeal cancers along with other factors like tobacco use and alcohol consumption.HPV has a synergestic action rather than action alone[21].In people without habits of tobacco use and alcohol consumption,bile reflux and exposure to human papilloma virus are considered to have a significant contribution. High risk types of HPV 16 and 18 have been found to have role in laryngeal carcinoma[96].

Tobacco and alcohol

The main causative agent in laryngeal cancer has been identified as tobacco smoking[4].The risk is proportional to the intensity and duration of tobacco or alcohol consumption, and the risk decreases slowly after cessation but does not return to the baseline rate for at least 20 years. The risk varies with the type of tobacco exposure (e.g., cigar vs. cigarette, filtered vs. Non filtered cigarettes), But the most important factors are the amount of tobacco consumed and the duration of exposure. Processed tobacco contains at least 30 known carcinogens. Tobacco smoke contains a high concentration of reactive oxygen species and more than 50 known carcinogens and procarcinogens.[22] Increased consumption of alcohol and tobacco has a multiplicative effect in causing laryngeal cancers[23]-[24]. Stable mutations can be caused by the DNA lesions induced by tobacco smoke carcinogens. Initiation of carcinogenesis is by oncogenes and tumor suppressor genes. At the same time, cells provide self-protection processes by carcinogen detoxication, DNA repair and apoptosis.There occurs competition between the cell protection processes and the mutation pocessses[25].A metanalysis of 14 studies on effects of tobacco and alcohol in aerodigestive cancers by Zeka A *et al*, tobacco appeared to have a much stronger effect on the larynx than on any of the other aerodigestive sites.Alcohol's effect was strongest on the pharynx.The study confirmed the multiplicative effects of tobacco and alcohol[26]. Significant dose-response trends for tobacco use were observed for both supraglottic and glottic cancers, with a potentially more important effect for supraglottic cancer.The frequency and duration of the tobacco use were the significant factors[27].Every incremental increase in pack years of smoking increases the risk of laryngeal cancers, likewise “heavy” drinkers are more prone than “social” drinkers[19].

Reflux disease and laryngeal carcinoma

The relation between Gastroesophageal reflux disease (GERD) and upper aerodigestive tract carcinoma was initially suggested by Gabriel and Jones in 1976[28].There is controversial evidence regarding relation between gastroesophageal reflux disease and laryngeal carcinoma[29]. The effect of reflux disease is demonstrated more clearly in studies involving laryngeal cancer patients who were non smokers.In a retrospective study by Morrison on laryngeal cancers, he noted that 47% of lifelong non-smokers had GERD signs and symptoms[30].Similarly, in a study by Mercante et al,he found that 25% of nonsmoking cancer subjects had GERD, whereas only 5% of nonsmoking control subjects had GERD[31].The metaanalysis by Mohammed *et al* concluded that GERD is two times more common in laryngeal subjects than in control population[29]. Epithelial growth factor receptor(EGFR) role in laryngeal squamous cell carcinomas have already been studied.Increased expression of EGF has been seen in response to the bile reflux on the esophageal epithelial cells and similar effect maybe expected in laryngeal mucosa as well[32]. The greater distance between the laryngeal mucosa and the direct origin of gastric acid increases the time limit(>20 years) for the development of damaging action[33].

Occupation and laryngeal cancers

In a study by Paget-Bailley *et al*,99 publications relating to occupational exposure and laryngeal cancers were analysed.Exposure to polycyclic aromatic hydrocarbons , engine exhaust, textile dust, and working in the rubber industry were found to have increased meta-relative risk. No significant association was noted with exposures to wood dust, formaldehyde, and cement dust[34].Increased incidence of laryngeal squamous cell

carcinoma is seen in nickel and chromate refining workers[35].There is probable association between asbestos and laryngeal cancers[36].

Diet and laryngeal carcinoma

There is an increased risk for laryngeal carcinoma with low intake of fruits and vegetables.Low intake of vitamin C, beta-carotene and vitamin E were also associated with increase risk for laryngeal cancers[37].Direct association with laryngeal cancers were noted with animal products and the animal unsaturated fatty acids .Vegetable unsaturated fatty acids and the starch-rich patterns did not show any significant laryngeal cancer risk[38].

Laryngeal Cancer and genetics

Mutations maybe produced by activation of proto-oncogenes and inactivation of tumour suppressor genes.The cell turnover is maintained by an equilibrium between growth promoting and growth restraining signal transduction and the natural cell loss.Mutations occur in the form of deletions, rearrangements, point mutations, translocations or reduplications.Aggregation of these genetic alterations give rise to multistep carcinogenesis[39].

Familial predisposition and laryngeal cancers

Incidence of upper aerodigestive tract cancers is higher in first degree relatives of head and neck cancer patients as compared to cancer free controls[90].The intermediate metabolites formed in an attempt to detoxify the carcinogens are more carcinogenic than the carcinogens itself.An efficient enzyme system is essential to detoxify the danger

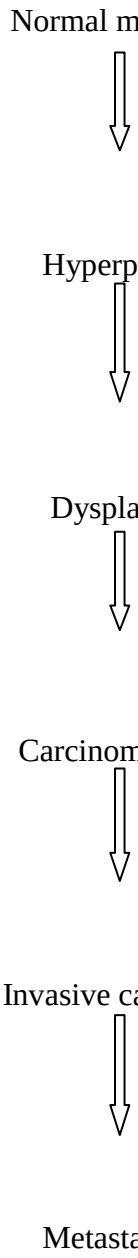
compounds.Glutathione S-transferase and *N*-acetyltransferase 1 are important enzymes in this context. Acetylating action of variety of carcinogenic aromatic amines is done by *N*-acetyltransferase 1.A Japenese study on a series of oral squamous cell carcinomas revealed increased risk for people with a particular polymorphism of N-acetyltransferase1[91].

GST family consists GST α,μ (M), π (P) and θ (T).Polymorphism in several members of the GST family has been analysed in HNSCC. GSTMI AB genotype may be associated with a lower risk for all HNSCC.GSTM3 BB genotype is specifically associated with lower risk of laryngeal cancers.GSTP 1 AA genotype is specifically associated with a lower risk of oral/pharyngeal cancers[40].Lack of GSTMI gene in induvidals have been shown to be at an increased risk for all types of HNSCC[41].

Molecular biology of laryngeal cancers

The molecular biology of laryngeal squamous cell carcinoma is a complex system and no single entity is responsible for carcinogenesis.The characteristic features of cancer are increased cell proliferation and decreased cell death.This is brought about by inactivation of tumour suppressor genes,activation of oncogenes or both.Progressive accumulation of genetic alterations lead to selection of a clonal population of transformed cells and lead to cancer[42].The precise number of genetic alterations needed before development of cancer is difficult to define.However Renan in 1993 put forth a statistical model in which he suggested between six to ten genetic alterations have to accumulate for carcinogenesis[43]. Knudson put forth his model of the "two-hit" hypothesis in 1971. He described a model in which two copies of the parentally inherited Rb gene were inactivated either by mutation or by loss of chromosomal material, leading to

development of hereditary or sporadic retinoblastoma[44] Califano and associates used microsatellite analysis and correlated allelic imbalance due to chromosomal loss and gain with varying grades of dysplasia in premalignant lesions.He defined the progression of normal mucosa to invasive carcinoma which is demonstrated by the following flowchart[39]



There were clonal, genetic changes in even the earliest of lesions.The first genetic alterations to occur in the progression to cancer were loss at 9p21 or 3p and the

corresponding inactivation of p16 and p14/aRF and putative 3p tumour suppressor genes. The model demonstrates the increased abberations in chromosomes as we go from normal mucosa to invasive carcinoma.There is loss of 3p and 9p as hyperplasia progresses to the stage of dysplasia.Dysplasia is classified into mild,moderate and severe grades. 17p loss result in transformation from mild dysplasia to moderate dysplasia whereas p53 mutation, 11 q13 amplification cause severe dysplasia.Loss of 17p13, 3p25, 3p14, 8q, 13q 14q cause progression of dysplasia into carcinoma insitu.Invasive carcinoma results from carcinoma insitu by amplification of 4q, 6p, 8p, 18q loss, 3q.Overexpression of matrix metalloproteinases MMP-2 and MMP-9 causes metastasis.There is overexpression of Epidermal growth factor(EGFr) seen in the first stage of hyperplasia of cells.

The concept of field cancerization was described by Slaughter and colleagues.They recognized histopathologic changes in the epithelia surrounding the invasive tumours and increased incidence of second primary tumours.

WHO Classification of laryngeal tumors

WHO has classified laryngeal tumours based on the histology into non-neoplastic lesions, pre-malignant lesions and primary laryngeal malignancies[1]. Primary laryngeal malignancies were again subdivided into Epithelial,malignant salivary gland tumors,neuroendocrine tumors,malignant soft tissue tumors,malignant tumors of bone and cartilage,haematolymhoid tumors.The WHO classification of primary laryngeal malignancies is listed below.

Primary Laryngeal Malignancies

Epithelial

Squamous cell carcinoma (SCC)

Verrucous SCC

Spindle cell carcinoma

Adenoid SCC

Basaloid SCC

Clear cell carcinoma

Adenosquamous carcinoma

Giant cell carcinoma

Lymphoepithelial carcinoma

Malignant salivary gland tumors

Adenocarcinoma

Acinic cell carcinoma

Mucoepidermoid carcinoma

Adenoid cystic carcinoma

Carcinoma ex pleomorphic adenoma

Epithelial-myoepithelial cell carcinoma

Salivary duct carcinoma

Neuroendocrine tumors

Carcinoid tumor

Atypical carcinoid tumor

Small cell carcinoma

Malignant paraganglioma

Malignant soft tissue tumors

Fibrosarcoma

Malignant fibrous histiocytoma

Liposarcoma

Leiomyosarcoma

Rhabdomyosarcoma

Angiosarcoma

Kaposi's sarcoma

Malignant hemangiopericytoma

Malignant nerve sheath tumor

Alveolar soft part sarcoma

Synovial sarcoma

Ewing's sarcoma

Malignant tumors of bone and cartilage

Chondrosarcoma

Osteosarcoma

Hematolymphoid tumors

Lymphoma

Extamedullary plasmacytoma

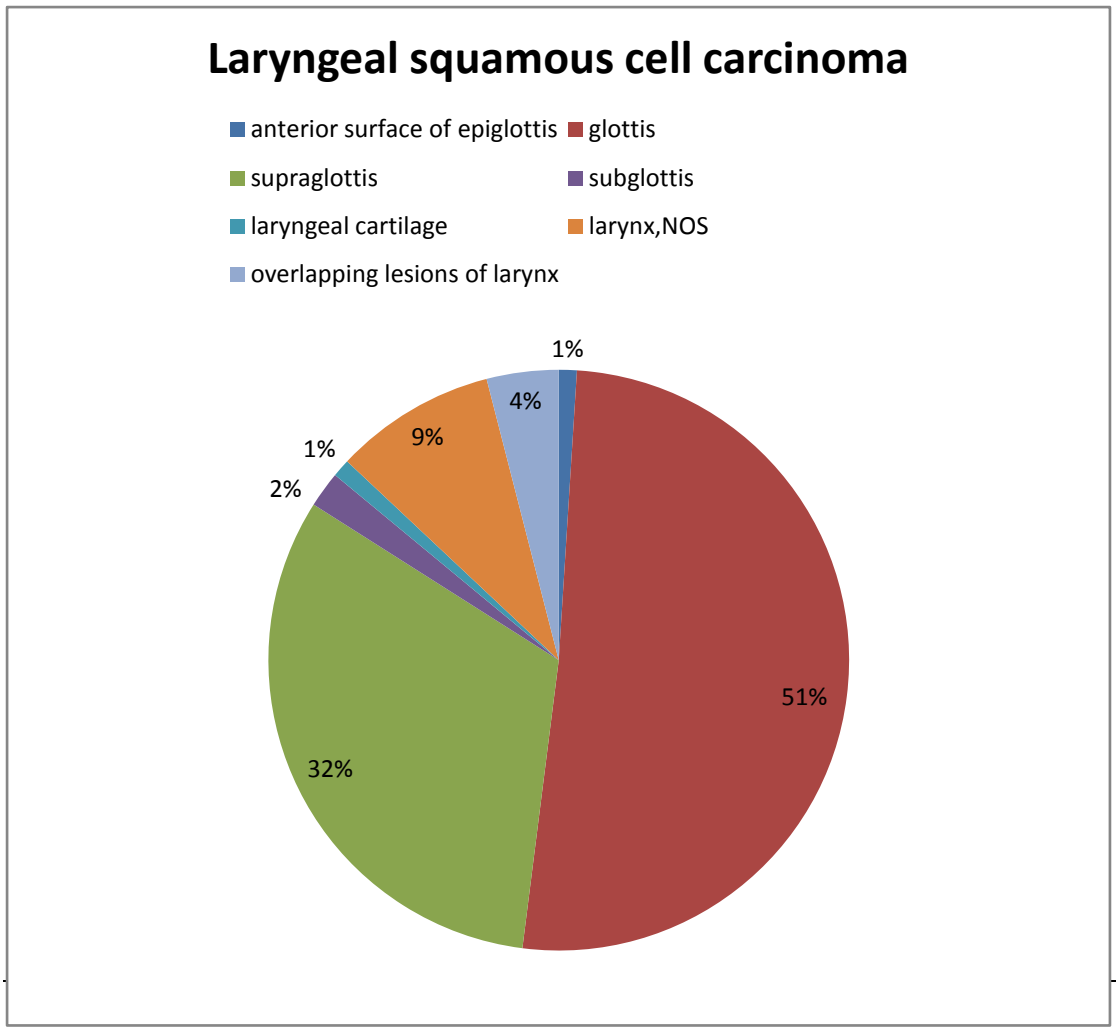


Fig.9 Distribution of laryngeal squamous cell carcinoma

Squamous Cell Carcinoma of the Larynx

Among the malignant tumors of larynx,squamous cell carcinoma is the most common.It accounts for 85-95% of laryngeal cancers.Squamous cell carcinoma may be macroscopically exophytic or endophytic. Microscopically it has 'prickle'cells and keratin whorls.The second most common malignancies among laryngeal cancers are lymphomas.

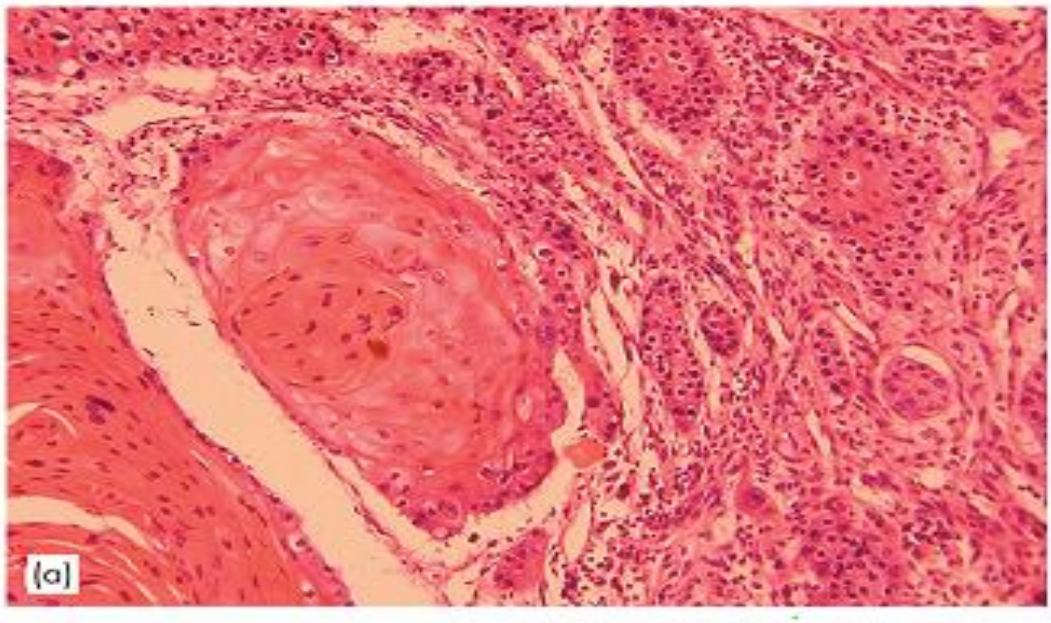


Fig.10 Squamous cell carcinoma microscopy

Pathology of laryngeal squamous cell carcinoma

The characteristic feature of squamous cell carcinoma is squamous differentiation.It is defined by the formation of keratin and/or the presence of intercellular bridges[45]. SCC is graded by its histologic appearance into three categories: well, moderately, and poorly differentiated. Well-differentiated SCC resembles normal squamous epithelium and contains basal-type cells and squamous cells with keratinization and intercellular bridges. The nuclei are hyperchromatic and irregular in size and shape (pleomorphic), and the nuclear-cytoplasmic ratio is reduced. Atypical mitoses are rare. Moderately differentiated SCC has less keratinization, more atypical mitoses, and more nuclear pleomorphism. Intercellular bridges are present. Poorly differentiated SCC has minimal keratinization, minimal intercellular bridges, and numerous atypical mitoses[87].

The histologic grade has been reported as having prognostic value.However, grading is subjective, and sampling error may influence the grading pattern.SCC invades the underlying tissue by breaching the basement membrane.The pattern of invasion can be expansive when there is well defined margins or infiltrative when there is ill defined margins. *SCC in situ* is the term assigned to a lesion in which the entire thickness of the epithelium shows the cellular features of carcinoma without invasion of the underlying stroma. Microinvasive SCC refers to SCC in which limited tumor invasion is confined to the area just deep to the basement membrane.

Necrotising sialometaplasia and pseudoepitheliomatous hyperplasia are two entities which needs to be distinguished from squamous cell carcinoma.Immunohistochemistry is used to differentiate from these entities[46]. Epithelial markers such as cytokeratin and epithelial membrane antigen are expressed in squamous cell carcinoma.

Clinical presentation and staging

The symptoms of laryngeal SCC depend on the site from which the lesion arises.Acording to the site of origin the presenting symptoms vary. Supraglottic tumors may present with dysphonia,dysphagia, odynophagia, otalgia, stridor, dyspnea, or hemoptysis. There is rich lymphatic supply for the supraglottis.Hence patients with supraglottic SCC may also present with metastatic cervical adenopathy, without obvious laryngeal symptoms. Supraglottic SCC usually metastasizes to levels II, III, and IV. Levels I and V are involved by metastases rarely and only when other nodal levels are also involved[88].

The cardinal symptom of glottic SCC is hoarseness of voice which develops early in the natural history of the disease as the normal vibratory characteristics of the vocal cord are

altered by even a small lesion. Therefore patients with glottic SCC usually present with earlier stages of disease[47]. Glottic tumors remain localized in the glottis for prolonged periods, owing to the natural barriers to tumor spread (ligaments, membranes, and cartilages) and to the relative paucity of glottic lymphatics.If the early symptoms are ignored or attributed to other diagnoses, symptoms of advanced disease such as dyspnea and stridor may arise.SCC of the subglottis often presents with advanced-stage disease.Dyspnea and stridor are the most common symptoms of subglottic SCC. Because their onset is usually gradual and insidious, subglottic SCC may be misdiagnosed as asthma or other pulmonary diseases.

Distant metastases from laryngeal SCC include not only hematogenous metastases to distant organs, but also lymphatic metastases to nodal groups outside the neck.The most common site for distant hematogenous metastases is the lung. The mediastinum is the most common site for distant lymphatic metastases[89].The liver and skeletal system (ribs, vertebrae, and skull) are affected less often.

TNM staging of laryngeal cancers

Supraglottis

T1 Tumor limited to one subsite of supraglottis with normal vocal cord mobility

T2 Tumor invades mucosa of more than one adjacent subsite of supraglottis or glottis or region outside the supraglottis (e.g., mucosa of base of tongue, vallecula, medial wall of pyriform sinus) without fixation of the larynx

T3 Tumor limited to larynx with vocal cord fixation and/or invades any of the following: postcricoid area, pre-epiglottic tissues, paraglottic space, and/or minor thyroid cartilage erosion (e.g., inner cortex)

T4a Tumor invades through the thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of neck including deep extrinsic muscle of the tongue, strap muscles, thyroid, or esophagus)

T4b Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures

Glottis

T1 - Tumour limited to vocal cord(s) (may involve anterior and posterior commissure) with normal mobility.

T1a – Tumour limited to one vocal cord. (Fig.11)

T1b – Tumour involves both vocal cords

T2 – Tumour involves supra glottis and / or subglottis and /or with impaired vocal cord mobility

T3 – Tumour limited to larynx with vocal cord fixation and/or invades paraglottic space and / or minor thyroid cartilage erosion.

T4a – Tumour invades through thyroid cartilage and / or invades tissue beyond the larynx (eg: trachea, soft tissues of neck including deep extrinsic muscles of the tongue, strap

muscles, thyroid or oesophagus)

T4b – Tumour invades pre vertebral space, encases carotid artery or invades mediastinal structures

Subglottis

T1 Tumor limited to the subglottis

T2 Tumor extends to vocal cord(s) with normal or impaired mobility

T3 Tumor limited to larynx with vocal cord fixation

T4a Tumor invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx (e.g., trachea, soft tissues of neck including deep extrinsic muscles of the tongue, strap muscles, thyroid, or esophagus)

T4b Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures

<u>Regional lymph nodal staging</u>

NX Regional lymph nodes cannot be assessed.

N0 There is no regional nodes metastasis.

N1 Metastasis is in a single ipsilateral lymph node, 3 cm or less in greatest dimension.

N2 Metastasis is in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or metastasis

is in multiple ipsilateral lymph nodes, none more that 6 cm in greatest dimension; or metastasis is in bilateral or contralateral lymph nodes, none greater than 6 cm in greatest dimension.

N2a Metastasis is in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension.

N2b Metastasis is in multiple ipsilateral lymph nodes, none more that 6 cm in greatest dimension.

N2c Metastasis is in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.

N3 Metastasis is in a lymph node more than 6 cm in greatest dimension.

Distant Metastasis (M)

MX Distant metastasis cannot be assessed.

M0 There is no distant metastasis.

M1 There is distant metastasis.

Stage	**T**	**N**	**M**

Stage	T	N	M
0	Tis-	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	T4b	Any N	M0
	Any T	N3	M0
IVC	Any T	Any N	M1

Adopted from AJCC: Laryngeal. In: Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 57-67.

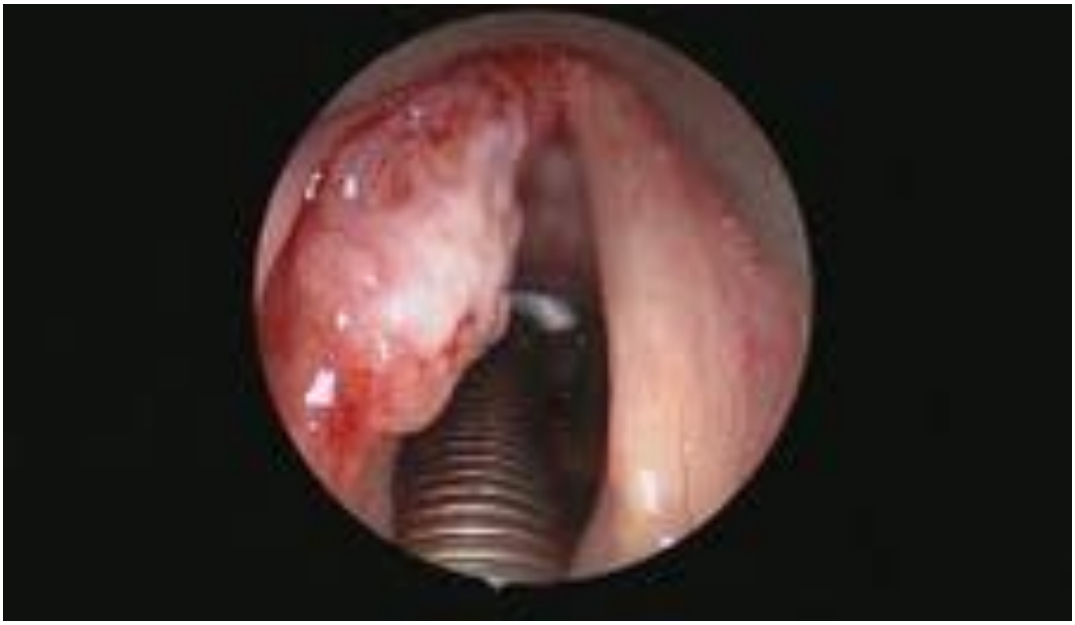


Fig.11 T1a glottic carcinoma

Prognosis and prognosis predictors in laryngeal cancers

The prognosis of laryngeal cancer patients depends on disease factors and patient factors.The 5 years survival rate is 64%, the rate for individual sites being 47% for supraglottic SCC, 79% for glottic SCC, and 30%-50% for subglottic SCC[48].Clinical staging of the disease is an important prognosis predictor of the disease.In TNM staging,with increase in T and N,the prognosis becomes poor.N value is more important factor than T value[49].The histological grading pattern,invasion pattern and peineural or vascular invasion may influence the survival and locoregional control[50].Epidermal growth factor overexpression predicts chemosensitivity and radiosensitivity.Disease free survival is predicted by overexpression of cyclin D1/D2.Overexpression of both has the worst prognosis[51].In a metaanalysis conducted for HPV in head and neck cancer,HPV related oropharyngeal cancers had a better prognosis compared to non HPV types[52].

Human papilloma virus

Papilloma viruses are icosahedral non-enveloped viral particles belonging to the taxonomic family papillomaviridae. They are DNA viruses.It has a circular double stranded DNA molecule of approximately 8 kb. The number of HPV types identified has reached 96 and will soon exceed 100.The first PV types were isolated about 30 years ago.[53]They are divided into high risk types,intermediate type and low risk types according to cervical carcinogenesis.[2] All open reading frame (ORF) protein-coding sequences are restricted to one strand.

Functionally,the genome is divided into three regions [54](Fig.12)

(1)A non-coding upstream regulatory region of 400 to 1,000 bp, which is known as noncoding region or the long control region (LCR), or the upper regulatory region.

This region contains the p97 core promoter along with enhancer and silencer sequences that regulate DNA replication by controlling the transcription of the ORFs. (2) The second is an early region consisting of ORFs E1,E2, E4, E5, E6, and E7, which are involved in viral replication.(3) third is a late region, which encodes the L1 and L2 structural proteins for the viral capsid.

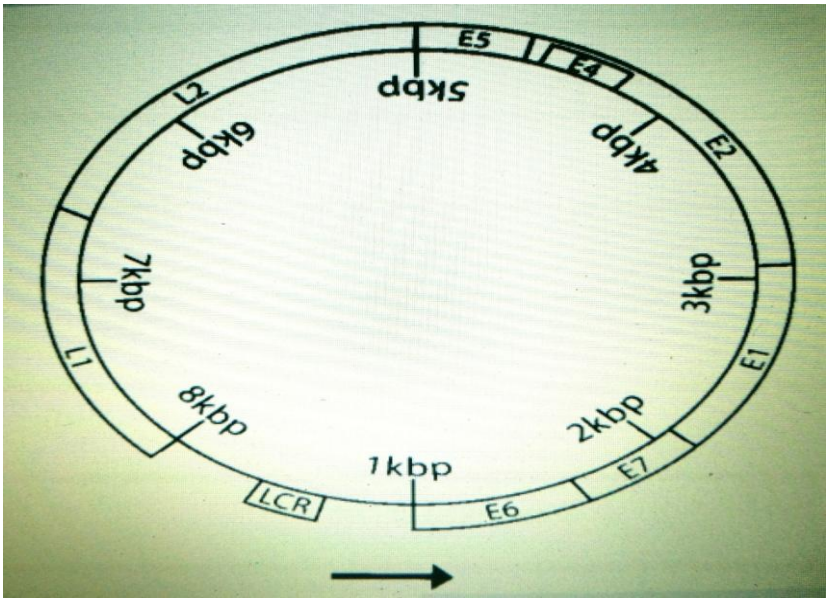


Fig.12 HPV genome

E1 to E7 are seven early open reading frames which encode proteins involved in DNA replication, transcription, and cellular transformation.L1 and L2,the capsid proteins are encoded by late open reading frames.Early genes E1 and E2 are expressed as proteins that bind to DNA and act as transcriptional activators or repressors, thus regulating virus transcription and genome replication. The E4 gene is involved in maturation and release of papillomavirus particles and is expressed relatively late in virus replication.E6 and E7 are described as viral oncogenes.They encodes proteins which bind to the tumour suppressor proteins. E6 induces degradation of tumor suppressor protein p53 by encoding a protein that binds to it. E7 encodes a protein that binds to retinoblastoma protein(Rb) [87]. Long control region (LCR) is the non-coding region which contains regulatory sequences that respond to steroid receptor hormones.[55]

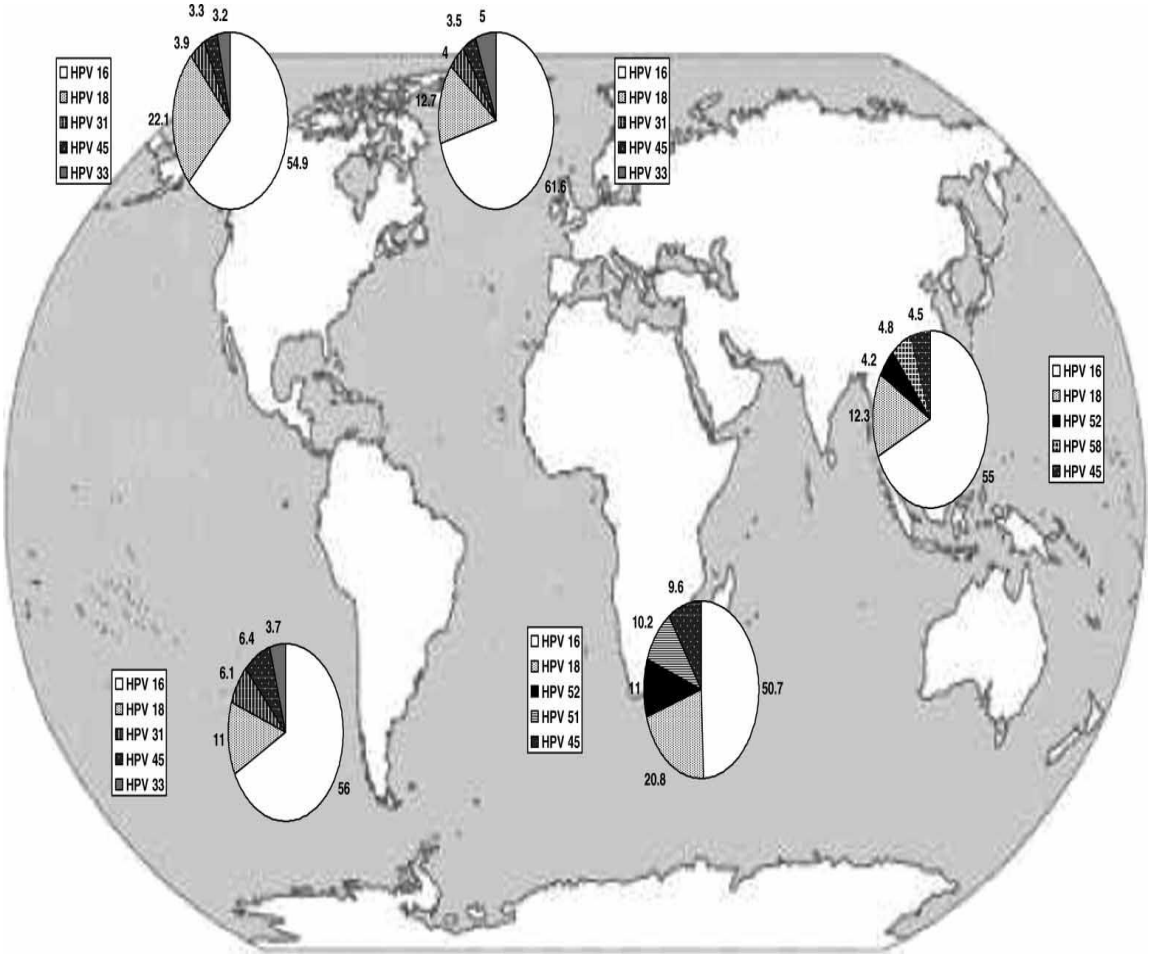


Fig.13 Geographical distribution of HPV virus[56]

HPV 16 and 18 together account for nearly 70% of cases of cervical cancer worldwide, but the relative distribution may vary according to ethnic and regional conditions. In Asia the third most frequent type is HPV 58 and then HPV 52[3](Fig.13).

HPV is an epitheliotrophic virus of almost 8000 base pairs.They are organized in early (E) and late (L) transcriptional genes. HPV is an obligatory intranuclear virus.The infection can access basal and parabasal cells in 3 different sites: at the site of mucosal injury, metaplastic epithelium, or the squamocolumnar junction.[57] An HPV genotype is defined as a distinct one when the nucleotide sequence of its L, E6, and E7 genes differs from that of any other by at least 10%.pRb causes cell cycle arrest in mid to late G 1

phase where it is underphosphorylated by negative regulation of cell proliferation.[58]. Wild-type p53 acts as a cell cycle checkpoint after DNA damage.It induces G 1 arrest or apoptosis and maintain genomic stability.

The papillomaviruses were originally clumped together with the polyomaviruses in one family, the *Papovaviridae*. This was because both groups had nonenveloped capsids and the common circular double-stranded DNA genomes. But it was later recognized that the two virus groups have different genome sizes, genome organizations, and no major nucleotide or amino acid sequence similarities.Hence the International Committee on the Taxonomy of Viruses (ICTV) have now officially recognized them as two separate families, *Papillomaviridae* and *Polyomaviridae.* The L1 ORF is the most conserved gene within the genome.This gene has therefore been used for the identification of new PV types.For a new PV isolate to be recognized,the complete genome has to be cloned and the DNA sequence of the L1 ORF should differ by more than 10% from the closest known PV type. Differences between 2% and 10% homology define a subtype and a variant described by less than 2 %. This definition was agreed upon between all PV scientists working on PV taxonomy and diagnosis at the International Papillomavirus Workshop held in Quebec in 1995.The genus of papillomavirus contain alpha-,beta-,gamma-,delta-,epsilon-,zeta-,eta-,thota-,lota-,kappa-,lambda-,mu-,nu-,xi-,pi-papilloma viruses.[3]

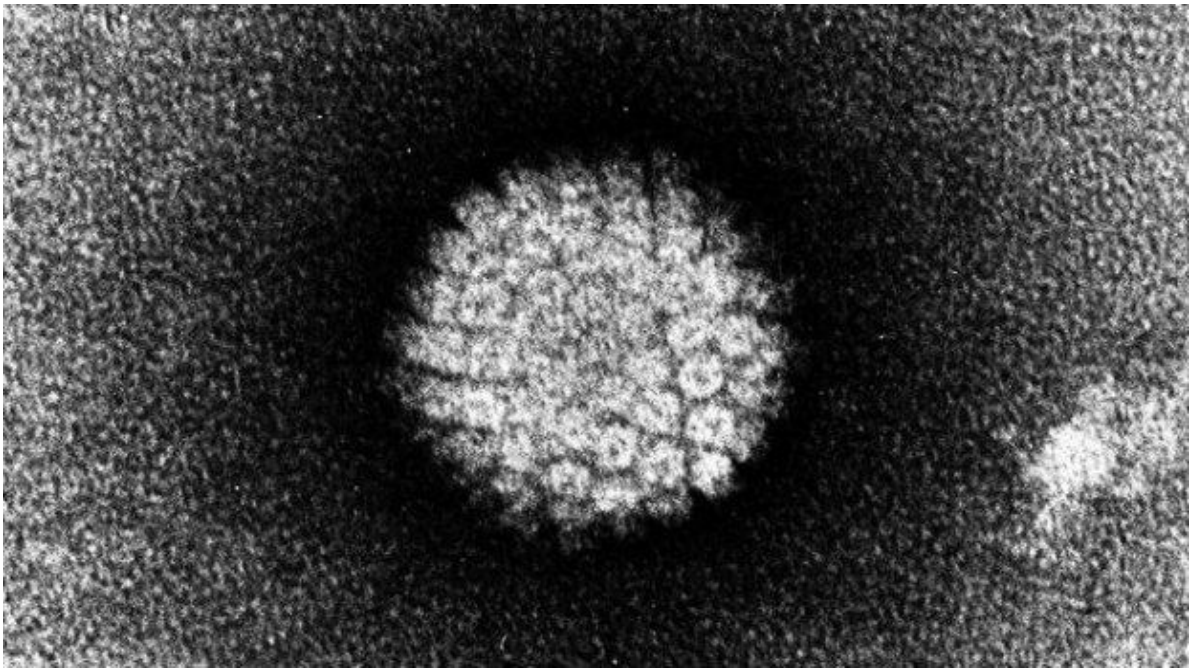


Fig.14 Human papilloma virus, under electron microscope(*Adopted from National Institutes of Health)*

Infections caused by HPV virus Benign lesions

Human papilloma virus is the causative organism for a wide range of diseases starting from common warts to head and neck squamous cell carcinoma and cervical carcinomas.HPV are known to cause laryngeal papillomas, anogenital condylomas, skin cancer in patients with

epidermodysplasia verruciformis[59] and anal cancer. Type 1, 2, and 4 are associated with common warts and plantar warts. Human papilloma virus associated with flat warts are Types 3, 10, 28, and 41. Types 5, 8,9, 12, 14, 15, 17, 19-25, 36, 46, and 47 are found in are epidermodysplasia verruciformis (EV) .Veneral warts or condyloma acuminate(CA) are caused by low risk type HPV 6 and HPV 11[60].

Laryngeal papillomatosis is the most common benign neoplasm of the larynx accounting for 84% of benign tumours[61](Fig.15).It was first described by Morrell Mackenzie in 1880.The condition is more frequently seen in children and is clinically divided into juvenile and adult onset laryngeal papillomatosis.Rarely,there can be malignant transformation for the papilloma lesions.It is seen in 3-7% of cases[62]Laryngeal papillomatosis is notorious for its recurrence.HPV 6 and HPV 11 are the most common causative organism for laryngeal papillomatosis eventhough other types have also been implicated.Most studies reveal HPV 11 disease to be more aggressive than HPV 6[63].The means of transmission in children is believed to be from mother's HPV infected genital tract and in adults through orogenital spread[64],[65].Hoarseness is the most common symptom at presentation because vocal folds are the first and predominant site of papilloma.The child's voice may be described as hoarse or weak from the time of birth. Stridor is often the second clinical symptom to develop, beginning as an inspiratory noise and becoming biphasic with progression of the disease. Other presenting symptoms maybe less commonly, chronic cough, recurrent pneumonia, failure to thrive, dyspnoea, dysphagia, or acute life-threatening events may be the presenting symptoms. After presentation, the disease may undergo spontaneous remission or persist in a stable state requiring only periodic surgical treatment. At the other extreme, RRP may become extremely aggressive, requiring frequent surgical treatment prompting early institution of medical adjuvant therapy. The adjuvant treatment modalities are antiviral therapy,photodynamic therapy,anti reflux therapy,indole-3-carbinol,retinoids and celecoxib[66].Antiviral therapy include alpha-interferon[67],ribavarin,cidofovir and acyclovir.

Malignant degeneration is a rare but fatal transformation.About twenty pediatric cases with malignant transformation have been reported.Radiating the papillomas increase the

risk of malignant transformation.Adult onset laryngeal papillomatosis is commonly seen in young patients in the age group of 18-39 years.

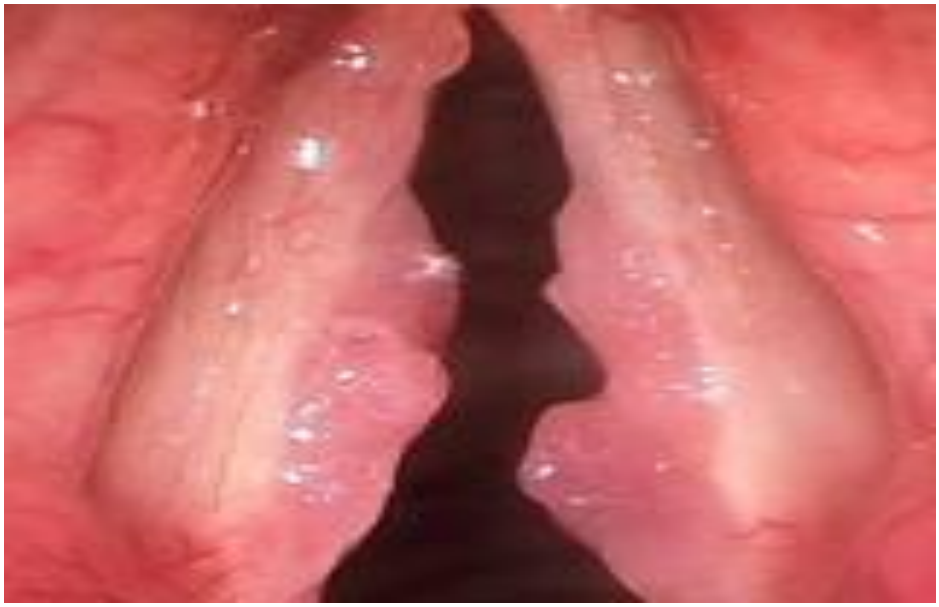


Fig.15 Laryngeal papillomatosis

HPV and oropharyngeal cancers

The oropharynx was the first head and neck site found to be related to HPV related cancer.The cryptic epithelium of the tonsil and tongue base acts as a reservoir for HPV and acts similar to the action in cervical carcinogenesis[68].The viral reservoir provides increased access to the basal epithelial layer.In developed countries,HPV now contribute to 45%-90% of oropharyngeal squamous cell carcinoma cases[69].Recent studies reveal oropharyngeal cancers,more specifically those of tonsils and base of tongue are associated with high risk human papilloma virus infection[70].In a multinational study conducted by the International Agency for Research on Cancer (IARC), only 18% of oropharyngeal tumors were HPV positive[71],Incidence rates for HPV related oropharyngeal cancers are higher in men than in women and oral sex has been the principal risk factor for such

cancers[72].As in other HPV associated cancers,HPV 16 is the commonly detected genotype[73].

The clinical presentation of HPV related OPSCC cases are a little different from that of non HPV related cases.HPV related cases are usually young,without a history of addictions like alcohol or tobacco. They are usually married and college educated and more commonly white[74].Factors which increase the exposure to HPV such as number of oral or vaginal sexual partners,increase in age and infrequent use of barriers[5].

As only high risk types are responsible for the OPSCC,there is role for vaccination in these cancers.Vaccines promote neutralising antibodies that prevent the entry of virion particles.It do not halt the progression of existing lesions.Hence vaccination is only effective before the infection is established[75].

Patients with OPSCC often have TNM staging with a high N and low T[76]. Poorly differentiated, nonkeratinizing, and with basaloid morphology is the usual histological finding[68].

The rate of progression and chances of locoregional spread were lower among HPV related than non HPV related cases.The former had better survival rates compared with non-HPV related cases[68].In a systematic review and metanalysis was conducted to study HPV in orophaaryngeal and non-oropharyngeal cancers by Mehanna *etal.*The study concluded increase in HPV positive cases over the last decade in Europe compared to North America.Non oropharyngeal cancers showed an overall prevalence of 21%.Non oropharyngeal cancers also showed a declining non-significant trend in HPV relation[77].

HPV and cervical cancer

Cervical cancer is the second most common cancer in women worldwide[75].In early 1980s Harold zur Hausen, a German virologist first demonstrated the link between HPV and cervical cancer.He was awarded Nobel prize in Medicine or physiology in 2008 for his discovery.World Health Association, along with the European Research Organization on Genital Infection and Neoplasia and the National Institutes of Health Consensus

Conference on Cervical Cancer, recognized HPV as an important cause of cervical cancer in 1996.HPV 16 and 18 are the most common genotypes causing cervical cancers.Other common types are 31 and 45[54].The tools used in the screening and the diagnosis of cervical neoplastic lesions are papanicolaou testing (Pap smear) and HPV DNA testing[4].A high HPV-DNA background prevalence, combined with an early age at sexual initiation, high number of partners of both men and women, and an important frequency of sexual contacts with prostitutes increases the incidence rate of cervical cancer for a given population . Other risk factors for cervical cancer are oral contraceptives, smoking, other sexually transmitted diseases, poor hygiene, and cervical inflammation[78].Strong relation between human papilloma virus and cervical cancer along with the knowledge about the natural history of the virus has led to the development of prophylactic vaccines for cervical cancers[79].Human papillomavirus L1 self assembling like particles are the contents of these vaccines.They induce strong neutralising antibody like action against papillomavirus infection.These antibodies block the virions from entering the basal layer of the epithelial cells[80].Two vaccines are available the quadrivalent vaccine (Merck, Whitehouse Station, NJ

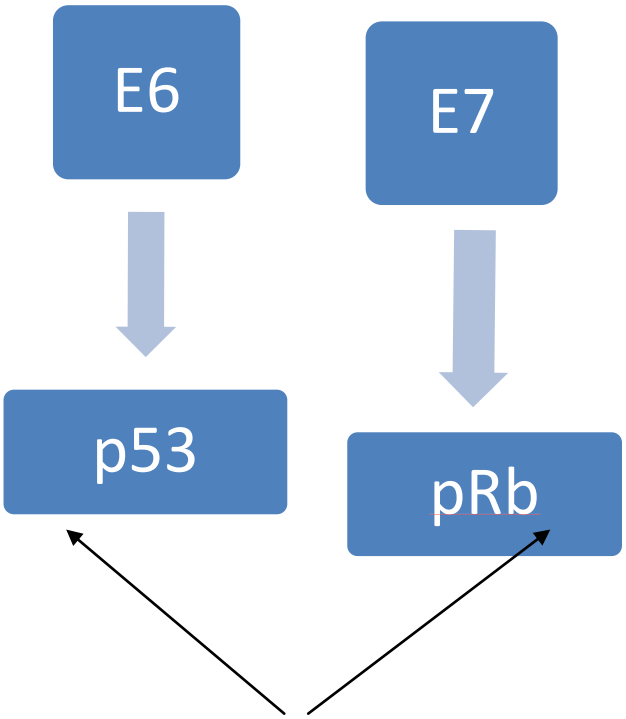
USA) and the bivalent vaccine (GlaxoSmithKline, Rixensart, Belgium).The quadrivalent vaccine contains virus-like particles to human papillomavirus types 6, 11, 16, and 18.Bivalent vaccine contains virus-like particles to human papillomavirus types 16 and 18.The quadrivalent vaccine is marketed as Gardasil by Merck,USA and the bivalent vaccine is marketed as Cervarix by GlaxoSmithKline,Belgium[79](Fig.16).

Fig.16 HPV Vaccines

These HPV vaccines have prophylactic action only and not therapeutic action.They are not effective in an existing disease but prevents the disease[81]. It is important to generate cell mediated immunity against HPV infected cells to control of cervical cancer and treat established HPV infections.It is possible only by development of therapeutic vaccines.Two early proteins of HPV, E6 and E7 oncoproteins, are the preferred targets because they are consistently expressed in virtually all cervical cancer cells and are necessary for the induction and maintenance of HPV-associated disease. Immune responses to these proteins are the basis of development of peptide-based vaccines[82].Overlapping long peptides are the new area of interest in therapeutic vaccines.They can crossover the obstacle of MHC restriction which is a drawback of peptide vaccines.This is brought about by broadening the range of antigenic epitopes through inclusion of immunogenic peptides or peptides that direct CD4+ T-helper or CD8+ cytotoxic immune responses[83].

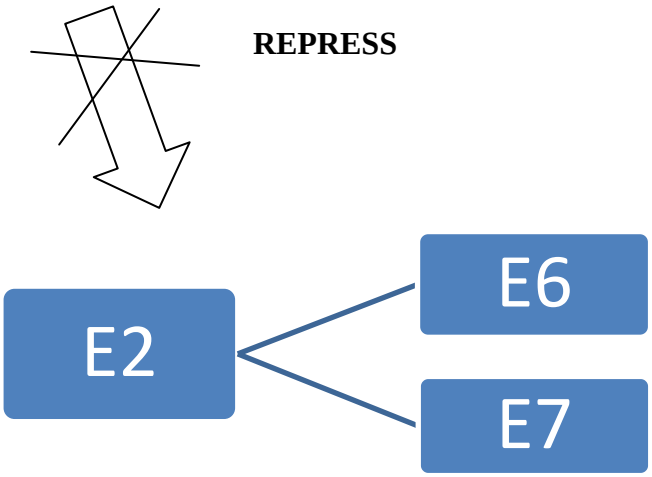
Other than cervical cancer,HPV is also known to cause cancer of the anus,vulva,penis and vagina.Current data shows HPV associated with 90%–93% of anal cancers,36%–40% of penile cancers, 40%–64% of vaginal cancers, and 40%–51% of vulvar cancers[2].

MODE OF ACTION OF HPV



INACTIVATE

INTEGRATION OF HOST & HPV GENOME



The HPV proteins associated with cancer are E6 and E7 . There are six early (E1, E2, E3, E4, E6, and SE7) and two late (L1 and L2) proteins in the HPV genome.When a host cell is infected,two early proteins exons E1 and E2 are expressed first.Early 6 and 7 proteins inactivate tumour suppressor proteins.E6 inactivate p53.E7 inactivate pRb.Expression of E6 and E7 is repressed by high levels of E2.Integration of host and HPV genome,disrupts E2 function.This prevents the repression of E6 and E7[2].

Previous studies on HPV and laryngeal cancers

In a study by Brandsma[93] JL et al,6 patients of veruccous carcinoma of larynx were studied for HPV 16 type related sequences .They used the Southern and DNA dot blot hybridization for HPV DNA.All the 6 patients were positive for HPV 16 type related sequences.

In a study by Bauman[95] et al,38 patients with laryngeal cancer were studied for presence of HPV DNA. HPV DNA was detected in 6 of the 38 lesions.HPV DNA was detected in 16 % of cases. HPV types 16, 26, 31, 39,and 52, and p16 tumor suppressor protein expression was confirmed in 10 representative cases.

In a study by Morshed K[96], et al 3 groups were studied. Ninety three primary laryngeal squamous cell carcinoma (LSCC)tissue samples were collected. Forty nine specimens of normal mucosa were collected.The control group had 22 specimens of laryngeal nodules. Thirty three of the 93 samples from LSCC were positive for Human papillomavirus (35.5%). Four of 49 samples of the normal mucosa showed HPV(8.2 %).HPV was not detected in any of the sample from the control group. Twenty-eight of 33 (81.8%) were positive for HPV-16.6 of 33 (18.2%) were positive for HPV-18. 5 of 33 (15.1%) were positive for HPV-33. Multiple infections were also found. 5 of 33 (15.1%) showed

multiple infections. Three samples were positive for HPV-16 and HPV-33, 2 samples for HPV-16 and HPV-18.

Atula et al studied the relationship between HPV infection and epithelial laryngeal malignancies. 27 laryngeal carcinoma cell lines from 22 patients were studied. Seven of 27 (26%) cell lines and were found to harbour high-risk HPV. Seven of 12 (58%) tumour samples were found to harbour high-risk HPV.

In a study by Almadori [98]et al the presence of HPV DNA was studied in 45 fresh squamous cell carcinoma (SCC) specimens and in 29 normal mucosa specimens collected from 45 primary laryngeal SCC patients.They used polymerase chain reaction.PCR with consensus primers that detect HPV types 6, 11, 16 and 18 were used.20 %,that is 9 out of 45 patients were HPV positive.In normal laryngeal mucosa,in four of the 29 specimens (14%) ,the presence of HPV was detected.

In a study by Kreimer et al,a prevalence of HPV DNA of 24 per cent of laryngeal squamous cell carcinomas was detected.

Liu et al determined the prevalence and genotypes of HPV infection in laryngeal cancer specimens.84 specimens from pathologically confirmed LSCC patients were studied for the presence of viral DNA and possible virus integration into the cellular genome. HPV L1 general primer amplification was used. HPV DNA was detected in 23 of the 84 LSCC samples,that is 27.4%. HPV16 were found in all 23 L1 positive samples.

In a study by Gungor [99]et al Human papilloma virus deoxyribonucleic acid was detected in seven of 95 cases of laryngeal squamous cell carcinoma,that is 7.36 per cent.

In a meta-analysis including 55 studies addressing HPV prevalence and its association with laryngeal cancer the overall HPV prevalence in laryngeal cancer was found to be 28.0% . HPV-16 was the most common subtype, with a prevalence of 19.8% . A significant association was found between HPV infection and laryngeal SCC risk, with a summary OR of 5.39[1].

In a study by Jacob et al, the prevalence of HPV infection in India was found to be 34 percent of invasive laryngeal squamous cell carcinoma.Jacob et al studied the cellular manifestations of HPV in laryngeal cancers. The frequency of HPV infection in various neoplastic and non-neoplastic laryngeal tissues were investigated.The association of HPV with expression of the tumor suppressor protein p53 and the proliferating cell nuclear antigen (PCNA) .The methods used were PCR for HPV detection and immunohistochemisty for expression of PCNA and p53.Six normal laryngeal tissues, 16 laryngeal papillomas and 44 invasive carcinoma tissues were studied. None among the normal laryngeal tissues showed the presence of HPV. Thirteen out of the 16 papillomas were positive for HPV and 15 (34%)out of the 44 invasive cancers were HPV positive.Significant correlation was noted between type of laryngeal neoplasm and p53 accumulation as well as presence of HPV and p53 accumulation and PCNA expression indicating that HPV positive tumours showed significant p53 accumulation and increased proliferation.

From the above studies the paucity of literature in the Indian scenario is well noted.As there is vast difference in the social ,religious and cultural backgrounds when compared to the West,it is important to have more local(Indian) studies on the relationship between HPV and laryngeal cancers.Hence this study is aimed at analysing the association between HPV and laryngeal cancers.

MATERIALS AND METHODS

The purpose of the study is to find the relation between human papilloma virus and laryngeal cancers.

Type of study: Case control study

Period of study: 2 years

Setting:

The study was conducted in the ENT outpatient department and in the ENT operating room at Christian Medical College,Vellore between November 2011 and July 2013.

Cases and controls were evaluated in the ENT outpatient department and explained about the study in detail and were given an information sheet for clarifications. Informed valid consent was taken.Tissue was taken for the study when patient was posted for a direct laryngoscopic/microlaryngoscopic biopsy of the lesion.

Dates of significance in the study are when patient is diagnosed clinically with carcinoma larynx,date of direct laryngoscopic/microlaryngoscopic biopsy of the lesion which is done as part of standard protocol for confirmation of the diagnosis of laryngeal cancer. follow up of the result of the PCR analysis.

Eligibility criteria:

1. Clinically diagnosed cases of carcinoma larynx based on clinical and flexible laryngoscopy examination.
2. Control group will be patients with benign lesions of vocal cord like vocal cord polyps or nodules. Cases and controls will be 1:1 ratio

Exclusion criteria:

1. Post Radiation therapy cases
2. Recurrent cases of carcinoma
3. Laryngeal papillomas

Sample size calculation

From the previous studies in the literature the average prevalence of HPV in laryngeal

carcinoma is 25%

$n=2pq(Z\alpha + Z\beta)^2/(P1-Po)^2$
P0=0.05
P1=Po(OR/Po)/Po(OR-1)+1
P=P1+Po/2
q=1-p
By applying the formula n=73

Hence, in each arm 73 cases should be studied.

But the average number of microlaryngoscopy for cases of laryngeal cancers in CMC is 3-4 per month. Hence the aim was to study 30 cases and 30 controls. As there can be

inadequacy of tissue for PCR analysis in some patients,it was planned to study 40 cases and 40 controls.

Method

New clinically diagnosed cases and controls were recruited for the study. All clinically diagnosed cases of laryngeal malignancies underwent a flexible fibreoptic laryngoscopy in our outpatient department. The findings were carefully noted and extend of lesion, site and airway assessed. Patients with T2 and above lesions also underwent imaging– contrast enhanced computed tomography(CT) of the neck. Patients were explained about the study in detail and patient information sheet was given to the patient .Informed valid consent was obtained from patients who were willing to participate in the study. The proforma for the study was filled in the OPD and patient details were obtained. Patients participated from all the 3 units of our department .Patients were posted for microlaryngoscoy and biopsy of the lesion.

MICROLARYNGO SCOPY-ENT OPERATING ROOM SETTING

Patient is taken under general anaesthesia for microlaryngoscopy and biopsy of the lesion.The steps described in performing a direct laryngoscopy/microlaryngoscopy are as follows:

STEP 1. Patient positioned supine in Boyce’s position with head close to the head end of the bed.

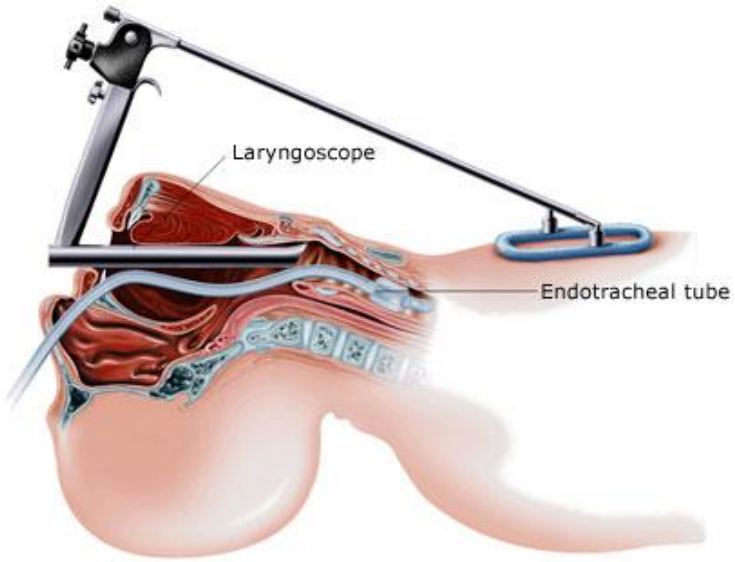
STEP 2. GA was induced by the anesthesia staff.

Patient is intubated using a smaller sized appropriate endotracheal tube to assist the surgeon with more operating space.

STEP 3. Draping of the operative field.

The eyes are carefully taped to prevent corneal abrasions and other injuries. The head is draped with sterile towels.

STEP 4. Check for loose teeth.Insert a tooth guard to protect the dentition.Saline soaked gauze piece maybe used for edentulous patients to prevent mucosal trauma to the alveolar ridge.



STEP 5. Perform oropharyngeal examination.

STEP 6. Continue oropharyngeal examination.

The laryngoscope is gently introduced pressing into the tongue and tongue base .Suction is used to suction out the secretions and saliva.Structures like soft palate,uvula is identified and scope advanced to visualise epiglottis.

STEP 7. Examination of larynx.and hypopharynx

Epiglottis is lifted with the tip of the scope and scope is advanced to examine aryepiglottic folds. The aryepiglottic folds should be followed on both sides to the arytenoid cartilages. The interarytenoid space should be inspected.Both pyriform sinuses and postcricoid region are inspected. Withdraw the scope to the epiglottis, maintaining the tip of the scope inferior to the tip of the epiglottis. Pressure is applied to the tongue base to inspect the false vocal folds and the vocal processes. Vocal folds are viewed by continued base of tongue pressure application.The laryngoscope is fixed using a chest suspension.

STEP 8. Biopsy of the suspected laryngeal lesions is done and tissue taken separately for HPV study.

Tissue is taken for biopsy using suitable miicrolaryngoscopy forceps –straight or upturn and separate tissue is taken from the lesion and is transferred to the VTM tubes.Tubes are transported to the virology lab using ice containers.

STEP 9. Topical anesthetic is used to reduce laryngeal spasm

STEP 10. Remove the laryngoscope and the tooth guard.

STEP 11. Good oral suction needs to be given.

STEP 12. Extubation by anaesthetist.

Sample collection:

Sample tissue was collected in viral transport medium and transported in an ice container to the virology lab.Viral transport medium is a balanced isotonic solution at physiological pH. It maintains the virus in the viable state.It contains fetal calf serum and antibiotics

Once received in lab, the samples were transferred from the VTM tube to a 1.5ml eppendorf tube and stored at -80^{0}C until further testing in -80 degree freezer

DNA Extraction protocol:

The DNA extraction kit used was DNeasy® Tissue kit: (Qiagen GmbH, Hilden, Germany).The principle of DNA extraction is column based separation. Tissues were cut into small pieces weighing 25mg and its digested by adding ATL buffer and Proteinase K at 56C.Once digested, an equal amount of AL (Lysis buffer) buffer and Ethanol were added.

The DNA gets precipitated and its washed twice by adding buffers (AW1 and AW2).The DNA was then eluted by adding elution buffer. The extract containing the DNA was stored at -20C until further testing.

Polymerase Chain Reaction:

The extracted DNA undergoes PCR.A known positive control was used for PCR and beta-globin serves as internal control.Primers used were:

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 was interpreted as positive or negative.Beta globin positive indicates that the mode of sample collection and whether the sample was adequate for analysis.

Sequencing:

If sample was positive for HPV, the amplified PCR products were 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 were run on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, CA, USA).

Finally, the data was analyzed using Bioedit software version 7.0.5.3 and study sequences compared to the GenBank HPV sequences.

WHO recommended CHUV assay had to be used in case of 2 samples which were not able to be sequenced using routine methods.

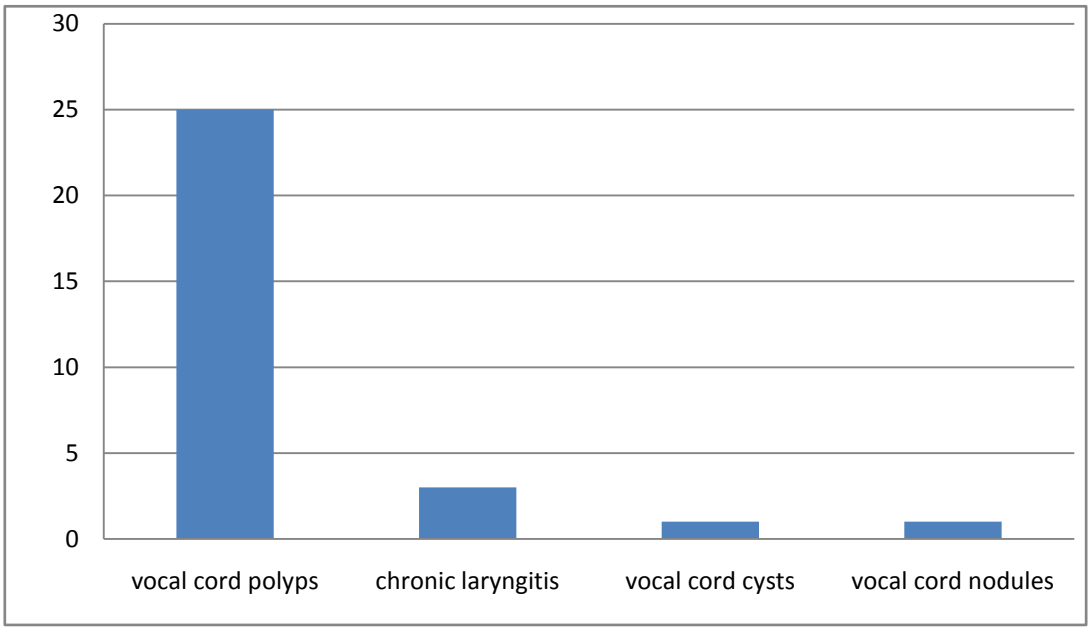
PGMY-CHUV assay was developed at the RRL, Institute of Microbiology, CHUV, in Lausanne. It is an in-house PCR reverse blotting hybridization (RBH) assay.

The assay is relatively cheap (3-4$ per sample, excluding DNA extraction) given the hybridization membrane with covalently linked probes can be reused more than or equal to10 times (up to 400 samples).

RESULTS

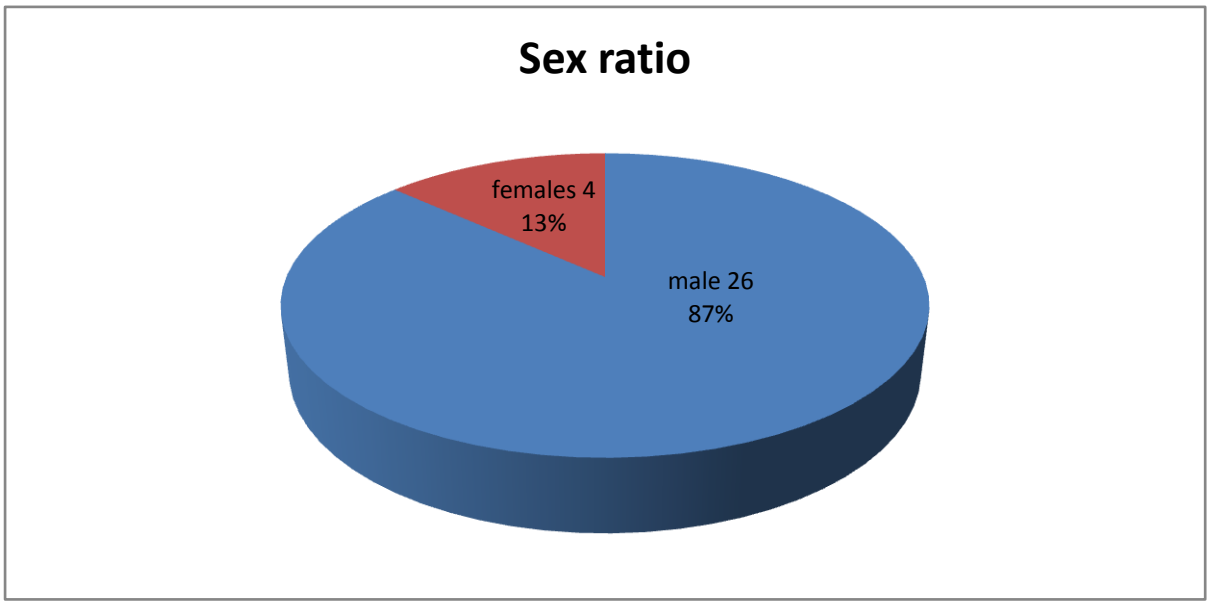
The targeted sample size of the study was 30 cases and 30 controls .We studied a total of 69 patients.Nine patients were excluded as the histopathological reports did not fufill the required criteria.

A total of 30 controls were included in the study.They comprised vocal cord polyps,vocal cord nodules,vocal cord cysts and chronic laryngitis. The various types of vocal cord benign lesions studied were as follows:

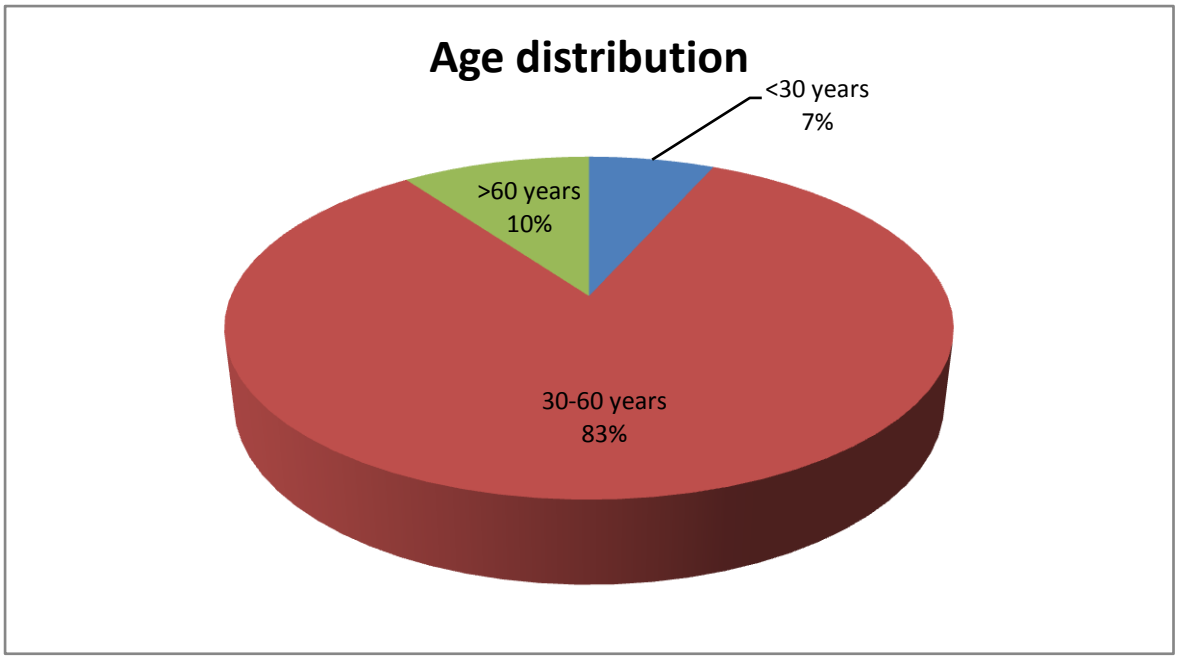


Vocal cord polyps formed the major part of the control study group forming 83% of the controls.The other benign lesions studies were chronic laryngitis,vocal cord cysts and nodules.

Sex ratio of the control population:

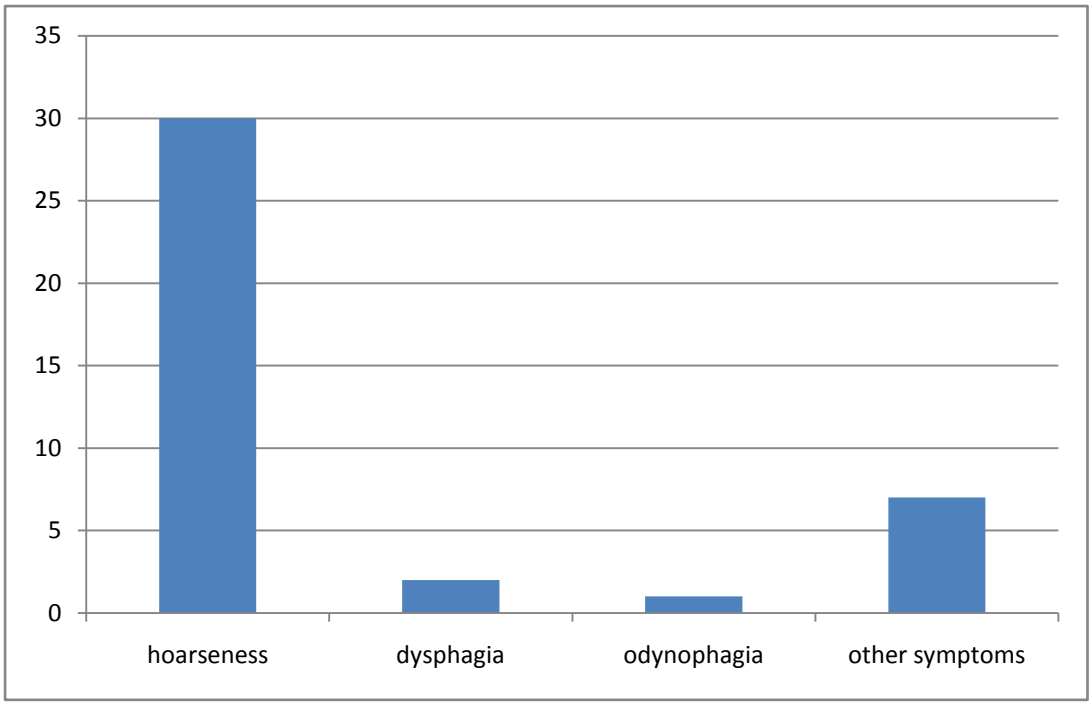


Males comprised 87 % of the study control population.All the four female patients had vocal cord polyps.

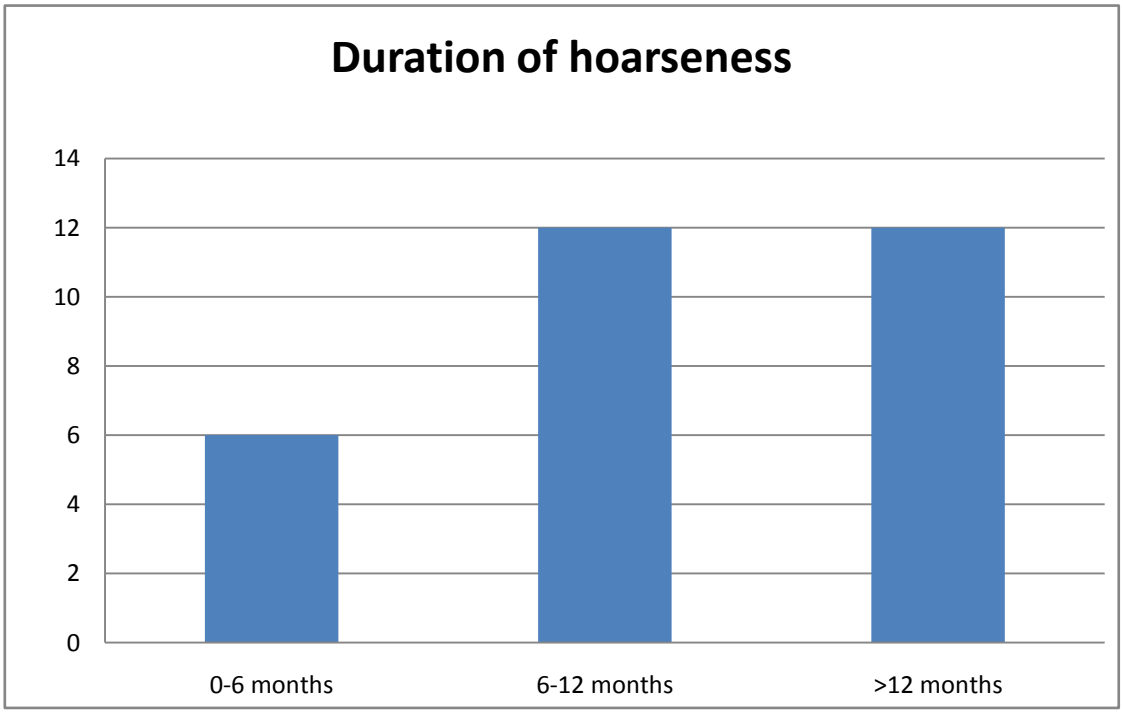


Middle aged people formed most of the study group with benign lesions.

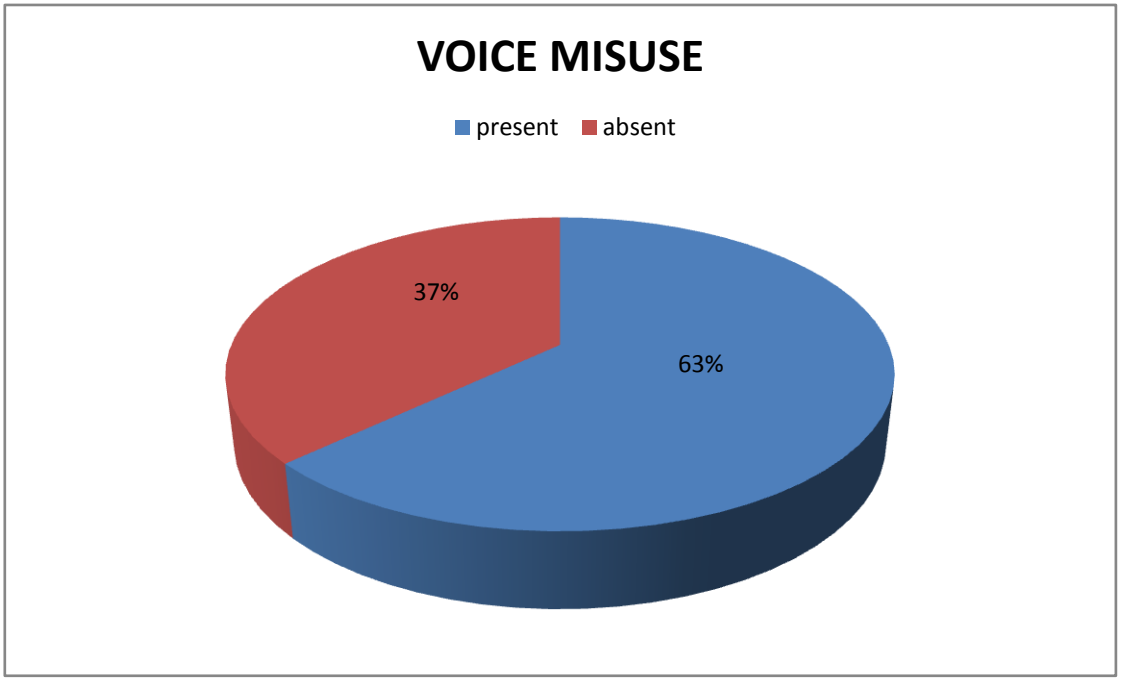
The chief presenting complaint of all the patients in the control group was hoarseness though the duration was variable. Other symptoms were mild dysphagia,foreign body sensation throat voice fatigue and occasional odynophagia.The following table shows the presenting complaints pattern:



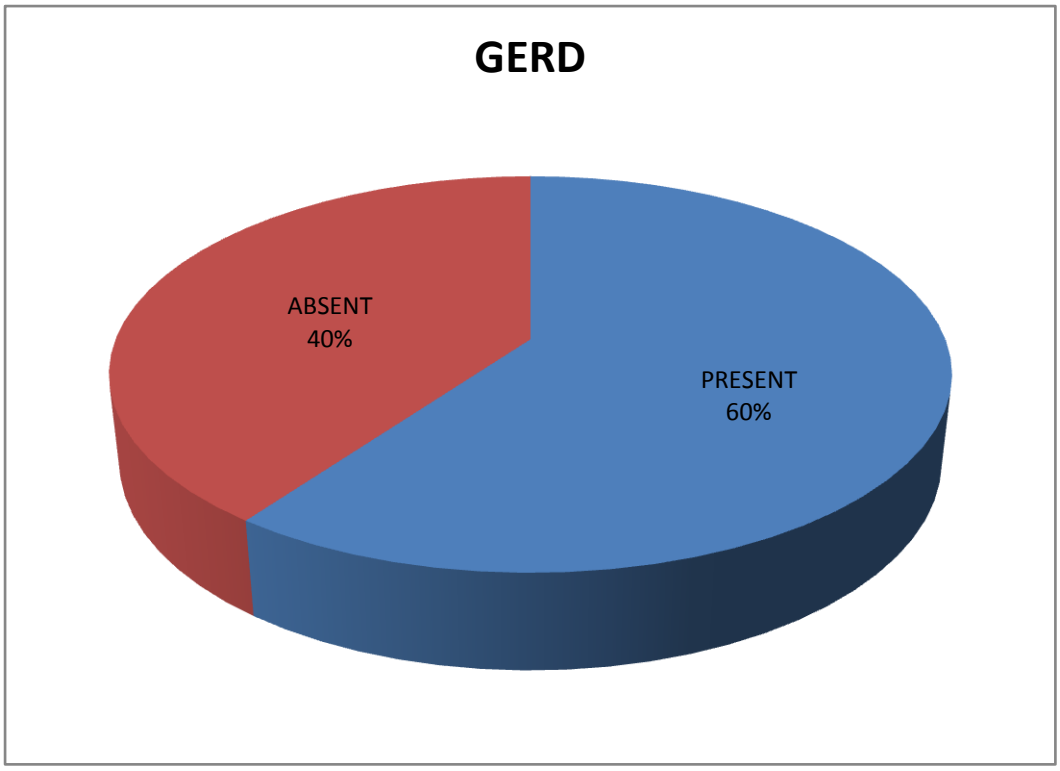
The duration of hoarseness were variable.Majority of patients had a duration of hoarseness for more than 6 months.The maximum duration was 8 years and minimum was of 1 month duration.



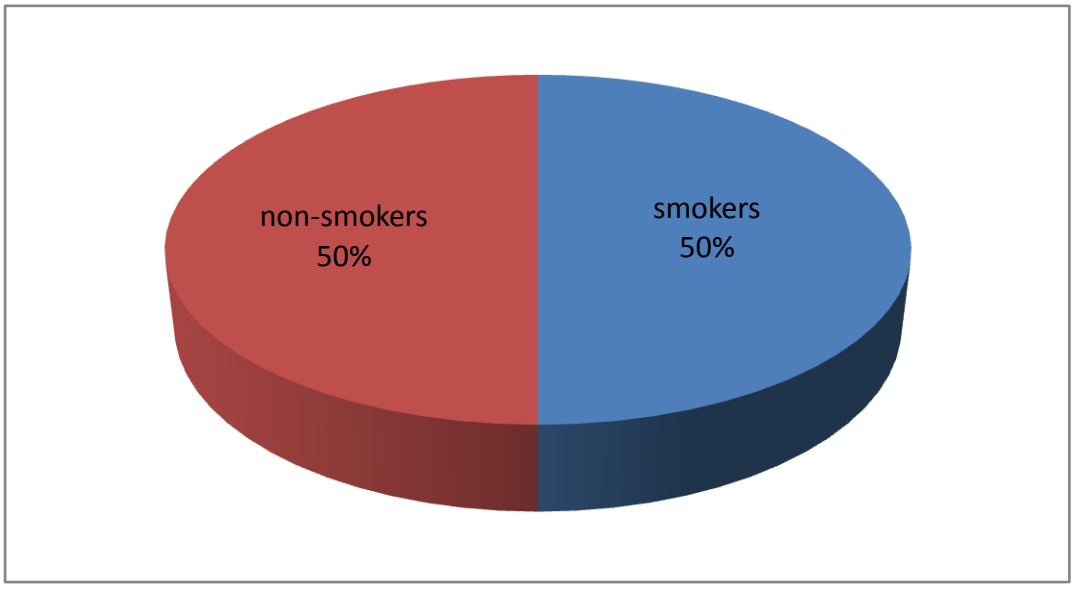
Majority of the patients with benign vocal cord lesions gave history of vocal misuse. Vocal misuse is improper voice usage such as speaking too loudly or at an abnormally high or low pitch. Most of them were occupation related



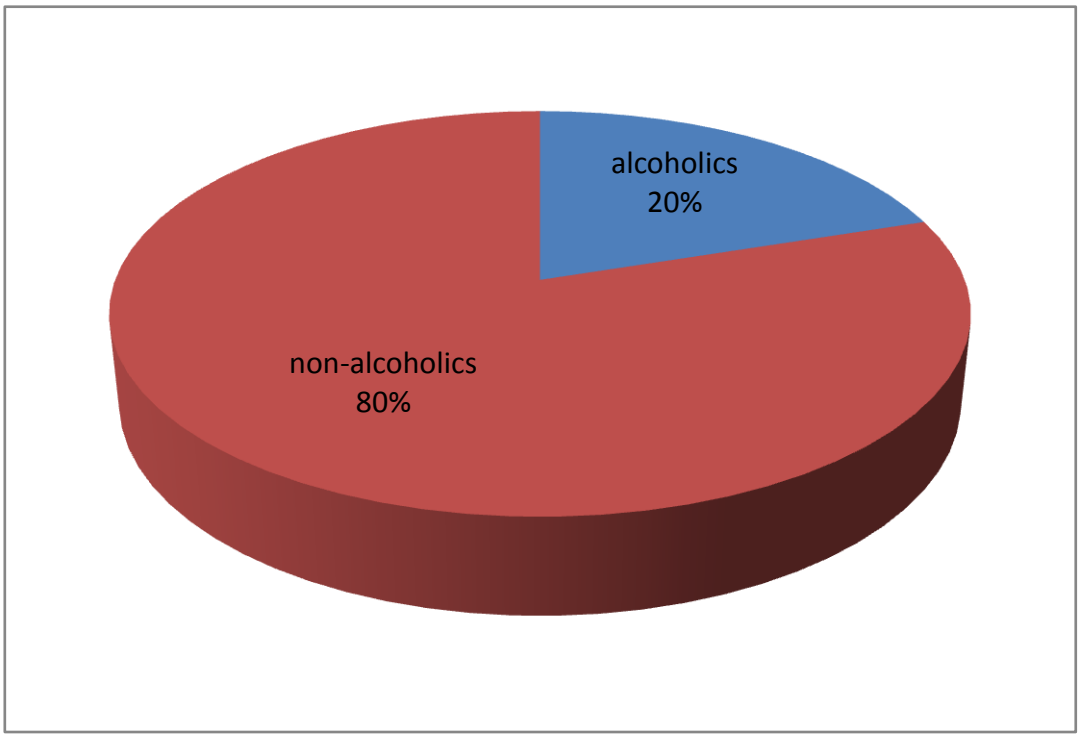
The prevalence of GERD in benign lesions were as follows:



The prevalence of smoking in benign lesions were as follows:



The prevalence of alcohol intake in the control population were as follows:

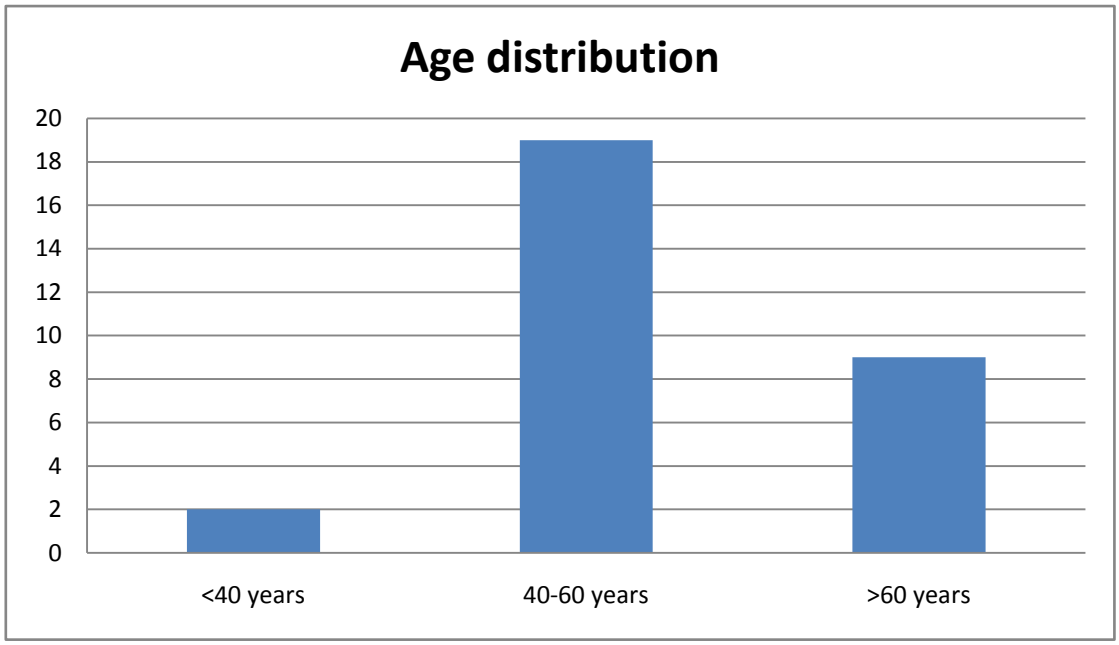


Hence voice misuse was present in 63% of patients with benign lesions stressing its role in causing vocal cord lesions. A history of reflux disease was also found in majority of patients. However smoking and alcoholism were less common among the control population.

We studied 30 cases of squamous cell carcinoma of the larynx.

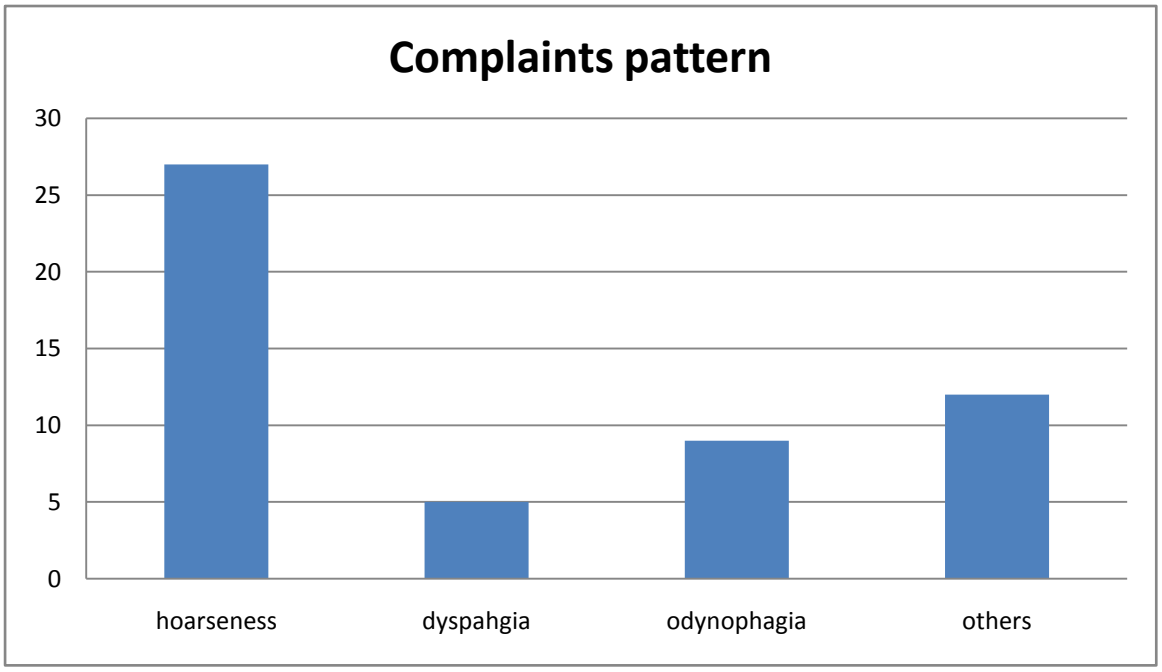
All the cases studied were males.

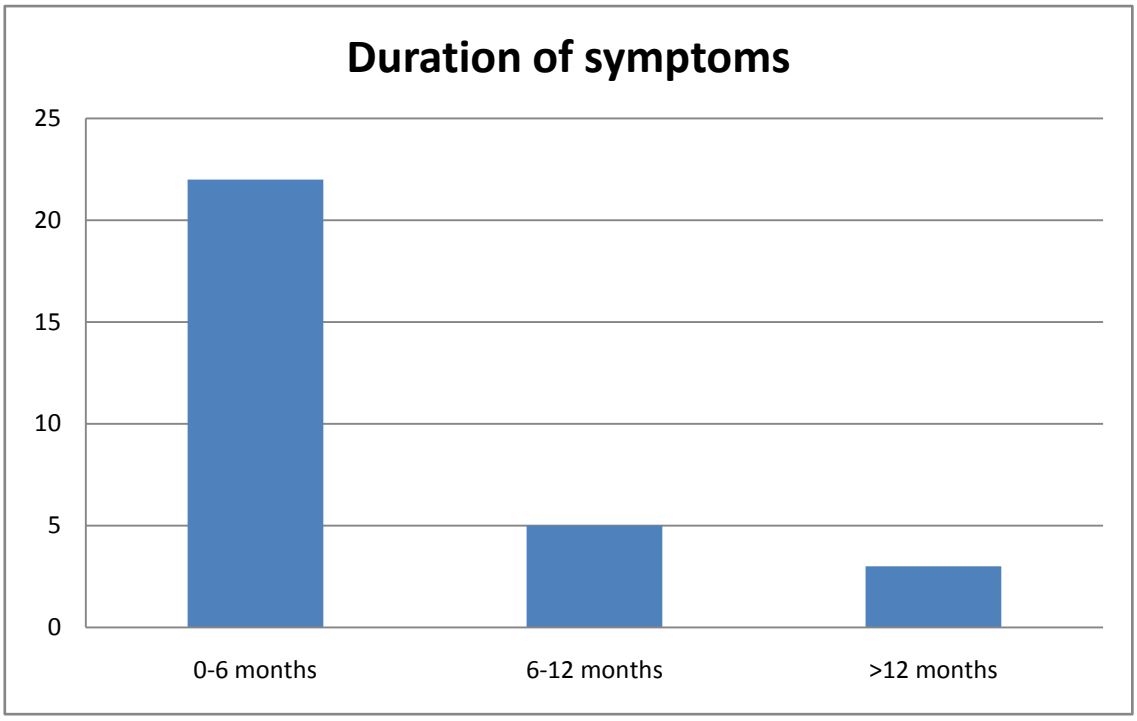
The commonest age group was 40-60 years. The lowest age group studied being 37 years and the oldest being 78 years.



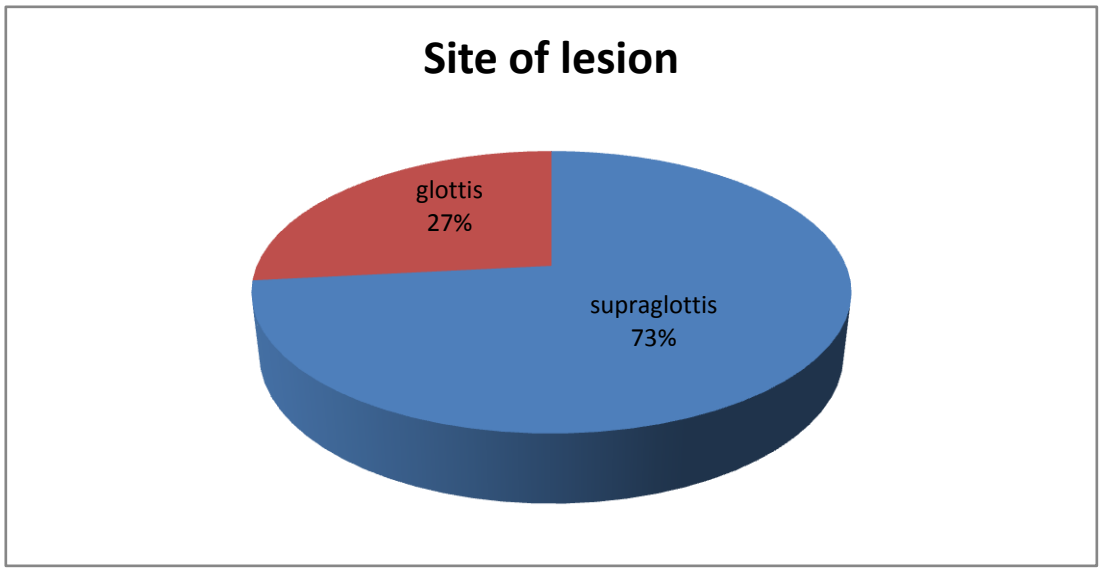
The commonest site of origin of the lesions was supraglottis accounting to 73% of cases followed by glottic lesions forming 27%.There were no lesions from the subglottis as site of origin.

The symptom pattern of the cancer group were as follows:

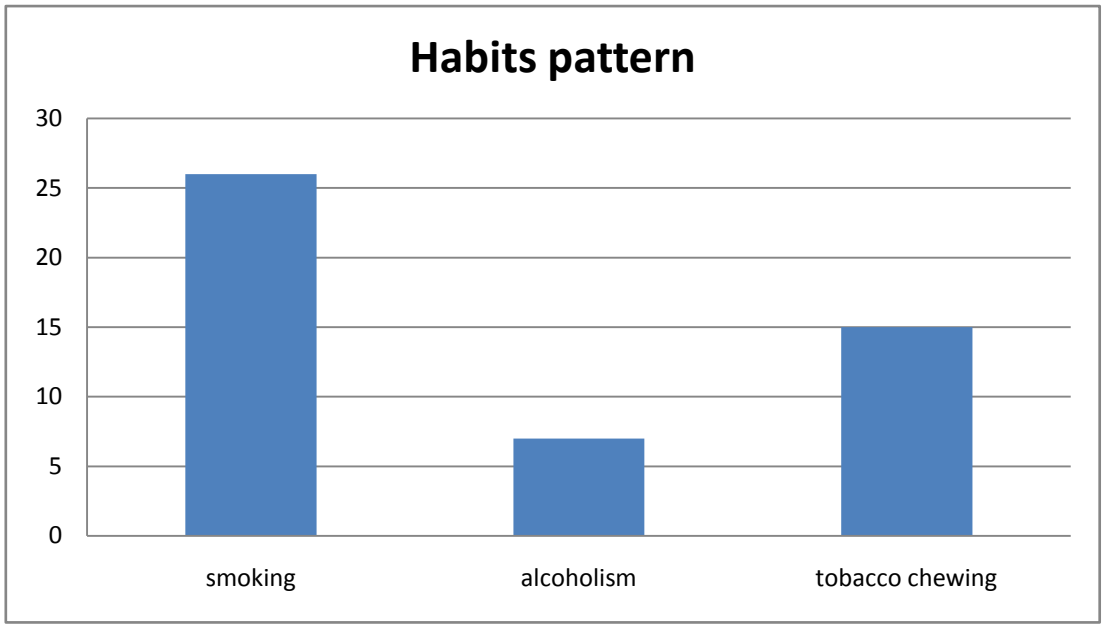




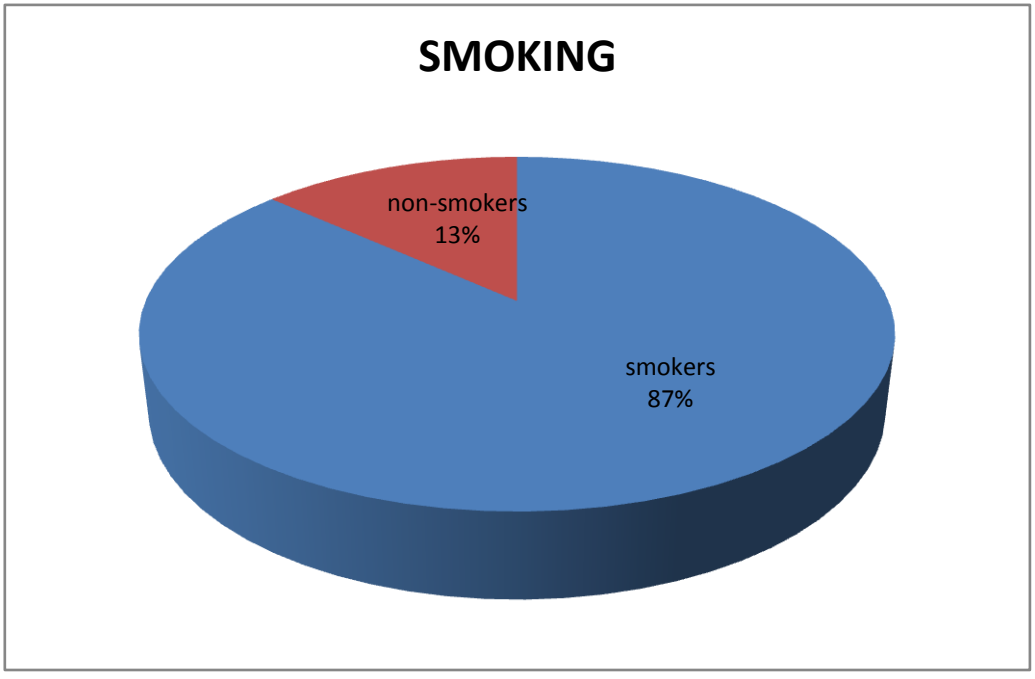
.The site of origin ratio was as follows:



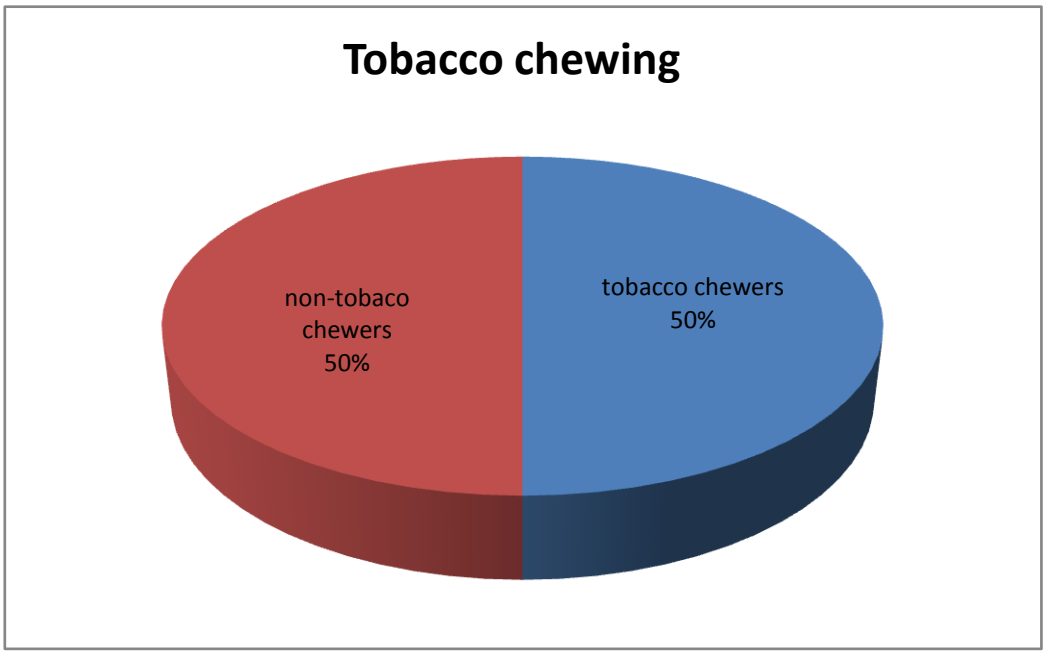
Ninety percent of the cases were chronic smokers.Alcohol consumption was prevalent in only 23% of cases.Alcohol intake and smoking were present in 10% of cases.



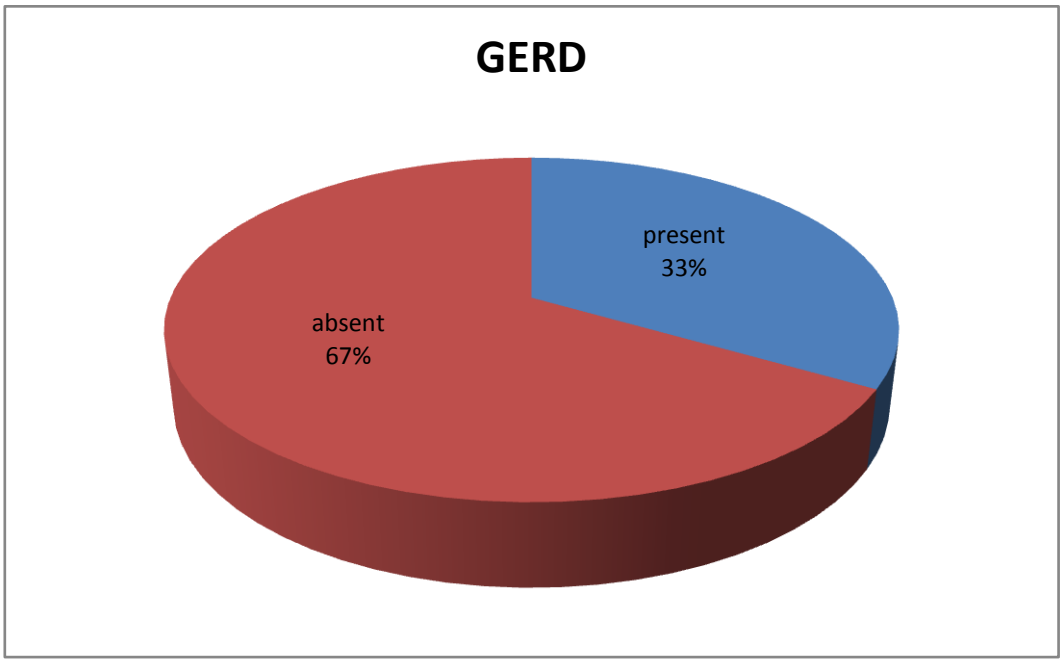
.The percentage of smokers were as follows:



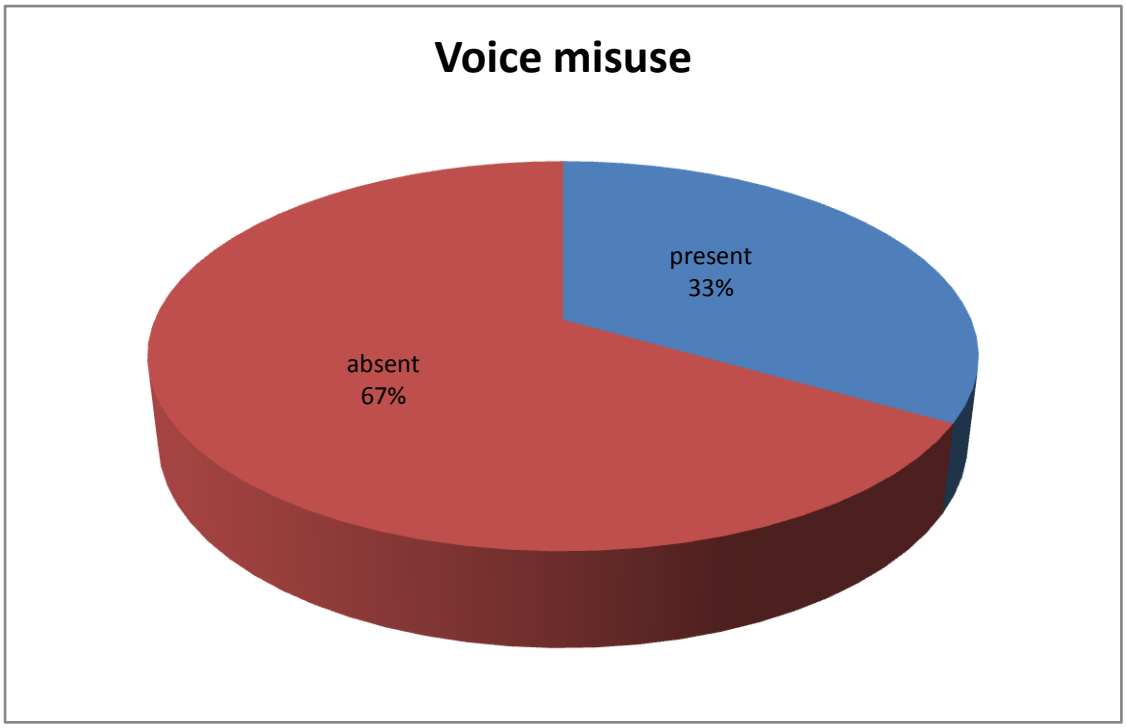
Fifty percent of the cases were paan chewers



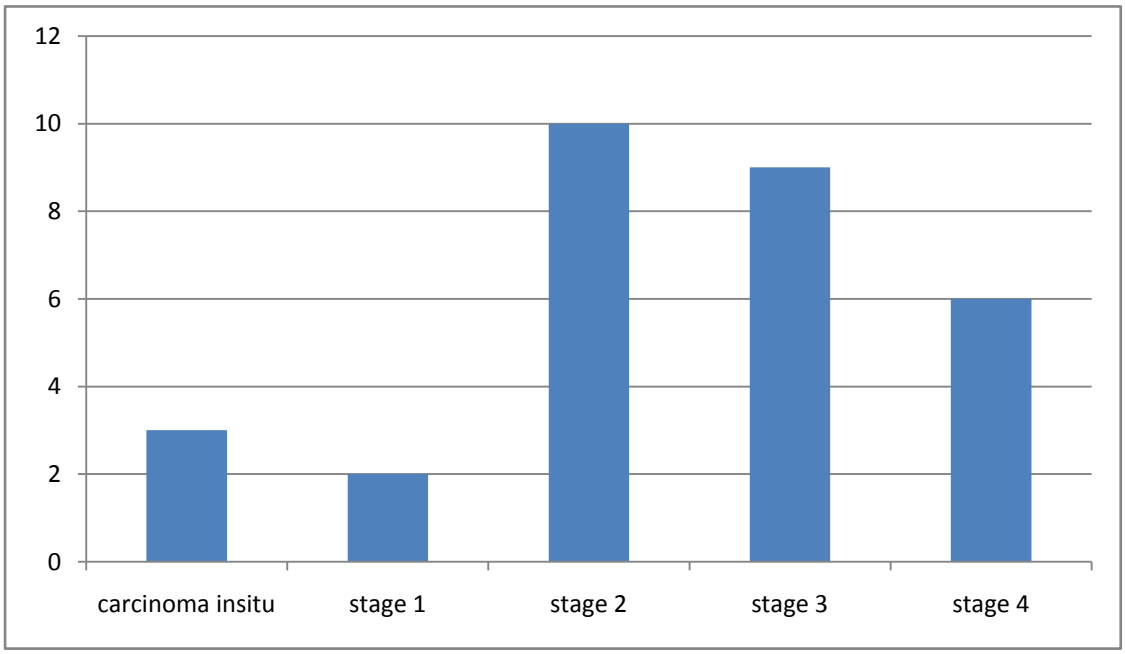
GERD in laryngeal cancer patients



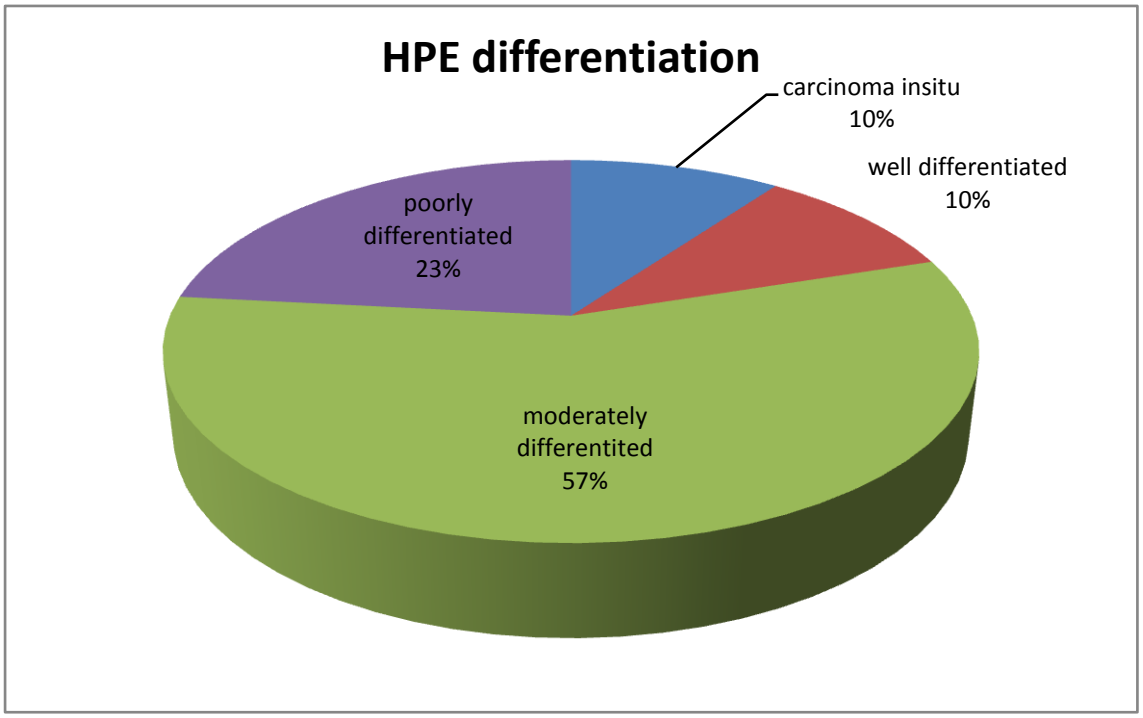
Voice misuse in laryngeal cancers



The staging pattern of the thirty cases were as follows:



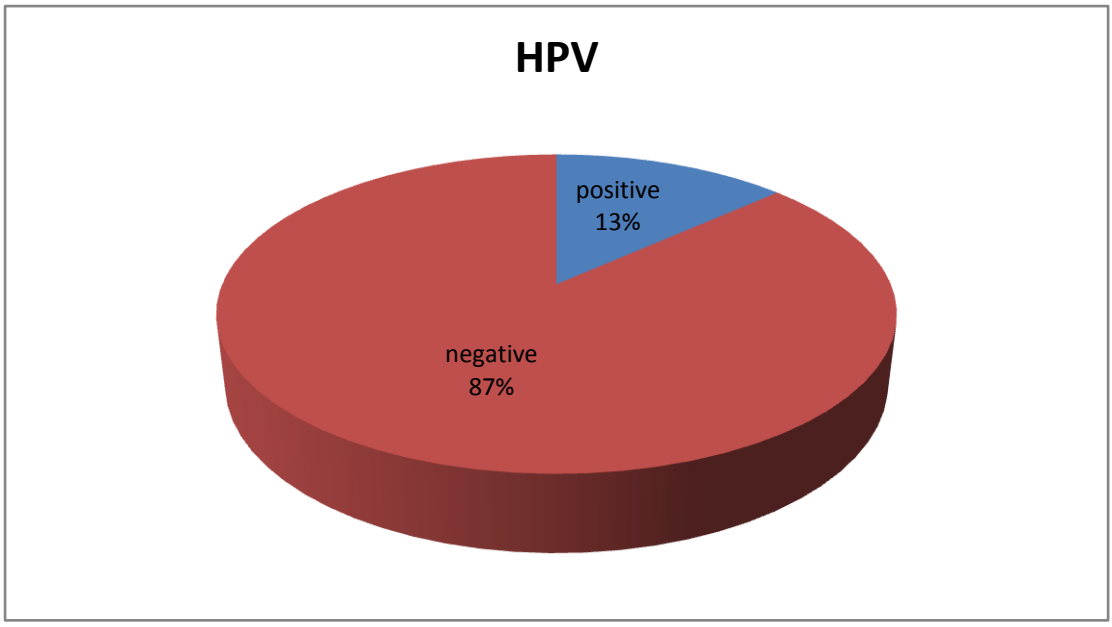
Moderately differentiated squamous cell carcinoma formed the majority of histopathological type followed by poorly differentiated and well differentiated type. Only three patients had carcinoma insitu type variety.



HPV and laryngeal cancers

Out of the thirty cases of squamous cell carcinomas of the larynx which were

studied, four were positive for HPV. One was HPV 16 type and another one HPV 11 type. Two other positive cases were not able to be sequenced probably due to low viral load.All the positive cases were males. All were in the age group of 40-60 years.Three cases were chronic smokers. Hence the percentage of positive HPV cases were:



None among the 4 HPV positive cases had a history of laryngeal papillomatosis in the past.

Given below are the clinical details of the 4 HPV positive cases:

Case 1:

Hospital no.:340507f

Age: 46 years

Chief complaint: Hoarseness of voice since 24 months

Other symptoms: Odynophagia and intermittent breathing difficulty and stridor

Habits: nil

Reflux disease: Absent

Voice misuse: Absent

Nasopharyngolaryngoscopy findings:

A mucosa covered growth seen on the right aryepiglottic fold,right arytenoid and medial wall of right pyriform sinus.Right hemilarynx fixed.

Microlaryngosopy findings:

Smooth mucosa covered friable growth involving right aryepiglottic fold, right arytenoids, pushing the right arytenoids medially blocking the glottis, right ventricle and right vocal cord involved, left vocal cord and subglottis not seen clearly. Posterior pharyngeal wall and bilateral pyriform sinus free of tumour, postcricoid region is free of tumour.

Histopathology report: Biopsy No.38987/12:

Moderately differentiated squamous cell carcinoma,soft tissue biopsy,supraglottis

TNM Staging:T4aN1M0

Stage 4 disease

HPV Status: Positive

HPV Type: HPV 16 type

Treatment received: Total laryngectomy with partial pharyngectomy with right selective neck dissection with right hemithyroidectomy and primary trachea-oesophageal puncture under GA followed by adjuvant chemoradiation.

Case 2:

Hospital Number:124925f

Age : 59 years

Chief complaint: Hoarseness of voice since 4 months

Habits: Smoking

Reflux disease: Absent

Voice misuse: Absent

Nasopharyngolaryngosopy findings:

Irregular growth involving bilateral vocal cords extending into anterior commissure. Bilateral vocal cords mobile

Microlaryngoscopy findings:

Irregular growth right false cord, right ventricle, right true cord, anterior commissure and left cord as well as immediate subglottis. Wedge of right false cord removed with LASER dissection. Type 3 LASER cordotomy done. Rest of larynx and hypopharynx normal.

Histopathology report: Biopsy No. 3720/12

Poorly differentiated carcinoma, probably squamous, biopsy, right vocal cord

TNM Staging:T2N0Mx

Stage 2 disease

HPV Status:Positive

HPV type:Not able to sequence

Treatment recieved:Radiotherapy-single modality

Case 3

Hospital Number:097279f

Age:48 years

Chief complaint:

Hoarseness of voice and noisy breathing since 3 months

Habits:Smoking

Reflux disease-Absent

Voice abuse-Absent

Nasopharyngolaryngosopy report:

Proliferative growth involving entire length of left vocal cord,anterior commissure and anterior third of right vocal cord with subglottic extension.

Microlaryngoscopy findings:

Proliferative growth involving entire length of left vocal cord,anterior commissure and anterior third of right vocal cord, subglottis on both left and right side and anteriorly..Growth extends to left ventricle.Left ventricular band edematous.Right ventricular band,bilateral arytenoids,bilateral pyriform sinus and postcicoid region normal.

Histopathology report:Biopsy No.40837/11

Squamous cell carcinoma-moderately differentiated ,biopsy tissue from bilateral vocal cords.

TNM Staging:T3N0Mx

Stage 3 disease

HPV status: Positive

HPV Type: Not able to sequence

Treatment received: Radiotherapy

Case 4

Hospital Number:130857f

Age:62 years

Chief complaint:Hoarseness of voice since 6 months

Habits:Smoking

Voice misuse:Absent

Reflux disease:Absent

Nasopharyngolaryngoscopy report:

Growth involving entire length of right vocal cord upto anterior commissure.Malignancy glottis.

Microlaryngosopy findings:

Proliferative growth involving entire length of right vocal cord upto the anterior commissure.Anterior commissure,left vocal cord,right false cord,rest of larynx and hypopharynx,subglottis free of tumour.

Histopathology report:Biopsy No-5359/12

Well differentiated squamous cell carcinoma,right vocal cord,separately sent superior resection margin,free of tumour.

TNM Staging:T1aN0Mx

Stage 1 disease

HPV status:Positive

HPV type: HPV 11

Treatment received:Transoral LASER microsurgery and excision under GA

Statistical analysis

The etiological factors of laryngeal cancers studied were smoking, alcoholism, tobacco chewing ,laryngopharyngeal reflux and HPV.The role of HPV in laryngeal cancer was studied in detail.The role of each etiological factor was studied separately.Only smoking and tobacco chewing showed statistically significant association in causing laryngeal cancer with a p value of 0.010 and 0.012 respectively.The results of HPV in laryngeal cancers were statistically insignificant with a p value of 0.121.The statistical results were as follows:

Univariate risk

	Case n (%)	Control n(%)	p value
Smoking	26	16	0.010
Alcoholism	7	6	0.750
Tobacco chewing	15	6	0.012
Reflux disease	9	15	0.187
Voice misuse	10	18	0.069
HPV	4	0	0.121

	p value	Odds ratio
SMOKING	0.010	5.804
TOBACCO CHEWING	0.012	5.296

Statistical analysis showed significant association of smoking and tobacco chewing with laryngeal cancers.While smoking had an association p value of 0.010,tobacco chewing had a p value of 0.012.Results showed that 5 times increased risk of getting laryngeal cancers compared to non-smokers.It also showed that tobacco chewers had a 5 times increased risk compared with non tobacco chewers.

DISCUSSION

Laryngeal cancer is the most common head and neck cancer worldwide[94].It is important to study the various etiological factors causing laryngeal cancers because carcinogenesis can be prevented by controlling these factors. Various studies conducted in different parts of the world ascertain the fact that tobacco is a definitive causative agent for laryngeal cancer[21].Among tobacco users, smoking is the habit which has been shown to have maximum carcinogenic effect followed by tobacco chewing.[22] Alcohol also has a significant part in laryngeal carcinogenesis[20].Smoking and alcohol consumption appear to have a synergestic action compared to each of them alone.The morbidity and the mortality caused by laryngeal cancers is significant in that surgery and radiation therapy can cause disabilities in speech and swallowing affecting the individual as well as the family.The burden of laryngeal cancer can be brought down by understanding the various etiological agents in detail.The search for other causes other than habits of tobacco use and alcohol consumption is likely to be useful in the prevention of laryngeal cancers.

Traditionally,middle aged males who were chronic smokers or alcoholics were thought to be more prone to oropharyngeal and laryngeal cancers.However recent trends show a group of oropharyngeal cancer patients who were non-smokers and non-alcoholics and were from a younger age group with HPV positive status.Studies reveal that these patients seem to respond better to chemoradiation and have a better prognosis[68].Though HPV 16 screening is not mandatory at present ,it is expected to be made mandatory in the future for cases of oropharyngeal cancers as it is recommended by most cancer treatment guidelines. Hence it is important to study the HPV status of cancer patients so that these patients can be treated as a separate category since they respond better to treatment.The

outcome of treatment can be better predicted in these patients and the management plan can be modulated.

Newer studies are aimed at finding out the role of Human papilloma viruses in carcinogenesis and their impact on treatment outcome.It is now well established that HPV has a significant role in cervical and oropharyngeal cancers. Our study was aimed at studying prevalence of HPV in laryngeal cancers presenting to a tertiary Hospital during the period November 2011 to July 2013 .We studied 30 cases of histopathology proven laryngeal cancers and 30 patients with benign vocal cord lesions as disease controls. All 30 cases were squamous cell carcinomas of the larynx.

Laryngeal cancer is most common in middle aged men and our study also showed similar results[84]. All our cancer group patients were men. Nineteen patients out of thirty were in the age group of 40-60 years. Only 2 were below 40 years and 9 were in the age group of above 60 years. The increased prevalence of laryngeal cancer in the middle age group is attributable to the habit pattern of individuals. Most of them start the habits of smoking and alcohol intake at about 2^{nd} to 3^{rd} decades of life. Hence by the age of 40 years most of them would have crossed 20 pack years. Studies have shown that as the number of pack years increases the relative risk for carcinogenesis also increases[85].

Hoarseness of voice was the predominant symptom in the both the cancer group as well as the control group. Whereas 27 cancer patients presented with hoarseness as the chief symptom, all the 30 control patients all had hoarseness as the chief complaint. Other studies showed that the most common site for laryngeal squamous cell carcinoma is the glottic larynx[86].However in our study, the majority of the lesions were from the supraglottis accounting to 73%.Eventhough supraglottic lesions formed the majority, the

chief complaint of hoarseness in the cancer group in our study could be explained by the spread of the disease to the glottis. The duration of symptoms in the cancer group were less than 6 months in 22 patients ie 73 %.In the control group the duration of hoarseness was mostly more than 6 months.

Our study also confirmed the significant role of smoking as an etiological factor in laryngeal cancers. Twenty six out of thirty patients were smokers. However only 23% of the cancer group gave history of alcohol intake and it did not show significant role in causing laryngeal cancers in our study. Our study included patients from different conservative religious backgrounds where habits such as alcohol intake are prohibited. This maybe the reason why our study did not find alcohol intake as a major risk factor.

Tobacco chewing also proved to be an important risk factor in accordance with the literature. The association was found to be significant with a p value of 0.012.Our study showed that both smoking and tobacco chewing had 5 times increased risk of acquiring laryngeal cancers. The control group showed less patients with habits such as alcohol consumption and smoking. Only half of them were smokers and only 20% gave history of alcohol intake. Voice misuse was found in 63% of the control patients in our study whereas it was present in only 33% cancer group patients. This reinfores the role of voice misuse in vocal cord benign lesions.

The role of gastroesophageal reflux disease as a risk factor for laryngeal cancers has been studied in recent years. Some studies had shown increased risk compared to non-cancer group. However in our study, the association between GERD and laryngeal cancers was not statistically significant. It was present in only 33% of cancer patients whereas 60% of

the control group had symptoms suggestive of GERD.As per clinical evaluation, our study showed increased prevalence of GERD in the benign laryngeal lesions.

Histopathologically, moderately differentiated squamous cell carcinoma was the commonest variety followed by poorly differentiated and well differentiated variety. Among the four HPV positive cases also, 2 were moderately differentiated. The maximum number of cases were in stage 2 according to TNM classification followed by stage 3 and stage 4.

In our study group four cancer cases(13%) were positive for HPV among the 30 cases studied. None among the 30 control patients were positive for HPV. Baumann *etal* studied 38 carcinoma insitu or T1 lesions glottis and had 6 (16%)positive cases.In an Indian study by Jacob *etal*,15 cases were positive among the 44 cases of laryngeal cancers studied. In our study all the HPV positive patients were males and all of them were in the middle age group. One of them was HPV 16 type and another one HPV 11 type whereas two positive samples were not able to be sequenced. In a metaanalysis by Li *etal*,9 studies from the Asian subcontinent gave a prevalence of 25%[1].However our study showed decreased prevalence of HPV in laryngeal cancers and statistics showed the relationship to be insignificant(p value=0.121).

The patient positive for HPV 16 did not have any habits such as tobacco use or alcoholism. He did not have a history of reflux disease as well. As other common risk factors were absent in this patient it could be concluded that HPV was the causative agent for his disease. Other HPV positive cases were chronic smokers. However as 2 among them were not sequenced,it could not be serologically confirmed whether it was high risk or low risk HPV genotype. These two samples could not be sequenced despite

being tested by an alternate method, namely the PGMY CHUV assay[102]. The reason for this could be low HPV copy numbers that were not sufficient to be sequenced or typed. The successful sequencing would have given us a clearer picture of the genotype and hence the carcinogenicity.

HPV 11 is known to cause laryngeal papillomatosis.[63]The case positive for HPV 11 in our study had a well differentiated squamous cell carcinoma of the glottis in stage 1.Two among the 4 HPV cases were moderately differentiated squamous cell carcinomas histopathologically.The other two cases were well differentiated and another poorly differentiated, respecively. Among four positive cases, one each was in 1,2,3 and 4 stages.

There were some limitations in the study .The problem faced during the data collection was the collection of detailed sexual history of the patients.As the patients were from different conservative religious,cultural and social backgrounds they were reluctant and refused to answer questions related to their personal sexual history. Most of the patients were accompanied by one or two family members at all times and it was difficult to take detailed sexual history in their presence in an outpatient clinic setting. Another drawback was the inability to sequence the 2 HPV positive cases.The reasons for this have been described earlier.

In a meta analysis by Li X[1] et al including 55 eligible studies,the overall prevalence of HPV in laryngeal cancer tissues was 28%. High risk genotype HPV-16 was most frequently observed type, with a prevalence of 19.8%. High risk HPV type infection was detected in a total of 26.6% laryngeal cancer patients.The meta-analysis based on 12

eligible case-control studies suggests a strong association between HPV infection and laryngeal squamous cell carcinoma, with a summary odds ratio (OR) of 5.39.

The relation between HPV and cancer larynx has to be studied further to include a larger series of patients and to follow up the HPV positive cases long term to understand better the role of HPV infection in the treatment outcome and prognosis.HPV detection in normal laryngeal tissues has not been standardised and hence the general prevalence has not been determined[57]. It is important to study whether HPV is merely a "by-stander infection" or a "latent infection" in causing laryngeal cancers or whether it has a definitive carcinogenic role and whether it has a significant effect on the prognosis and treatment outcome of laryngeal cancers.

The development of vaccines against cervical cancers has had a significant impact in the management and prognosis of cervical cancers.In current times it has been postulated that this maybe soon applicable for oropharyngeal cancers. Further studies will be needed to see whether these can be translated to the management of laryngeal cancers.However the fact that no single HPV genotype have been identified in laryngeal cancers compared to HPV 16 in oroparyngeal cancers,is a limitation for translation of vaccines. At the current time the results of studies have been variable and the effect of HPV on the treatment outcome and prognosis of laryngeal cancers has yet to be comprehensively studied .Further studies are required before any changes in management are implemented in national treatment policies and laryngeal cancer management guidelines .

The role of HPV in laryngeal cancers need to be confirmed by detailed studies including larger group with long term followup . Health education regarding the etiology of HPV infection ie sexual transmission and the need for safe sex needs to be emphasised.

CONCLUSION

The role of Human papilloma virus in head and neck carcinogenesis is well established. Studies have already shown a significant association between HPV and oropharyngeal cancers[73] with HPV 16 causing more than 90% of cases.[5]However its role in laryngeal cancers is yet to be clearly established.The treatment outcome of cases with HPV related laryngeal cancers is still being currently studied .

There is significant paucity of case-control studies in literature that have looked at HPV and laryngeal cancers in the Asian subcontinent.We studied 30 cases of histopathologically proven laryngeal squamous cell carcinomas as cases and benign lesions of larynx such as vocal cord polyps,nodules or cysts as disease controls .

There were 4 positive cases of HPV in the laryngeal cancer group(13%) whereas there were no positive cases in the control group. This shows an increasing trend towards positivity in the case group compared to the control group. However the association between HPV and laryngeal cancers was found to be statistically insignificant (*p* value=1.21). One of the cases was genotyped as high risk HPV type 16 and another was HPV 11.The other two positive cases could not be sequenced even after using WHO recommended CHUV assay.

Our study also showed a significant association between tobacco chewing and laryngeal cancers in addition to smoking.This is in accordance with existing studies which have shown a similar correlation.

The technical limitation in sequencing positive HPV samples were a drawback for our study.Other limitation was the small sample size.The data collection in the form of detailed sexual history was another major drawback.

HPV subtyping needs to be done for all juvenile and adult-onset laryngeal papillomatosis in view of chances of malignant transformation.These patients need to be followed up regularly. HPV testing is strongly recommended in the workup of laryngeal cancer patients especially in the younger age groups without any co-existing risk habits such as smoking ,tobacco chewing and alcohol consumption. Further studies should be conducted including a larger group of patients to confirm the role of HPV in laryngeal cancers and to followup these patients to study the treatment response and survival .This can open new dimensions in the treatment and prognosis of laryngeal cancers.

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APPENDIX

1. Consent forms

2. Patient information sheet

3. Proforma

4. Data Analysis sheet

5. Colour plates

INFORMED VALID CONSENT

Study number-

Participant's name-

Date of birth/age

I, son/daughter/wife of has been explained in my own understandable language about the proposed study.I have been explained about the study which involves taking tissue from the lesion for PCR analysis during direct/microlaryngoscopic biopsy under GA which is done routinely for patients with my disease(clinically diagnosed laryngeal cancers/vocal cord polyp).I have been explained that there is no additional risk in the study.It has been explained to me that I am free to withdraw from the study any time I want and will not in any way compromise the treatment ,the ENT department is giving me.I understand that my identity and participation will not be revealed in any information released to third parties.I am giving this consent on my own free will.I have been explained about the study in a language familiar to me.I hereby give my full valid consent for the proposed study

Name Signature Doctor

Name of relative(guardian/parent)

Signature

Name of witness

Signature

PATIENT INFORMATION SHEET

I will be part of this study.As part of the study,I will be asked details about my disease. This study is aimed at finding out the prevalence of a virus named Human Papilloma Virus in cancer of the larynx and vocal cord polyps.I am clinically diagnosed with cancer of the voicebox/ vocal cord polyp.A test(biopsy)should be routinely done in which tissue is taken to test for features of cancer.A microscope assisted visualisation of the voicebox is done and tissue is taken for biopsy under general anaesthesia after taking written valid consent.This is done routinely for all patients with a similar diagnosis.During the process of taking tissue for biopsy,some extra tissue will be taken for this study.Tissue will be sent for a test called PCR analysis.There wont be any other risks involved in this study.I need not pay any extra money for the test.This study can help in studying the cause of laryngeal cancers. I can withdraw from the study at any moment if you feel so and that in no way will compromise my treatment at ENT department.My participation in the study will remain confidential and shall be known only to the investigators.For any queries I can contact:

Dr.Philip George

PG Registrar

CMC Vellore

PROFORMA

HUMAN PAPILLOMA VIRUS IN LARYNGEAL CANCERS

Serial No.:

Name:

Age:

Sex:

Hospital number:

Date of Admission:

Unit:

Chief complaint:

Other complaints:

Duration of symptoms:

Voice misuse:

GERD:

Habits:

Any previous treatment taken:

(surgery/ chemotherapy/ radiotherapy)

NPL scopy findings:

Surgery date:

Surgeons:

Microlaryngoscopy findings:

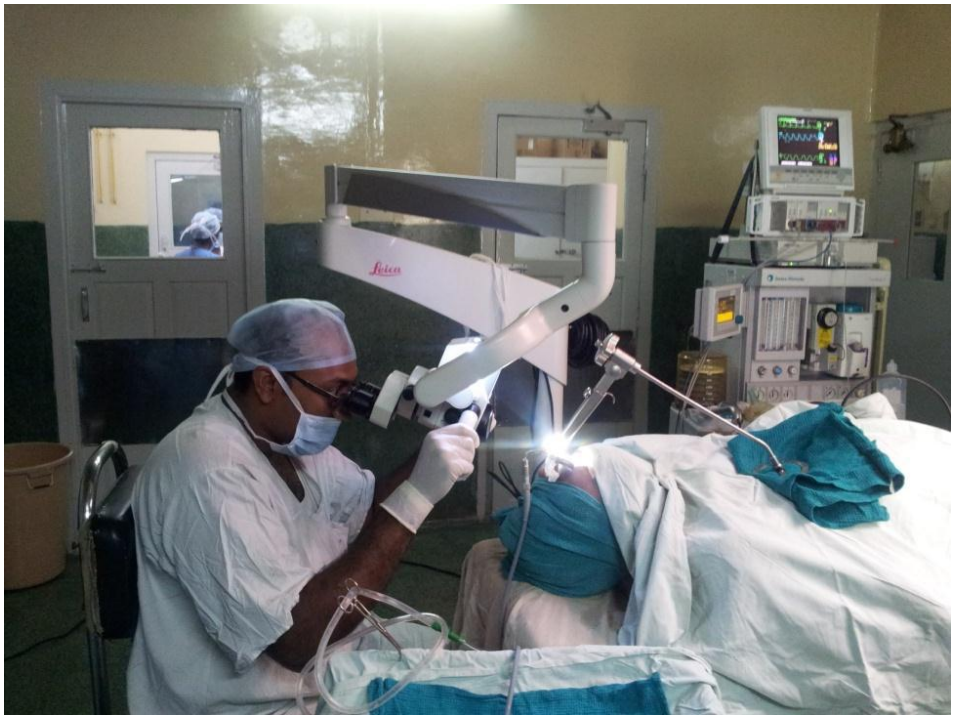
Biopsy no. and date:

Biopsy report:

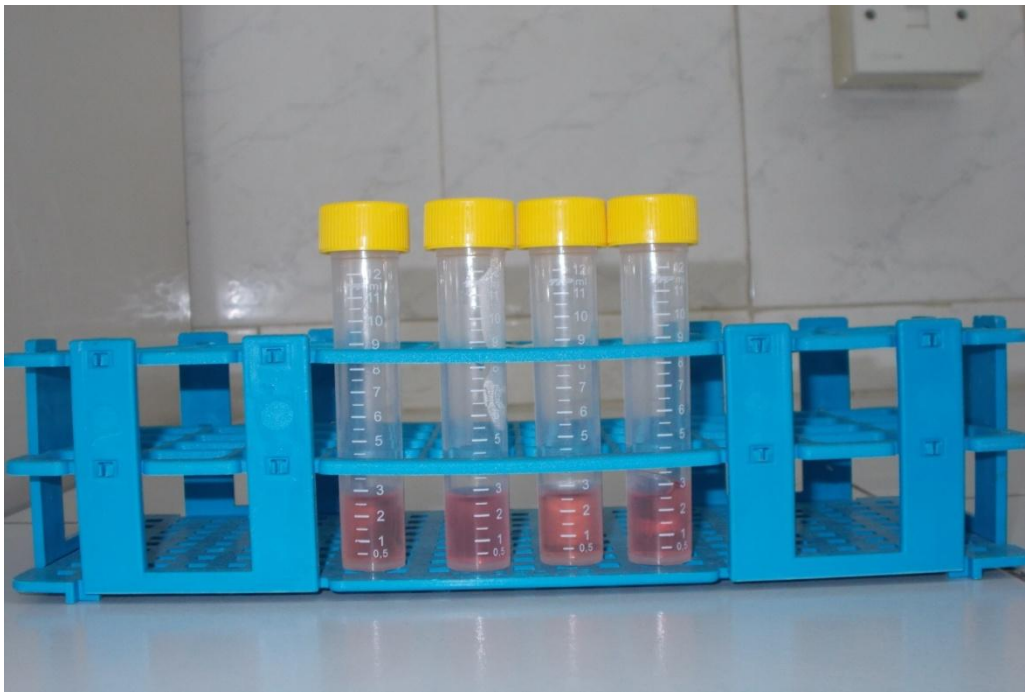
Final Diagnosis:

HPV report:

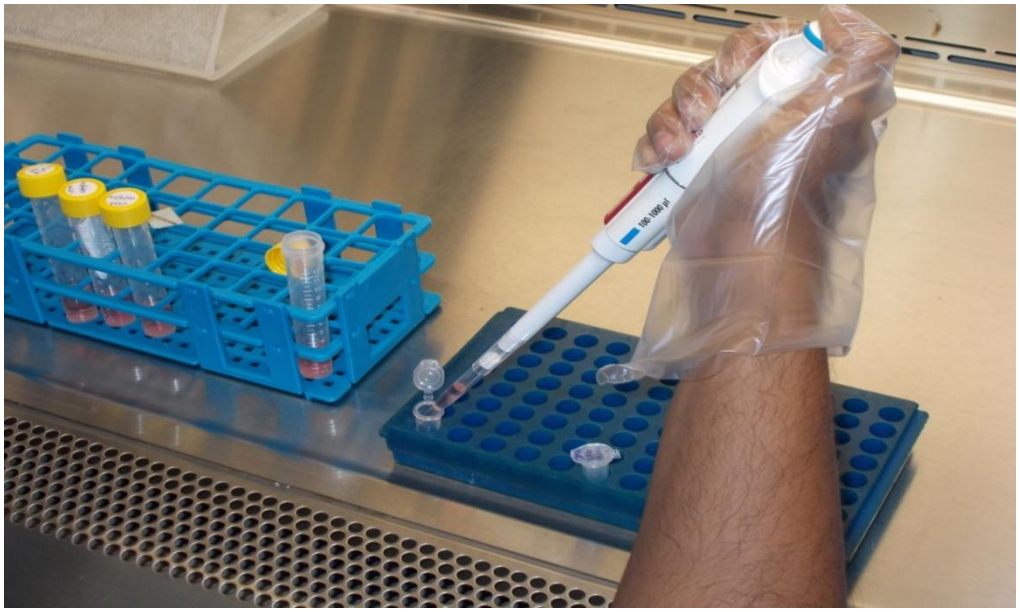
HPV type:



ML SCOPY OR SETTING



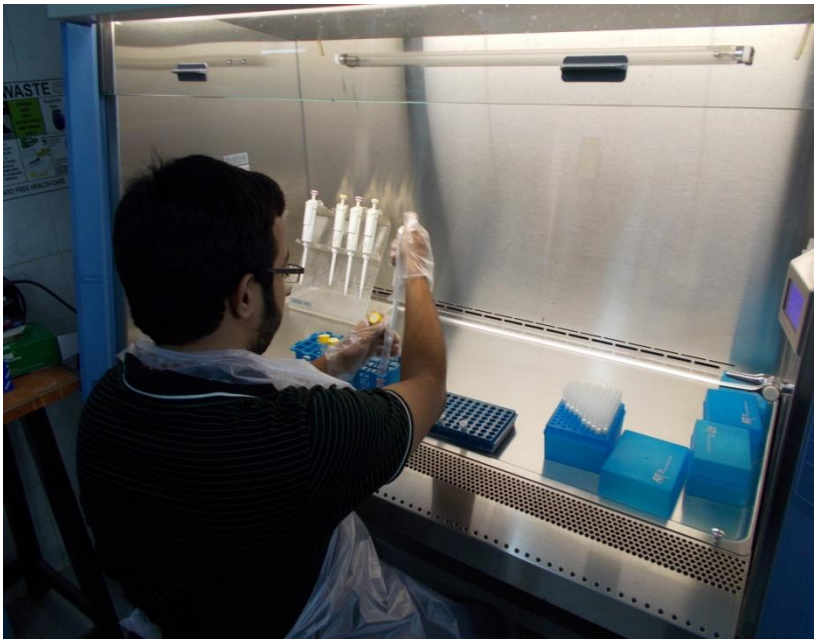
VTM TUBES



EPPENDORF TUBE

-80 DEGREE FREEZER

BIOSAFETY CABINET FOR DNA EXTRACTION



EXRACTION OF HPV DNA: DNeasy® Tissue kit

THERMAL CYCLER

SEQUENCER

serial	sex	age	hoarseness	dysphagia	odynophagia	other	duration	smoking	aloholism	tobacco	other1	gerd	voice
1	1	58	2	1	1	1	5	1	2	1	2	1	2
2	1	76	1	2	2	1	2	1	1	2	2	2	2
3	1	53	1	2	2	2	4	1	2	1	2	2	1
4	1	46	1	2	1	1	24	2	2	2	2	2	2
5	1	65	1	2	2	2	12	2	2	1	1	1	1
6	1	63	1	2	1	1	4	1	2	1	2	2	1
7	1	60	2	2	2	1	3	1	2	1	2	2	2
8	1	47	1	2	1	2	5	2	2	1	2	2	1
9	1	56	1	2	2	2	4	2	2	1	2	2	2
10	1	59	1	1	1	1	2	1	2	1	2	2	2
11	1	57	1	2	2	2	12	1	2	2	2	2	2
12	1	54	1	2	2	2	24	1	2	2	2	2	1
13	1	58	1	2	2	2	8	1	2	1	2	1	2
14	1	57	1	2	2	1	1	1	1	2	2	1	2
15	1	55	1	2	2	2	12	2	2	2	2	1	1
16	1	78	1	1	1	2	1	1	2	2	2	2	2
17	1	45	2	1	1	2	2	1	2	2	2	1	2
18	1	59	1	2	2	2	4	1	2	2	2	2	2
19	1	48	1	2	2	1	3	1	2	2	2	2	2
20	1	60	1	2	2	2	6	1	2	2	2	2	1
21	1	57	1	1	1	2	5	1	2	1	2	2	2
22	1	55	1	2	2	1	72	1	1	1	2	2	2
23	1	38	1	2	2	1	12	1	1	1	2	2	1
24	1	61	1	2	2	2	10	1	1	2	2	1	2
25	1	71	1	2	2	2	2	1	2	2	2	2	2
26	1	65	1	2	1	1	2	1	1	2	2	2	2
27	1	71	1	2	2	1	2	1	2	2	2	1	2
28	1	37	1	2	2	2	3	1	2	1	2	1	1
29	1	59	1	2	2	2	3	1	2	1	2	2	1
30	1	64	1	2	2	2	2	1	1	1	1	2	2

serial	sex	age	hoarseness	dysphagia	odynophagia	other	duration	smoking	aloholism	tobacco	other1	gerd	voice
1	2	42	1	2	2	2	4	2	2	1	2	1	1
2	1	56	1	2	2	2	6	1	2	1	2	1	1
3	1	58	1	2	1	1	6	2	2	2	2	2	1
4	1	32	1	2	2	2	7	1	1	2	2	2	1
5	2	47	1	2	2	1	4	2	2	2	2	1	1
6	1	27	1	1	2	2	24	1	1	2	2	2	1
7	1	47	1	2	2	2	1	1	1	2	2	1	1
8	1	62	1	2	2	2	3	1	2	2	2	2	2
9	2	57	1	2	2	1	2	2	2	2	2	1	1
10	1	46	1	2	2	2	6	1	2	2	2	1	2
11	1	26	1	2	2	2	60	1	2	2	2	1	1
12	1	60	1	2	2	2	8	1	2	2	2	1	1
13	2	35	1	2	2	1	8	2	2	2	2	1	2
14	1	35	1	2	2	2	6	2	2	2	2	2	2
15	1	39	1	2	2	2	9	2	2	2	2	2	1
16	1	36	1	2	2	2	12	2	2	2	2	1	1
17	1	50	1	2	2	2	23	1	2	2	2	1	1
18	1	49	1	2	2	2	12	1	2	2	2	1	2
19	1	49	1	2	2	2	12	1	2	2	2	2	1
20	1	58	1	2	2	2	24	2	2	2	2	2	2
21	1	47	1	1	2	2	6	2	1	1	2	2	2
22	1	45	1	2	2	2	6	2	2	1	2	1	1
23	2	3	1	2	2	2	8	1	1	2	2	2	1
24	1	37	1	2	2	1	96	1	2	2	2	2	2
25	1	33	1	2	2	1	36	1	1	1	2	2	2
26	1	25	1	2	2	2	36	2	2	2	2	1	2
27	1	37	1	2	2	2	4	1	2	2	2	2	2
28	1	62	1	2	2	1	2	1	2	2	2	2	2
29	1	42	1	2	2	2	72	2	2	1	2	1	1
30	1	54	1	2	2	2	12	2	2	2	2	2	1

serial	npl	date	ml	site	who	histopatho	tnm	staging	hpv
1	Malignancy glottis T3	2/25/2013	Ulceroprolif growth left vc false cord	2	1	3	T3NOMX	3	0
2	Malignanacy glottis with lesion hypopharynx	6/12/2013	growth left false cords ventricle r hpx	2	1	4	T3NOM0	3	0
3	Malignancy glottis	6/6/2013	prolif lesion right vc with antr commisu	2	1	2	T1N0MX	1	0
4	Malignancy supraglottis	11/14/2013	right aef arytenoids pushing right aryte	1	1	3	T4N1M0	4	1
5	Right vc polyp	7/9/2013	polyp anterior one third right vc	2		1	TisN0MX		0
6	Malignancy glottis	4/15/2013	growth anterior half of bilateral vcs	2	1	4	T4N0M0	4	0
7	Malignancy supraglottis	1/30/2013	up growth left epiglottis AEF arytenoid	1	1	3	T2N0MX	2	0
8	Malignancy glottis	1/18/2013	growth left vc,right true cord subglotti	2	1	3	T4N0M0	4	0
9	Malignancy glottis	2/4/2013	growth middle 1/3 right vc antr commisur	2	1	3	T3N0MX	3	0
10	Malignancy supraglottis	1/15/2013		1	1	3	T2NOMO	2	0
11	Malignancy glottis	6/26/2013	growth rt false cord,ventricle true vc	2	1	3	T3N2MX	4	0
12	Malignancy glottis	7/27/2013	growth rt true vc false vc AEF	2	1	3	T3N0MX	3	0
13	Malignancy glottis	6/7/2013	growth rt vc ant commissure ant 2/3 lt v	2	1	4	T2N0M0	2	0
14	Malignancy glottis	3/19/2013	irregularity left vc antr commisure	2	1	4	T2N0MX	2	0
15	Growth left vc	4/9/2012	thickened antr 2/3 left vc with keratoso	2		1	TisNOM		0
16	Malignancy supraglottis	12/5/2011	growth rt false cord,aef,arytenoid,true	1	1	3	T3N0MX	3	0
17	Malignancy supraglottis	1/16/2013	growth left arytenoid aef pfs,rt aryteno	1	1	4	T4N1M0	4	0
18	Malignancy glottis	2/2/2012	growth rt true cord false cord ventricle	2	1	4	T2N0MX	2	1
19	Malignancy glottis	12/19/2011	growth left vc antr comm.antr 1/3 rt vc	2	1	3	T3NOMX	3	1
20	malignancy glottis	2/16/2012	Growth rt vc antr comm lft vc	2	1	2	T1N0MX	1	1
21	Malignancy glottis	3/8/2012	growth left true false cord AEF ventricl	2		1	TisN0M		0
22	Malignancy supraglottis	5/7/2012	growth rt AEF False cord pfs ventriclevc	1	1	4	T3N0MX	3	0
23	Malignancy glottis	5/7/2012	growth entire left vc	2	1	3	T2N0Mx	2	0
24	Malignancy glottis	4/25/2012	ulcerative growth antr 2/3 rt vc	2	1	3	T2N0Mx	2	0
25	Malignancy glottis	4/22/2012	irregularity b/l vcs	2	1	2	T2N0MX	2	0
26	Growth left aef arytenoid pfs righ pfs postcricoid	7/6/2013		1	1	3	T3N1Mx	3	0
27	Malignancy glottis	7/12/2013	exophyti growth left vc to subglottis	2	1	3	T3N0MX	3	0
28	Malignany supraglottis	7/19/2013	malignancy supraglottis	1		3	T3N2CMx	4	0
29	Malignancy glottis	7/16/2013	irregular firm lesion right vc	2	1	3	T2N0MX	2	0
30	malignancy glottis	7/23/2013	proliferative growth entire length true cord antr	2		3	T3NOMX	3	0

serial	npl	date	ml	site	who	histopatho	tnm	staging	hpv
1	Right vc polyp	7/12/2013	broad based polyp rt junction 1/3-2/3	2		5			0
2	Left vc polyp	7/8/2013	broad based polyp left vcjunction1/3-2/3	2		5			0
3	Right vc polyp	7/8/2013	pinkish right v polyp at junction	2		5			0
4	Right vc polyp	6/18/2013	mid third right vc 2nd polyp antr commis	2		5			0
5	Right vc polyp	7/2/2013	bulge right vc junction of antr 1/3 2/3	2		7			0
6	Left vc polyp	7/2/2013	trilobed polyp anterior 1/3 left vc	2		5			0
7	gerd o r/o malignancy	7/2/2013	congested vcs with mild irregularity	2		7			0
8	Right vc polyp	7/14/2013	broad based polyp right vc	2		5			0
9	Right vc polyp	7/31/2012	polyp junction of ant 1/3 & post 2/3 rt	2		5			0
10	Left vc polyp	12/19/2011	broad based polyp left vc	2		5			0
11	Right vc olyp	1/25/2012	large bilobed right vc polyp at junction	2		5			0
12	Irregula medial surface b/l vcs	1/25/2012	irregular mrgins b/l vcs	2		7			0
13	Left hemorrhagi polyp	6/14/2013	polyp junction 1/3 &2/3 left vc	2		5			0
14	Right vc polyp	6/18/2013	broad based polyp right vc	2		5			0
15	Right v polyp	6/4/2013	right vc polyp antr 1/3rd	2		5			0
16	Right vc polyp	6/5/2013	cyst medial margin right vc	2		5			0
17	Polyp antr 1/3 postr 2/3 left vc	5/6/2013	polyp junction antr 1/3 postr2/3 left vc	2		5			0
18	Left vc polyp	4/15/2013	left vc polyp at 1/3-2/3 junction	2		5			0
19	Right vc polyp	1/23/2013	right vc polyp 1/3-2/3 junction	2		5			0
20	Right vc polyp	1/24/2013	right vc polyp antr 1/3-2/3 junction	2		5			0
21	Growth left vc	11/2/2012	right vc polyp antr 1/3 keratosis lft vc	2		5			0
22	?left intracordal cyst	1/23/2013	intracordal cyst left vc	2		6			0
23	Right vc polyp	12/18/2012	polyp anterior 1/3 right vc	2		5			0
24	Left vc cyst	1/22/2013	polyp anterior 1/3 left vc	2		5			0
25	Right vc polyp	2/7/2013	anterior 1/3 right vc polyp	2		5			0
26	right vc polyp	7/13/2012	Polyp anterior 1/3-2/3 junction rt vc	2		5			0
27	Right vc polyp	6/14/2013	polyp superior surface right vc	2		5			0
28	Left vc lesion vallecular cyst	7/4/2013	polypoidalesion antr comm 1/3 left vc	2		8			0
29	nil	6/15/2013	polypoidal growth medial surface lt vc	2		5			0
30	Left vc polp/polypoidal lesion	7/10/2013	Polypoidal lesion antr 1/3 left vc	2		5			0

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

March 8, 2012

Dr. Philip George
PG Registrar
Department of ENT
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
Prevalence of Human Papilloma Virus in laryngeal cancers in an Indian Population
Dr. Philip George, PG Registrar, ENT , Dr. Rajiv C.Michael, ENT Dr. Priya Abraham, Clinical Virology, Dr. Rupa Vedantam, Dr. Anand Job, Dr. Mary Kurien, ENT, Mr. Anantharam Raghavendran, Clinical virology.

Ref: IRB Min. No. 7659 dated 18.11.2011

Dear Dr. George,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Prevalence of Human Papilloma Virus in laryngeal cancers in an Indian Population" on November 18, 2011.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Informed Consent Form and Information Sheet (English, Tamil, Hindi and Bengali)
3. Cvs of Drs. Philip George, Rajiv C Michael, Priya Abraham, Anand Job, Mary Kurien, Mr. Anantharam Raghavendran.
4. A CD containing documents 1 - 3

The following Institutional Review Board (Ethics Committee) members were present at the meeting held on November 18, 2011 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore- 632002.

Name	Qualification	Designation	Other Affiliations
Dr. B.J.Prashantham	MA (Counseling), MA	Chairperson(IRB)&	Non-CMC

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

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Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

	(Theology), Dr Min(Clinical)	Director, Christian Counselling Centre	
Mr. Harikrishnan	BL	Lawyer	Non-CMC
Mrs. S. Pattabiraman	BSc, DSSA	Social Worker, Vellore	Non-CMC
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M.Phil, BL.	Legal Advisor, CMC.	
Dr. Gagandeep Kang	MD, PhD, FRCPath.	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Microbiology & Addl. Vice Principal (Research), CMC.	

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent and requires a copy of the final report.

A sum of ₹ 65,000/- (Rupees Sixty five thousand only) is sanctioned for 2 years.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

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