

**DISSERTATION ON**  
**“A STUDY OF UNDIFFERENTIATED HEAD AND NECK  
CANCERS AND THE ROLE OF IMMUNOHISTOCHEMISTRY”**

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## INTRODUCTION

Cancers of head and neck refers to neoplasm arising from below the skull base to the region of thoracic inlet. They are diverse group of diseases each with distinct epidemiologic, anatomic and pathologic features. They show wide variation in natural history, prognosis and treatment considerations.

India is one of the high incidence zones for head and neck cancers. In India, the most common head and neck cancers are those of oral cavity and pharynx. Age adjusted incidence for these sites in Indian population as follows

Males : 10.8 to 38.8/ 1 lakh population

Females : 6 to 15/ 1 lakh population

Some tumor groups challenge routine histopathological classification of Malignancies. Because of the absence of cell morphological differentiation that characterizes their lymphoid, epithelial or mesenchymal origin.

These are poorly differentiated or undifferentiated tumors and can occur with relative frequency in the head and neck. They can arise in mucosa as well as in salivary glands, soft tissues (or) lymphnodes.

The diagnosis and classification of such tumors are fundamental because suitable therapy and prognosis for each case depends upon precise histopathological diagnosis.

The introduction of the immunohistochemical method by Coons et al in 1942 has become a powerful complementary tool in tumor analysis. It has increased the possibilities for histogenetic diagnosis of undifferentiated tumors. Through the identification of specific cellular components of cell patterns, using a special panel of monoclonal or polyclonal antibodies, the immunohistochemical method has transformed the diagnosis of these tumors.

Diagnosis that used to be made on the basis of subjective information can now be accomplished using objective criteria. However there are only a few references in the literature to the immunohistochemical technique applied to the identification of undifferentiated head and neck tumors.

## **AIMS OF THE STUDY**

1. To know the distribution of tumors of head and neck with respect to the site of occurrence.
2. Evaluation of undifferentiated head and neck tumors and the way in which they were distributed according to tumor location.
3. Undifferentiated head and neck tumor incidence according to age and sex distribution.
4. To evaluate the results of the Immunohistochemical techniques in undifferentiated head and neck tumors.
5. To analyse the usefulness of the immunohistochemistry in determining the conclusive diagnosis of undifferentiated tumors, so that in the management of these tumors.

# REVIEW OF LITERATURE

## Malignant Tumors of the Head and Neck

### **UNDIFFERENTIATED TUMORS:**

Malignant neoplasm that composed of undifferentiated cells are said to be anaplastic. Lack of differentiation is loss of the structural and functional differentiation of normal cells. Cancer arise from stem cells in tissues, so that failure of differentiation accounts for undifferentiated tumors.

Anaplastic cells display marked pleomorphism hyperchromatic nuclei and large nuclei. Nuclear cytoplasmic ratio is 1:1 rather than 1:4 and 1:6. Anaplastic nuclei are variable and bizarre in size and shape. The chromatin is coarse and clumped. Mitosis is numerous and atypical. They loss normal polarity, grow in sheets with total loss of communal structures such as gland formation or stratified squamous architecture.

Because of the absence of cell morphological differentiation, it is difficult and impossible to characterize these tumors according to their cell of origin into lymphoid, epithelial or Mesenchymal origin by routine hematoxylin eosin staining technique.

These undifferentiated tumors constitutes 10% of all diagnosed tumors. In the head and neck, the undifferentiated tumor accounts for 3-6% with the exception of the lymphnodes most frequent locations of undifferentiated tumors in the head and neck region is, pharynx, nose and paranasal sinuses.

Immunohistochemistry is the application of immunologic principles and techniques to the study of cells and tissues.

## **MALIGNANT TUMORS OF NOSE AND PARANASAL SINUSES**

### **HISTOPATHOLOGICAL CLASSIFICATION**

- Basal cell carcinoma
- Squamous cell carcinoma
- Tumors of minor salivary glands
- Sarcomas
- Malignant melanoma
- Esthesioneuroblastoma
- Lymphoreticular neoplasms

- Plasmocytoma
- Adenocarcinoma
- Undifferentiated carcinoma
- Malignant neurogenous tumors

**Squamous cell carcinoma:**

- 50% begin in the maxillary antrum
- Every patient has signs of bony destruction when first seen
- Male preponderance
- Most cases the disease is localized
- Nasal vestibule tumors – indolent course – survival 80%
- Columella carcinoma - most aggressive form of nasal tumor
- High recurrence rate

### **Adenocarcinoma:**

- Uncommon tumor
- Common in people working in hardwood industry
- Histologically adenocarcinoma are best classified into high and low grade adenocarcinoma

### **Malignant melanoma:**

- This comprises 1% of nasal and paranasal sinus cancers.
- Histologically, there is no relationship to clark's skin classification
- Origin from the sinus mucosa is uncommon – if occurs survival is 0%, comparatively nasal melanoma has better response rate.
- Amelanotic melanoma quite frequently present as unilateral polyps, emphasizing the importance of sending all polypoidal material for pathological examination
- Biological behavior unpredictable
- Combination chemotherapy and immunotherapy have been used to treat melanomas.

## **Esthesioneuroblastoma (olfactory neuroblastoma)**

- This is a neuroendocrine tumor, until recently rarely described- only 400 cases reported.
- Resembles an anaplastic carcinoma and may remain undiagnosed unless the pathologist uses special marker
- Tumor arises in the upper part of nasal cavity from stem cells of neural crest origin, which differentiate into olfactory sensory cells.
- It is regarded as one of the primitive neuroectodermal tumors.
- Catecholamines can be demonstrated in olfactory neuroblastoma by fluorescent techniques after formaldehyde vapor (or) glyoxylic acid treatment.
- Biopsy shows nests of characteristic cells separated into compartments with rosette formation
- Sometimes tissue produces only sheets of densely packed uniform round cells and is mistaken for undifferentiated carcinoma
- Differs from sympathetic neuroblastoma as all ages are affected
- This is a slow growing tumor which may become very large

- Destructive and by its very nature must be regarded as involving the cribriform plate.

**Tumors of minor salivary glands:**

- The adenoid cystic carcinoma, muco epidermoid carcinoma can occur in the nasal sinuses.
- Vascular invasion, perineural invasion distant metastasis are frequent features.

**Nasal neoplasms - Immunohistochemistry**

Melanoma	S-100, Vimentin; HMB45
Esthesioneuroblastoma	Sustentacular pattern S-100
Lymphoma	Common Leucocytic Antigen(CLA)  Cluster Differentiation(CD)
Muscle derived	Muscle-specific actin/ desmin

## Treatment policy of sinonasal malignancy:

- Surgery alone (or) in combination with radiotherapy is required in the majority of case
- Lymphomas are treated by grading and assessment of spread and then by radiotherapy and chemotherapy.
- **Chemotherapy**

As part of triple therapy

E g: embryonal rhabdomyosarcoma

In combination with radiotherapy

Eg; disseminated lymphoma

### **As palliation**

Eg; Poorly differentiated squamous cell carcinoma with disseminated disease

- **Surgery**

3 operations :

1. Lateral rhinotomy/Medial maxillectomy

2. Total maxillectomy,

3. Craniofacial resection

- Esthesioneuroblastoma

This is a radiosensitive tumour

Craniofacial resection with radiotherapy

### **Malignant Tumors of Nasopharynx**

Carcinoma of the nasopharynx is a leading cause of death for large population in southeast asia

The age incidence curve is bimodal with a peak occurring between 15 to 25 years , another 60 to 69 years cases showing familial aggression have been reported.

The accumulated evidence strongly suggests that this tumour results from the combined action of

- Genetic Predisposition
- Environmental factors
- Epstein-Barr virus.

The virus can be demonstrated in the tumor tissue with insitu hybridization and immunohistochemical technique.

The virus has been found in all microscopic types of nasopharyngeal carcinoma including cases exhibiting glandular differentiation- although with different frequencies.

## **TUMOR TYPES**

### **Squamous cell carcinoma**

Carcinoma constitutes 85% of all malignant tumors of the nasopharynx. Nasopharyngeal carcinoma is divided into three types in the World Health Organisation(WHO) classification.

All are regarded as varieties of squamous cell carcinoma. The three types are

1. Keratinizing squamous cell carcinoma

Well differentiated

Moderately differentiated

Poorly differentiated.

2. Non-keratinizing carcinoma which is undifferentiated.
3. Undifferentiated carcinoma of nasopharyngeal type.

The nasopharyngeal carcinoma (formerly called transitional cell carcinoma, lymphoepithelioma or lymphoepithelial carcinoma) are now regarded as falling within the category of undifferentiated carcinoma of nasopharyngeal type.

THE LYMPHOCYTE INFILTRATE IN UNDIFFERENTIATED CARCINOMA CAN BE PREDOMINANT AND LEAD TO ERRORS IN DIAGNOSIS UNLESS IMMUNOHISTOCHEMICAL TECHNIQUES WITH LYMPHOCYTE MARKERS AND ANTICYTOKERATIN ANTIBODIES ARE USED TO PREVENT CONFUSION WITH LYMPHOMAS.

It is impossible to say from where the tumor arise. Most of the tumors arising from fossa of rosenmuller

### **Other malignant tumors**

- The second most common adult tumor is lymphoma. In 95% cases, it is non-Hodgkins lymphoma.

- Adenocarcinoma
- Adnoid cystic carcinoma
- Plasmacytoma
- Multiple myeloma
- Malignant melanoma can rarely occurs in nasopharynx.

In children Rhabdomyosarcoma is the commonest malignancy in nasopharynx and accounts for 30% of all malignancies in this site.

### **Treatment policy**

The majority of patients are treated radically.

### **Radiation Therapy**

This is the primary treatment modality for nasopharyngeal carcinoma

Radiation field must be large which includes oropharynx, retropharyngel node.

The nasopharynx is a midline structure with bilateral lymphatic drainage and so both sides of the neck should be treated even if only one

side has detectable lymphnodal enlargement. If the node is clinically involved, cervical node should be irradiated electively.

### **Chemotherapy**

Often with platinum based combination is being used increasingly frequently in conjunction with radiotherapy for nasopharyngeal carcinoma.

### **Surgery**

Has minimal role that too in recurrent tumors.

### **Immunohistochemical features**

Immunohistochemically, nasopharyngeal carcinoma shows reactivity for Keratin(always), epithelial membrane antigen (usually), CEA(occasionally).

Thus keratin is the most reliable marker for identification of this neoplasm. A population of S-100 protein positive dendritic cells may also be present.

## **Malignant tumors of the Oropharynx**

- The oropharynx is lined by squamous epithelium, so squamous carcinoma represents the most common tumor
- There is abundant lymphoid tissues in the palatine tonsil and also the lingual tonsil which can be affected in the head and neck lymphoma
- The soft palate is especially rich in minor salivary glands – minor salivary gland tumors can occur.

### **Tumor types**

- Squamous cell carcinoma-commonest malignancy - 90%
- Non-hodgkins lymphoma - 8%
- Minor salivary gland tumor - 2%

### **Prognostic factors**

- General condition/age
- TNM status
- Histology of the tumor
- Vascular/perineural invasion

## **Tumor Spread**

### **Lateral wall tumors (Tonsil)**

These are the commoner, often involves tonsil spread to retromolar trigone, to buccal mucosa, into the muscles of tonguebase. If erode deeply, they involve pterygoid muscles producing trismus. Inferiorly to the lateral pharyngeal wall, pyriform sinus, inferomedially to aryepiglottic fold, paraglottic space and posteriorly to the posterior pharyngeal wall.

### **Base of tongue tumors**

- Symptoms present only in the advanced stage
- Rapidly spreads through genioglossus muscle and across the midline, very quickly involve the entire tongue
- Spreads inferiorly into vallecula, the epiglottis and hence into the supraglottis and preepiglottic space.

### **Soft palate tumors**

almost exclusively appears on anterior surface spreads to nasopharynx, superior pole of the tonsil.

## **Treatment policy**

Treatment options: **squamous carcinoma**

### **Curative:**

Radiotherapy

Surgery

Surgery and post operative radiotherapy

### **Palliation:**

Radiotherapy

Radiotherapy/chemotherapy

Tracheostomy

## **Inoperability of oropharyngeal squamous cell carcinoma**

Poor general condition

Disease involving nasopharynx, tongue, larynx, direct neck extension.

Distant metastasis

Second primary

## **Lymphoma:**

Once the diagnosis of lymphoma was made, the disease should be staged and treatment may be with either chemotherapy (or) radiotherapy (or) combined modality of treatment. Most recurrences, if occurs, occur within 18 months. If there is a recurrence . the patient has only 10% chance of long time survival.

## **Malignant tumors of the Hypopharynx**

- Hypopharynx represents the lower most part of the pharynx
- Divided into 3 distinct sites
- Posterior pharyngeal wall – 10%
- The pyriform sinus – 55%
- The post cricoid space – 35%

## **Squamous cell carcinoma**

- Piriform sinus may involve lateral wall (or) medial wall -marginal
- More extensive, than they appear at clinical examination.
- Post cricoid tumor shows submucosal extension

- Maximal mural extension is medially 25mm and downward-20mm
- Lymphnodal metastasis more at presentation is seen in post cricoid and posterior pharyngeal wall tumors.

### **Other malignant tumors**

- Pseudosarcoma (or) spindle cell carcinoma,
- Plasmocytoma,
- Leiomyosarcoma,
- Adenoid cystic carcinoma

# Lymphoma

Some tumors which are not exclusively in the province of the head and neck surgeon and can occur in many other parts of the body, includes various types of lymphoma .

When they present with head and neck symptoms and signs, the head neck surgeon plays a pivotal role in obtaining tissue for diagnosis and in the treatment .

The lymphoid malignancy divided into

- Hodgkin's disease
- Non-Hodgkin's lymphoma

Malignant lymphoma can present initially as a mass in the sinonasal region, Nasopharynx, Tonsil. Nearly all cases are of Non.Hodgkin type. The large majority fall in three categories

- Natural killer (NK) /T-cell type
- B-cell type
- Peripheral Tcell type

## **NK /T-cell lymphoma**

This is a recently delineated distinct clinicopathologic entity which is highly associated with Epstein-Barr virus. Morphologically, it is characterized by a broad cytologic spectrum ranging from small or medium sized to large transformed cells. Necrosis is nearly always present.

Angioinvasion by tumor cells is a very frequent and diagnostically important feature and this is sometimes accompanied by epitheliotropism reminiscent of that seen in mycosis fungoides. An admixture of reactive histocytes some exhibiting erythrophagocytosis is a frequent feature, probably representing as expression of the virus-associated hemophagocytic syndrome. This type of lymphoma is referred to as **angiocentric lymphoma**.

The immunophenotype of NK/ T-cell lymphoma is characteristic. Positive for CD2 and CD56 and usually negative for surface CD3. Cytoplasmic CD3 can be detected in paraffin sections. There is no clonal rearrangement of the T-cell receptor gene. Tumors with similar phenotype occurring at other extranodal sites referred to as nasal type NK/T-cell lymphoma (in skin, GIT) Blastoid, Leukemic forms of this

lymphoma occur. CD56 positivity is a key diagnostic feature. This can be also seen in non-lymphoid neoplasms eg., Ewing's sarcoma.

High percentage of cases of lethal midline granuloma are due to NK/T-cell lymphoma.

### **B-cell Lymphoma**

B-cell lymphoma of the sinonasal region usually presents in a large cell lymphoma with a diffuse pattern of growth and a relatively monomorphic appearance. It is much more common in paranasal sinuses than in the nasal cavity. It constitutes the most common site of sinonasal lymphoma.

B-cell lymphoma with a diffuse undifferentiated appearance are the predominant form of sinonasal lymphoma in the pediatric population.

### **Peripheral Tcell lymphoma**

Peripheral Tcell does not express CD56 and usually lacks the necrotizing and angiocentric features of NK/Tcell lymphoma. The behavior of sinonasal lymphoma is difficult to assess because of the fact that one of its major types has been only recently defined, that some of the reported series do not include a thorough immune phenotypic

evaluation and that many cases of NK/Tcell lymphoma go unrecognized for years.70-80% of patients with Tcell lymphoma shows complete response rate of 75%. In case of B cell lymphoma overall survival rate is 52%.

### **Plasmacytoma**

Arising in the nasal cavity (or) nasopharynx may present primarily in the nose as a soft bleeding mass. microscopic examination shows a monomorphic infiltration by immature plasma cells. The majority of the patients with apparently solitary plasma cell tumors of the upper air passages in whom there is adequate follow up develop disseminated myeloma. Local control of disease usually be achieved with radiation therapy.

### **Angiotropic lymphoma**

In this condition the neoplastic lymphocytes are predominantly within the lumen of the vessels rather than infiltrating the vessel wall. This unusual type of lymphoma can present initially as an intranasal lesion.

### **Pseudolymphoma**

Lymphoid hyperplasia present as polypoidal intranasal mass.

## **Hodgkin's disease**

This is very rarely affects the head and neck region isolated occurrence reported. Peak age distribution is bimodal. 20 to 30 years one peak. another peak at old age.

Association with Epstein-Barr virus noted in some cases.

## **Histopathology**

The typical lymphnode architecture is affected by a mixture comprising predominantly normal cells and a lesser proportion of abnormal lymphoid cells. The normal cells – lymphocytes, histocytes, eosinophils, plasmacells and the malignant population Reed-sternberg cells, its mononuclear variant called **Hodgkin cell**.

**Reed-sternberg cell:** Large cell with eosinophilic cytoplasm. It contains 2, sometimes 4 (or) more mirror-image lobulated nuclei with darkly staining nuclear membrane.

## **B symptoms in Hodgkin's disease**

- Fever, Weight loss, Night sweats

## **The Revised European-American Lymphoma (REAL) classification**

### **B-cell neoplasm**

#### I- Precursor B-cell neoplasm

1. Precursor B-lymphoblastic lymphoma

#### II – Peripheral B-cell Neoplasms.

1. B cell chronic lymphocytic leukemia/ small lymphocytic lymphoma.
2. Lymphoplasmacytoid lymphoma
3. Mantle cell lymphoma
4. Follicular lymphoma grade I, II, III
5. Marginal zone B-cell lymphoma  
.Extranodal , .nodal
6. Splenic marginal zone lymphoma
7. Hairy cell leukemia
8. Plasma cytoma/myeloma

9. Diffuse large B cell lymphoma

10. Burkitt's lymphoma

11. High grade B-cell lymphoma, Burkitt like.

### **Tcell and postulated natural killer cell (NK) neoplasm**

#### **I . Precursor Tcell neoplasm**

1. Precursor T-lymphoblastic lymphoma

#### **II . Peripheral T cell and postulated NK cell neoplasm**

1. T cell chronic lymphatic leukemia/ lymphoma

2. Large granular cell lymphatic leukemia

. T cell type / natural killer cell type

3. Mycosis fungoides/ sezary syndrome

4. Peripheral T cell lymphoma unspecified

5. Angioimmunoblastic T cell lymphoma

6. Angiocentric lymphoma

7. Intestinal T cell lymphoma

8. Adult T cell lymphoma (HTLV-I)
9. Anaplastic large cell lymphoma(T and NK cell type)
10. Anaplastic large cell lymphoma Hodgkin's like.

### **Murphy staging system for Non-Hodgkin's lymphoma**

<b>Stage</b>	<b>Definition</b>
I	<p>A single tumor(extranodal) (or) single anatomical area (nodal) with the exclusion of the mediastinum or abdomen.</p>
II	<p>A single tumor with regional node involvement</p> <ul style="list-style-type: none"> <li>▪ Two or more nodal areas on the same side of diaphragm</li> <li>▪ Two single tumors with/without regional node involvement</li> <li>▪ On the same side of the diaphragm</li> </ul>
III	<p>Two single tumors on opposite side of the diaphragm</p> <ul style="list-style-type: none"> <li>▪ Two or more nodal areas above and below diaphragm</li> <li>▪ All primary intrathoracic tumor (pleural, Thymic)</li> </ul>

- All primary intraabdominal disease

IV Any of the above with initial CNS/ bone marrow  
Involvement.

### **Rye's classification of Hodgkin's disease**

1. Lymphocyte predominant (rare) - Nodular,Diffuse
2. Nodular sclerosing (common type) –Type I and II
3. Mixed cellularity (less common)
4. Lymphocyte depleted (rare)

### **Ann Arbor staging classification**

<b>Stage</b>	<b>Definition</b>
I	involvement of single extranodal site (or) single nodal  Region such as spleen, waldeyer's ring
II	2 or more lymph node region/lymphatic structure on the  Same side of diaphragm

Localized involvement of an extranodal organs or site and

Of one or more lymphnode regions on same side of

Diaphragm

III Involvement of lymphnode region/structures on both

Sides of diaphragm

IV Diffuse or disseminated involvement of one or more

Extranodal organs/ tissues with or without associated

Lymphnode involvement

**A or B A- Absence B- presence of constitutional symptoms**

- More than 10% weight loss in 6 months
- Unexplained fever during previous month
- Recurrent night sweat during previous month

**Treatment policy**

- Tumor characterized, classified and staged- the patient's age, performance status, comorbidity , also should be taken into consideration.
- Localized stage I low grade follicular NHL may be cured by  
Involved field radical radiotherapy.
- Even extensive disease has better cure rate. Simple chemotherapy with oral chlorambusil & prednisolone may be all that is required.
- The initial treatment for fit patients with high grade lymphoma nowadays usually chemotherapy and about half of adult patients may be cured.
- In children the cure rate is close to three quarters.
- The standard regimen **CHOP** – cyclophosphamide, doxorubicin, vincristine, prednisolone
- For patients with localized disease especially in the head and neck, this is followed by radio therapy.

## IMMUNOHISTOCHEMISTRY

Immunohistochemistry or Immunocytochemistry (IHC) is a method for localizing specific antigens in tissues or cells based on antigen-antibody recognition; it seeks to exploit the specificity provided by the binding of the antibody with its antigen at the microscopic level. IHC has a long history, extending more than half a century from 1940 when Coons developed an immunofluorescence technique to detect corresponding antigens in frozen sections.

However only since the early 1990s has the method found general application in surgical pathology. A series of technical developments in IHC have created sensitive detection systems. Among them is the **enzymatic label** (Horse radish peroxidase) developed by Avrameas and colleagues which in the presence of a suitable colorogenic substrate systems, allowed visualization of the labeled antibody by orthodox light microscopy.

One of the critical issues in the development of immunoperoxidase techniques was related to the need to achieve greater sensitivity from the simplest one step direct conjugate method to multistep detection techniques such as the Peroxidase-Antiperoxidase, avidin-biotin conjugate and Biotin

streptavidin methods, together with amplification methods and highly sensitive polymer based labeling systems .

The development of **hybridoma technique** facilitated the development of IHC and the manufacture of abundant highly specific monoclonal antibodies, many of which found early application in staining of tissues. Only when the IHC became applicable to routinely formalin fixed, paraffin embedded tissue sections did it usher in the “brown revolution”. The critical significance of rendering the IHC technique suitable for routine paraffin sections was illustrated by Taylor and colleagues, who in 1974, showed that it was possible to demonstrate antigens in routinely processed tissue .

**Enzyme digestion** was introduced by Huang and colleagues as a pretreatment to IHC staining to unmask some antigens that had been altered by formalin fixation. However the enzyme digestion method, while widely applied did not improve IHC staining of the majority of antigens as reviewed by Leong and colleagues. Another drawback of enzyme digestion was that it proved difficult to control the optimal “digestion” conditions for individual tissue sections when stained with different antibodies. These difficulties in

standardization provided a powerful incentive for the development of a new technique.

The **antigen retrieval** (AR) technique was developed by Shi and associates in 1991. In contrast to enzyme digestion the AR technique is a simple method that involves heating routinely processed paraffin sections at high temperatures before IHC staining procedures. An alternative method that did not use heating was developed for celloidin-embedded tissues. The intensity of IHC staining was increased dramatically after AR pretreatment, as demonstrated by various articles.

## **BASIC PRINCIPLES OF IMMUNOHISTOCHEMISTRY**

The object of all stains is to recognize micro chemically the existence and distribution of substances which we have been made aware of macro chemically. The basic principle of IHC as with any other special staining method is a sharp localization of target components in the cell and tissue based on a significant signal-to-noise ratio. Amplifying the signal while reducing the nonspecific background staining (noise) is the major strategy to achieve a satisfactory and practically useful result.

An **antibody** is a molecule that has the property of combining specifically with a second molecule, termed the **antigen**. **Antigen-antibody** recognition is based on three dimensional structure of protein or antigen, which is a critical issue in the understanding of the effectiveness of IHC as well as the mechanisms of AR. The term epitope corresponds to a cluster of amino acids residues that bind specifically to the paratope of an antibody. An **epitope** is a functional unit and not structural element of a protein and may be classified as continuous and discontinuous. The former are composed, of a continuum of residues in a polypeptide chain, whereas the latter consist of residues from different parts of a polypeptide chain, brought together by the folding of the protein conformation.

The development of hybridoma technique provided an almost limitless source of highly specific antibodies. Although monoclonal antibodies do not guarantee antigen specificity, since different antigens may share similar or cross reactive epitopes, the practical specificity reflected by IHC is excellent for most monoclonal antibodies tested. In contrast a polyclonal antibody is in fact an antiserum, which contains several different molecular species of antibody having varying affinities and even varying specificities against the different antigens or antigenic determinants As a

result polyclonal antibodies may give more nonspecific background staining in slides than the staining obtained using monoclonal antibodies.

Comparison of sensitivity and specificity between polyclonal and monoclonal antibodies indicate that polyclonal antibody may be more sensitive but less specific than monoclonal antibody. The reason may be that polyclonal antibody may recognize several different epitopes on a single antigen whereas a monoclonal antibody recognizes only a single type of epitope. Sophisticated amplification techniques, coupled with use of the AR technique have reduced the practical importance of this distinction. Although the specificity of monoclonal antibody has been questioned regarding cross reactivity with non-target molecules, most commercially available monoclonal antibodies are highly reliable for IHC, but again the ultimate specificity control should be the observation of the expected pattern of staining in control tissue sections, with the corresponding lack of unexpected or inexplicable staining reactions .

## **BLOCKING NON-SPECIFIC BACKGROUND STAINING**

There are two aspects to the blocking of background staining of tissues, nonspecific antibody binding and the presence of endogenous enzymes. Non specific antibody binding is generally more of a problem with polyclonal antibody, because multiple unwanted antibodies may exist in antiserum. The greater the optimal working dilution, the smaller the problem. If necessary it is advisable to pre incubate the tissue sections with normal serum from the same species of animal in order to occupy unwanted binding sites before incubation with primary antibody.

If enzymes similar to those used as a tracer are present in the tissue they may react with the substrate used to localize the tracer and give rise to problems in interpretation. Inhibiting the endogenous enzymes activity prior to staining can eliminate false positive reactions produced in this way. Peroxidase and substances giving a pseudo peroxidase reaction are present in normal and neoplastic tissues, e.g. leucocytes and erythrocytes and various methods have been described for the destruction of their activity.

Incubation in absolute methanol containing 0.5 percent hydrogen peroxide for 10 minutes at room temperature has been reported to

produce an almost complete abolition of endogenous peroxidase activity without affecting the immunoreactivity of antigens.

There are many types of alkaline phosphatase within the human body and most endogenous alkaline phosphatase activity can be blocked using a 1mM concentration of levamisole in the final incubating medium. The other commonly used enzyme labels glucose oxidase and bacterial beta-2-galactosidase do not present a problem.

## **IMMUNOCYTOCHEMICAL METHODS**

### **Traditional Direct Technique**

The primary antibody is conjugated directly to the labels. The advantage of the directly labeled antibody is that they are simple to use as they only require only one application of the reagent, followed by appropriate chromogen substrate solution. The disadvantage is that the sensitivity when compared to 2 or 3 stage techniques is low. While the most popular direct conjugates are those which are labeled with Fluorochrome. Horse radish peroxidase and alkaline phosphatase directly labeled antibodies are occasionally used.

### **New Direct Technique: (Enhanced Polymer one-step staining method)**

A large number of primary antibody molecules and peroxidase enzymes are attached to a dextran polymer backbone. The advantages of this technique are it is rapid, especially frozen sections immunohistochemistry and sensitive enough to demonstrate small amount of antigen.

### **Indirect Techniques:-**

The unconjugated primary antibody is applied, followed by a labeled antibody directed against the first antibody. Horse radish peroxidase labeling is most commonly used and with appropriate chromogen substrate is a more sensitive technique than the equivalent direct method.

### **Avidin-Biotin Techniques**

These methods rely on the marked affinity of the glycoprotein avidin for biotin, a low molecular weight vitamin. Avidin is present in egg white and is composed of four subunits which form a tertiary structure possessing biotin binding hydrophobic pockets. The oligosaccharide residues present in egg white avidin and its charged properties are reported as giving it some affinity for some tissue components and the result is non specific binding. Also some tissues such as liver contain large amounts of biotin and this can

cause further background problems. A similar molecule streptavidin can be extracted from the culture broth of the bacterium *Streptomyces avidini*. The lack of oligosaccharide residues and neutral isoelectric point is said to give streptavidin advantages over the chicken egg variant.

Biotin (vit H) is easily conjugated to antibodies and enzyme markers. Up to 150 biotin molecules can be attached to one antibody molecule, often with the aid of spacer arms. By spacing the biotins, the larger glycoprotein avidin has room to bind and maximize its strong affinity for biotin.

Variants of avidin-biotin system include peroxidase and alkaline phosphatase either directly bound to avidin or streptavidin. Alternatively the enzymes are biotinylated and 75% of avidin-binding sites are occupied by the biotinylated label forming the avidin-biotin complex. .

### **Hapten Labeling Technique:-**

Bridging techniques using haptens such as dinitrophenol and arsenilic acid have been advocated.(13) In this technique the hapten is linked to the primary antibody and a complex is built up using an anti-hapten antibody and either hapten-labeled enzyme or hapten labeled PAP complex.

### **Immuno Gold silver staining Technique (IGSS)**

The use of colloidal gold as a label for immunocytochemistry was introduced by Faulk and Taylor. It can be used in both direct and indirect methods and has found wide usage in ultrastructural immunolocalisation. It is not widely used in light microscope IHC even after the advantages of silver development reported by Holgate et al in 1983 (14). In this method the gold particles are enhanced by the addition of metallic silver layers. To produce slow forming metallic silver with a tolerance for natural light, the technique uses silver lactate as the ion supplier and hydroquinone as the reducing agent in a protective colloid of gum Arabic at PH 3.5. The method is generally accepted to be more sensitive than the PAP technique but suffers from the formation of fine silver deposits in the background and can be confusing when trying to identify small amounts of antigen.

## **Antigen Retrieval**

A simple heat induced AR technique is now widely applied in pathology. Successful application of the AR technique for routine IHC staining of formalin fixed tissues has rendered the search for alternative fixatives to replace formalin less urgent.

Heat mediated Ag Retrieval – Commonly employed antigen retrieval methods include microwave oven, pressure cooker, steamer, autoclave.

## **MATERIALS AND METHODS**

A study was made of **30 biopsies** performed from the period of April 2006 to March 2007 from the head and neck regions in the ENT department, Upgraded Institute of Otorhinolaryngology, Madras Medical College, Chennai – Histopathologically that were diagnosed as undifferentiated tumors.

### **Type of study**

Prospective study

### **Period of study**

April-2006 to March-2007

### **The Inclusion criteria**

- Tumors located in the head and the neck
- Histopathological diagnosis of undifferentiated tumors in sections stained using hematoxylin – Eosin (HE)
- Sufficient quantity of material in the paraffin section for Immunohistochemical technique to be performed.

## **The Exclusion criteria**

- Tumors with evident differentiation seen in sections stained using HE
- Specimens with insufficient material for the immunohistochemical technique
- Tumors affecting the skin of head and neck region
- Secondary necknodes with unknown primary lymphnode biopsies.

All the biopsies utilized were fixed in formalin 10%, embedded in paraffin and stained with hematoxylin – eosin (HE). They all had a diagnosis of undifferentiated tumors as seen under optical microscopy.

These tumors are distributed according to their site of occurrence, into **8 groups**, oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, nose, paranasal sinuses, others.

These biopsies applied to an immunohistochemical panel with monoclonal antibodies. In accordance with the avidin-biotin peroxidase complex method (ABC) and with respect to the patient's age and tumor location.

### **IMMUNOHISTOCHEMICAL PANELS EMPLOYED**

<b><i>Epithelial</i></b>	Cytokeratin
<b><i>Lymphoid</i></b>	CD - 3
	CD - 20
	CD - 45
<b>Mesenchymal</b>	S - 100 protein

### **IMMUNOCYTOCHEMICAL STAINING TECHNIQUES**

#### **Washes**

Between each step, sections require washing to prevent one reagent contaminating another. Gently wash with Tris buffered Saline (TBS) indicates several brief washes flooding the slide, followed by draining and wiping around the section to remove excess buffer.

## **Buffer**

0.005M Tris-buffered saline

Distilled water	10 liters
Sodium chloride	80.0g
TRIS (hydroxymethyl methylamine)	6.05g
M HCL	44 ml

If necessary, adjust final pH to 7.6 with either 1M HCL or 0.2M TRIS solution.

After the appropriate pretreatment steps, the following method employed

## **Avidin-biotin Technique**

In these techniques either peroxidase or alkaline phosphatase may be used as the enzyme label.

Labelled avidin/avidin-biotin complex technique for monoclonal antibodies

## **Method**

1. Bring sections to TBS.
2. Drain and wipe off excess TBS around section]

3. Incubate in optimally diluted primary antibody for 30-40min
4. Gently wash slides with TBS
5. Incubate in optimally diluted biotinylated bridge reagent for 30min
6. Repeat step 4
7. Incubate in optimally prepared labeled avidin/avidin-biotin complex for 30min.
8. Repeat step 4
9. Incubate in DAB substrate solution
10. Wash in running water, counterstain in hematoxylin, dehydrate clear and mount.

The final diagnosis was achieved after new microscopic analysis in conjunction with sections stained using the hematoxylin – Eosin Technique. The results were distributed according to patients age, tumor location

## RESULTS AND ANALYSIS

The following are the results and analysis after a study of 30 biopsies of undifferentiated tumors and the application of Immunohistochemical techniques.

### AGE AND SEX DISTRIBUTION

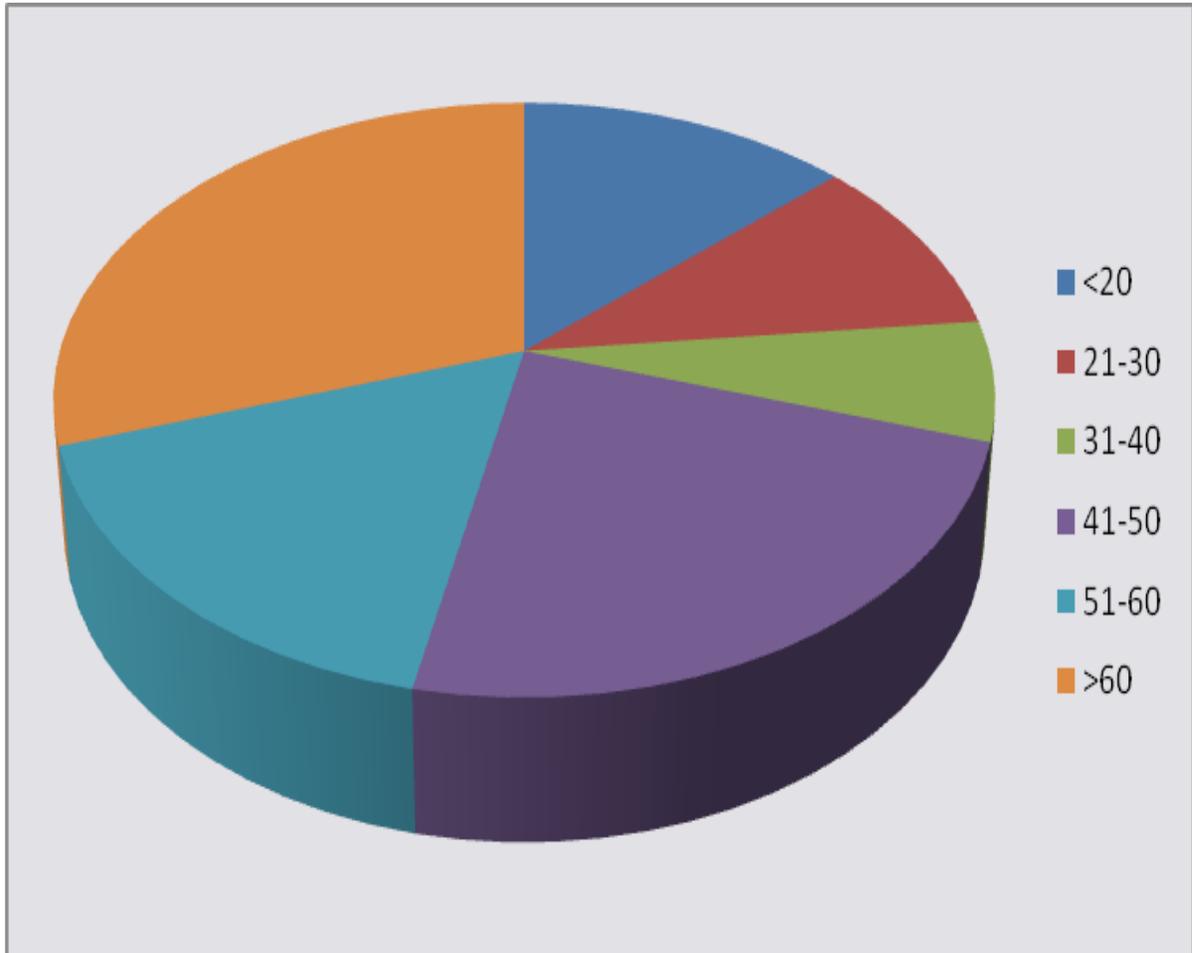
Age incidence and sex distribution of the patients with undifferentiated head and neck cancer was tabulated and analysed.

TABLE – I

<i>Age incidence</i>	<i>Sex</i>		<i>Total</i>	<i>Percentage</i>
	<i>Male</i>	<i>Female</i>		
<b>20&gt;</b>	1	3	4	13.3%
<b>21 to 30</b>	2	1	3	10%
<b>31to 40</b>	-	2	2	6.5%
<b>41 to 50</b>	5	2	7	23.33%
<b>51 to 60</b>	3	2	5	16.67%
<b>&gt; 61</b>	9	-	9	30%
<b>Total</b>	20	10	30	100%

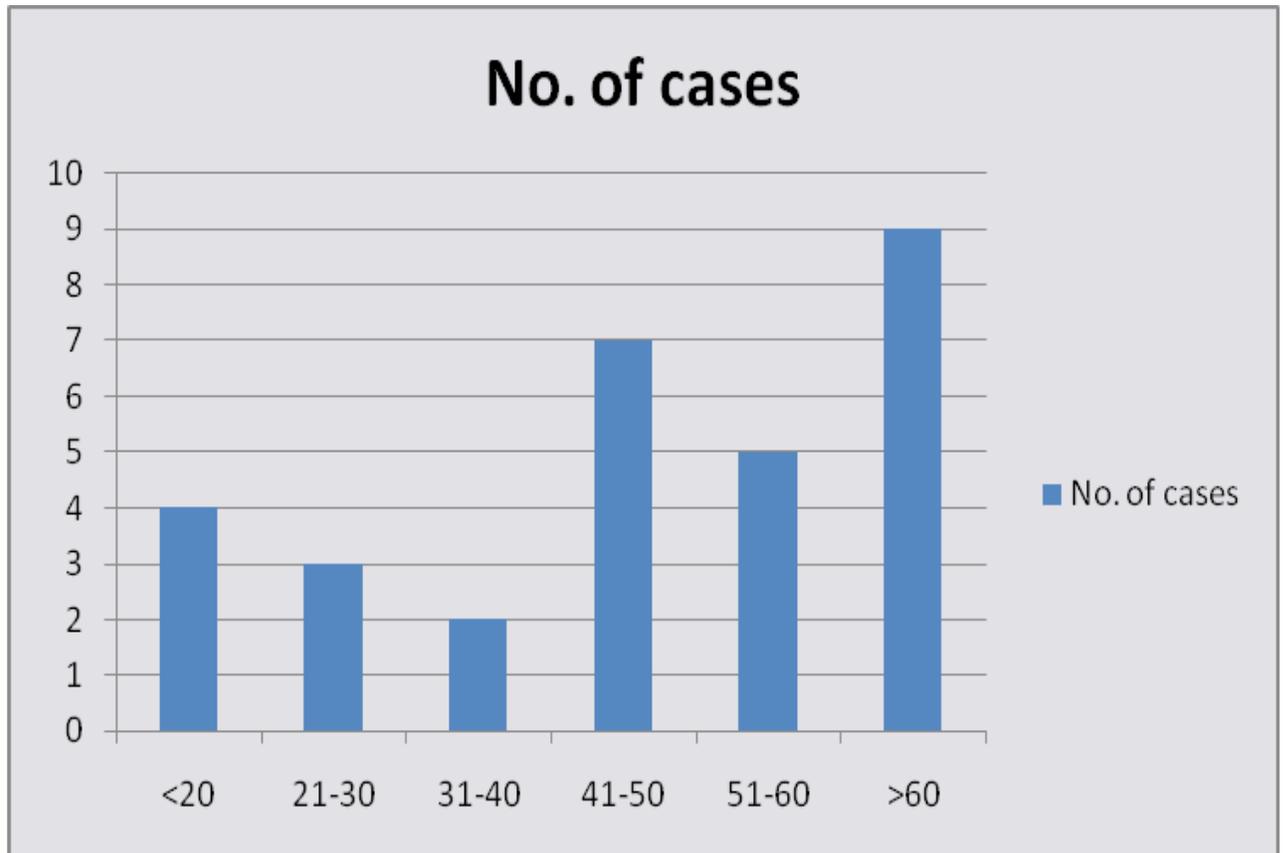
### UNDIFFERENTIATED HEAD AND NECK CANCERS

## AGE INCIDENCE



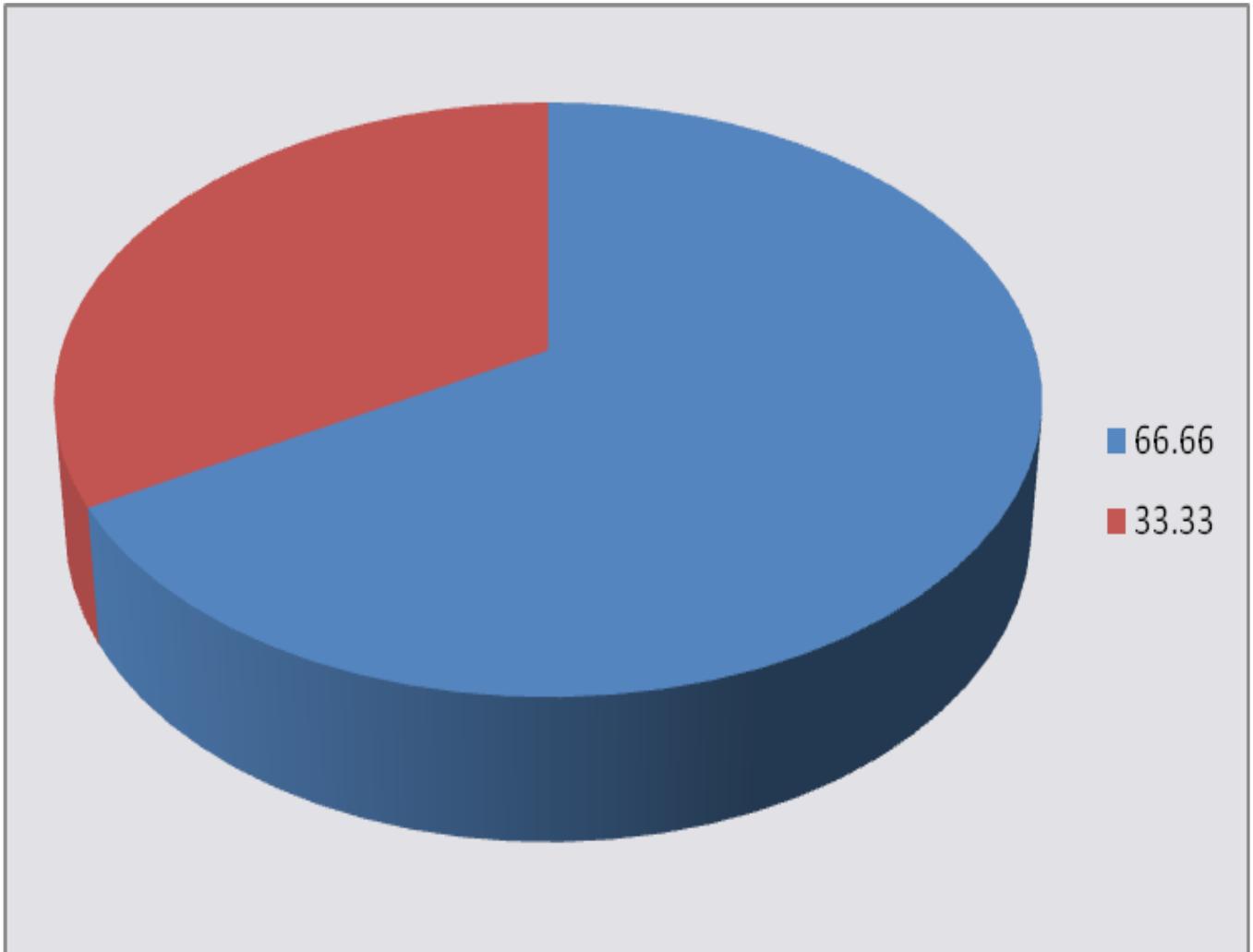
## UNDIFFERENTIATED HEAD AND NECK CANCERS

## AGE DISTRIBUTION



## UNDIFFERENTIATED HEAD AND NECK CANCERS

## SEX INCIDENCE



**BERNARD INSTITUTE OF RADIOLOGY AND ONCOLOGY  
STATISTICS**

<b><i>Head and Neck</i></b>	<b><i>Male</i></b>	<b><i>Female</i></b>	<b><i>Total</i></b>
<b>Lip</b>	4	4	8
<b>Alveolus</b>	18	9	27
<b>Tongue</b>	70	29	99
<b>Floor of mouth</b>	16	4	20
<b>Cheek</b>	64	68	132
<b>Palate</b>	25	7	32
<b>Salivary gland</b>	14	10	24
<b>Oropharynx</b>	82	31	113
<b>Nasopharynx</b>	18	8	26
<b>Laryngopharynx</b>	87	59	146
<b>Larynx</b>	88	30	118
<b>Ear</b>	4	1	5
<b>Nasal cavity</b>	13	8	21
<b>Paranasal sinuses</b>	12	7	19
<b>Other sites</b>	32	19	51
<b>Total</b>	547	284	841

Among Head and neck tumors , undifferentiated tumor constitutes 3.57% , where males constitute 3.66% and females 3.52%

## SITE OF OCCURRENCE

### CLINICAL CLASSIFICATION OF PATIENTS

TABLE - III

<i>SITE</i>	<i>Male</i>	<i>Female</i>	<i>Total</i>	<i>Percentage</i>
Oral cavity	1	2	3	10
Oropharynx	7	0	7	23
Nasopharynx	3	3	6	20
Hypopharynx	2	2	4	13
Larynx	1	1	2	6.5
Nose	1	2	3	10
Paranasal Sinuses	3	-	3	10
Others	2	-	2	6.5
<b>Total</b>	<b>20</b>	<b>10</b>	<b>30</b>	

- Pharynx constitutes 56% of those, Oropharynx 23% which suggests The overall incidence of oropharyngeal tumor is high rather than its higher percentage. Because the percentage of undifferentiated oropharyngeal tumor compared with other oropharyngeal tumor is 6.19%.

**PROPORTION OF UNDIFFERENTIATED TUMORS OF HEAD  
AND NECK**

**TABLE - IV**

<b>LOCATION</b>	<b>CANCER</b>	<b>UNDIFFERENTIATED CANCER</b>	<b>PERCENTAGE</b>
<b>Oral cavity</b>	179	3	1.68%
<b>Oropharynx</b>	113	7	6.19%
<b>Nasopharynx</b>	26	6	23.08%
<b>Hypopharynx</b>	146	4	2.74%
<b>Larynx</b>	118	2	1.69%
<b>Nose</b>	21	3	14.29%
<b>Paranasal sinus</b>	19	3	15.79%

- Naso pharynx has highest proportion of undifferentiated tumor .  
that is 23%

## IMMUNOHISTOCHEMISTRY RESULTS

TABLE - V

<i><b>Tumor Location</b></i>	<i><b>Carcinoma</b></i>	<i><b>Lymphoma</b></i>	<i><b>Others</b></i>	<i><b>Inconclusive</b></i>	<i><b>Total</b></i>
<b>Oral cavity</b>	3	-	-	-	3
<b>Oropharynx</b>	2	2	-	3	7
<b>Nasopharynx</b>	2	2	2	-	6
<b>Hypopharynx</b>	3	-	-	1	4
<b>Larynx</b>	-	1	-	1	2
<b>Nose</b>	1	1	1	-	3
<b>Paranasal sinus</b>	2	1	-	-	3
<b>Others</b>	-	-	1	1	2
<b>Total</b>	13	7	4	6	30

- In case of nasal cavity, S-100 positive olfactory neuroblastoma diagnosed.
- More numbers of lymphoma diagnosed in Oropharynx and Nasopharynx , 2 each.

**TUMOR TYPES IN ACCORDANCE WITH  
AGE OF PATIENTS**

**TABLE - VI**

<i>AGE</i>	<i>Carcinoma</i>	<i>Lymphoma</i>	<i>Others</i>	<i>Inconclusive</i>	<i>Total</i>
<b>20&gt;</b>	1	2	1	-	4
<b>21 to 30</b>	1	2	-	-	3
<b>31to 40</b>	1	-	-	1	2
<b>41 to 50</b>	2	1	2	2	7
<b>51 to 60</b>	1	1	1	2	5
<b>&gt; 61</b>	7	1	-	1	9
<b>Total</b>	13	7	4	6	30

- In younger age – Lymphoma is the dominant group.
- For above 60 years of age – carcinoma is the dominant type

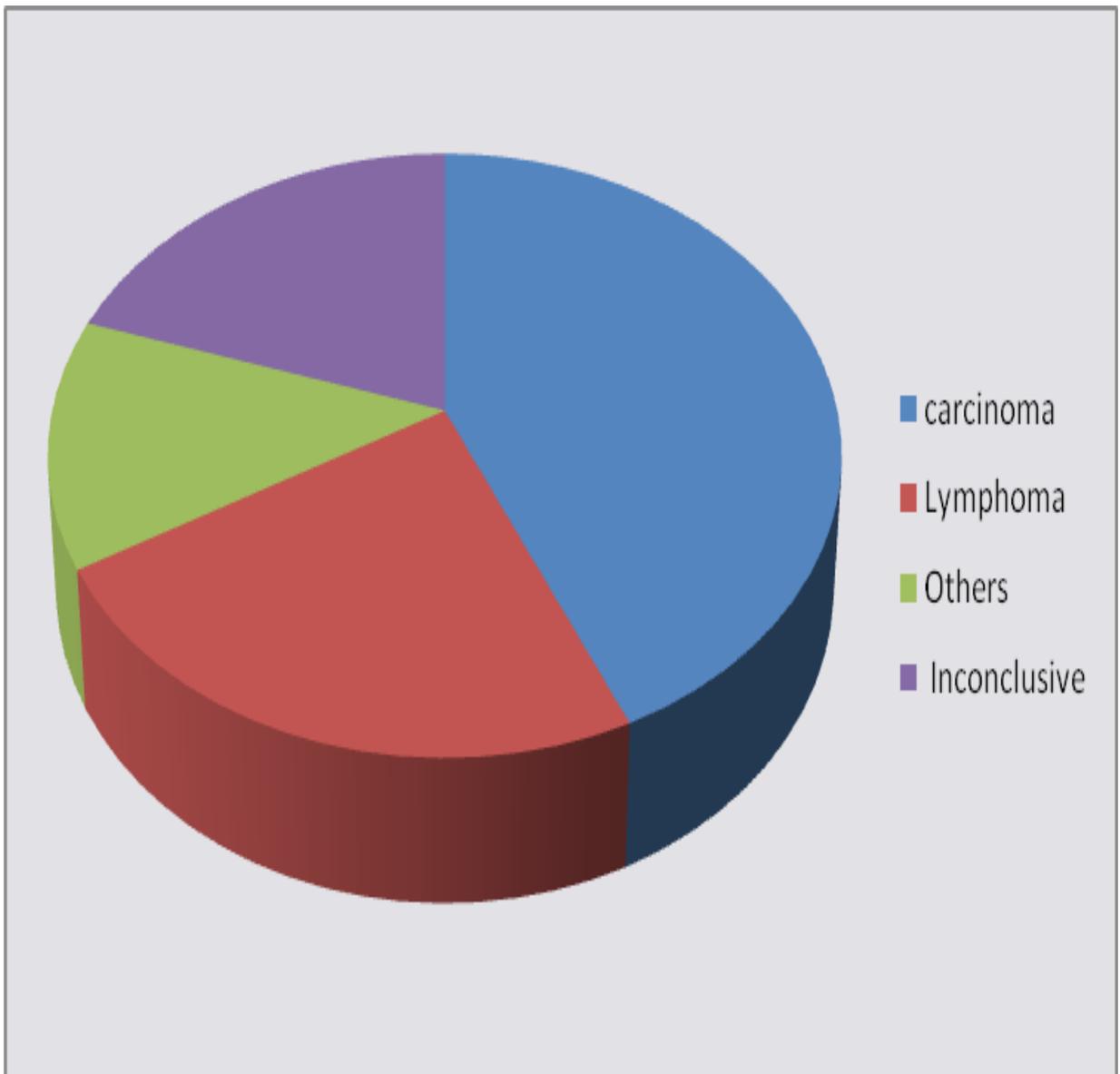
## PERCENTAGE OF TUMOR TYPES

TABLE VII

<i>Type of Tumors</i>	<i>No. of cases</i>	<i>Percentage</i>
<b>Carcinoma</b>	13	43.33 %
<b>Lymphoma</b>	7	23.33 %
<b>Others</b>	4	13.33 %
<b>Inconclusive</b>	6	20 %

- Carcinoma is the major group followed by lymphoma
- Either conclusive or suggestive diagnosis of undifferentiated tumor was achieved on this study is 80%
- Inconclusive diagnosis – that is not positive for any of the immunohistochemical panel (or) positive for carcinoma, lymphoma and mesenchymal markers, because of multiple epitopes is the main cause in this study.

## PERCENTAGE OF TUMOR TYPES



**IMMUNOHISTOCHEMICAL MARKER POSITIVITY WITH  
RELEVANCE TO TUMOR LOCATION**

**TABLE VIII**

<i><b>Tumor location</b></i>	<i><b>CK</b></i>	<i><b>CD3</b></i>	<i><b>CD20</b></i>	<i><b>CD45</b></i>	<i><b>S-100</b></i>
<b>Oral Cavity</b>	4	-	-	-	1
<b>Oropharynx</b>	2	-	2	2	-
<b>Nasopharynx</b>	4	-	3	3	-
<b>Hypopharynx</b>	3	-	-	-	-
<b>Larynx</b>	-	1	-	1	-
<b>Nose</b>	1	-	1	1	1
<b>Paranasal sinus</b>	2	1	-	-	-
<b>Others</b>	2	-	2	-	1
<b>Total</b>	18	2	8	7	3

- Cytokeratin is indicative of the carcinoma
- CD20, CD45 positivity indicates the lymphoma which is of B-cell variety.
- CD3 positivity indicates T cell lymphoma (or) natural killer cell lymphoma

**CONCLUSIVE DIAGNOSIS BY  
IMMUNOHISTOCHEMICAL TECHNIQUES ACCORDING  
TO TUMOR LOCATION**

**TABLE IX**

<b>Location</b>	<b>Conclusive</b>	<b>Inconclusive</b>	<b>Total</b>	<b>Conclusive percentage</b>
<b>Oral Cavity</b>	3	-	3	100%
<b>Oropharynx</b>	4	3	7	57.14%
<b>Nasopharynx</b>	6	-	6	100%
<b>Hypopharynx</b>	3	1	4	75%
<b>Larynx</b>	1	1	2	50%
<b>Nose</b>	3	-	3	100%
<b>Paranasal sinus</b>	3	-	3	100%
<b>Others</b>	1	1	2	50%

- 100% conclusive diagnosis was achieved in cases of oral cavity, Nasopharynx, nose and paranasal sinuses in this study
- In Larynx and oropharynx, the percentage of conclusive diagnosis is around 50%

## **DISCUSSION**

The poorly differentiated (or) undifferentiated tumors challenge routine histopathological classification, because of the absence of cell morphological differentiation. The diagnosis and classification of such tumors are fundamental, because suitable therapy and prognosis for each case depends upon precise histopathological diagnosis.

The immunohistochemical technique has revolutionized surgical pathology knowledge. The introduction of this method by Coons et al in 1942 has become a powerful complementary tool in tumor analysis.

There are only few references in the literature to the immunohistochemical technique applied to the identification of undifferentiated head and neck tumors.

### **Undifferentiated cancer incidence**

Head and neck tumors constitute about 35% of all diagnosed cancers. Undifferentiated tumors in the head and neck **represents 3.57%** of all tumors diagnosed in this study (30 cases out of 847 consecutive biopsies).

Walter Adriano bianchini (2003) in his study found the undifferentiated cancers constitutes 1.1% of all tumors diagnosed 43 cases out of 3,840.

### **Sex Incidence**

There were **20 male (66%)** , **10 female(33%)** cases in a proportion of 2:1 was found in this study

which was supported by AlbinaMaria Albermani in his study. Male and female ratio is 67.5% and female is 32.5%. Head and Neck cancers found predominantly in males than in females. Indian text books describe that most of the head and neck cancer occurs predominantly in males.

### **Age Incidence**

Head and Neck cancers and undifferentiated head and neck cancers were found predominantly in males.

Patients age ranged from 17 years to 72 years

These tumors were most prevalent in **6<sup>th</sup> decade of life(30%)** followed by 4<sup>th</sup> decade of life(23%)

Robert.J.Carpenter in his study (1989) found that in head and neck cancers, 60% of his patients in 5<sup>th</sup> and 6<sup>th</sup> decade

Walter adriano bianchini and Jarge Rizzato Paschaal (2003) in their study, the prevalence rate of undifferentiated carcinoma is in the 7<sup>th</sup> decade of life(34.9%)

Age of the patient ranged from 2 years to 89 years.

### **Incidence of site of undifferentiated cancer**

Abemayor.E; Kessler DJ, Ward PH (1987) in their study the commonest site of occurrence of head and neck tumor was pharynx and neck(21%) , lymphnode(18%), paranasal sinus(14%)

Walter Adriano bianchini (2003) in their study the commonest site of occurrence in Lymphnode (20.9%), pharynx(16.3%), Nose(11.6%) and PNS (9%)

In this study highest incidence was in **Oropharynx (23%)**, **Nasopharynx (20%)** followed by Nose and **paranasal sinuses (20%)**, hypopharynx(13%)

### **Proportional incidence of the undifferentiated cancer**

When compared to the other tumor types of the same site, the proportion of undifferentiated head and neck cancer is as follows.

**Nasopharynx(23%), Paranasal sinus(15.8%), nose(14%).** These data was supported by Gallo.O. Graziani et al (1993) – Nasopharynx(18%), Paranasal sinus(21%), nose (18%).

Subsite wise the more number of undifferentiated tumors found in posterior 1/3 of a tongue and nasopharynx.(six patients)

### **Type of tumors diagnosed by Immunohistochemical methods**

The immunohistochemical technique was usseful in conclusive diagnosis of the tumors in the oral cavity (100%), nasopharynx(100%), nose and nasopharynx(100%).

Milroy CM. Ferlito A et al (1995) in his study , the conclusive diagnosis was as follows : oral cavity(100%), Paranasal sinus(100%), Lymphnodes(89.9%) and Nasopharynx(57%).

Galter et al (1985) and Coindre et al (1982) demonstrated greater incidence of lymphomas in undifferentiated tumors in general.

Darrouzet .V et al (1989) in his study , the most frequent tumor is carcinoma(39%)

Walter Adriano Bianchini et al(2003) in his study , the most frequent tumor is carcinoma(27.9%) followed by lymphoma(20.93%)

In this study, the commonest tumor type diagnosed is **carcinoma (43%) followed by lymphoma (23%)**

Lymphoma was commonly found in the patients below the age of 30 years (4 cases ) -57%.

Carcinoma found in patients above 40 years of age (10 to 15 cases) – 76.92%.

Lymphomas commonly found in the oropharynx(Tonsil) and Nasopharynx, except 1 case, all are B cell Lymphoma.

### **Sensitivity of the Immunohistochemistry**

Abemayor et al (1987) in his study using immunohistochemistry, the conclusive diagnosis was achieved in 86.4% patients. It was not possible in 13.6%.

Walter Adriano Bianchini et al (2003) in his study, using immunohistochemistry, the diagnostic guidance was obtained in 81.4% of patients(35 of 43). It was not possible in 18.6%( 8 of 43)

In this study, conclusive diagnosis obtained in **80% (24 of 30)** of the patient. The result was inconclusive in 6 of 30 patients (20%)

The failure is probably due to limitations of the technique, lesser number of immunohistochemical panel of markers, Antigen changes during tissue fixation, true absence of cellular differentiation.

### **Immunohistochemical Markers (Panels)**

Gallo .O et al (1993) used 19 immunohistochemical markers as panel to study the undifferentiated cancer of nose and paranasal sinuses.

Walter Adriano et al (2003) used about 23 panel of immunohistochemical markers.

In this study, **5 immunohistochemical markers was used.** Cytokeratin positive cancers is the common pattern. 18 of 30 (60%), CD3 (8 of 30),CD45 (7 of 30), S-100(3 of 30).

Most of the head and neck tumors are of epithelial in origin,

## CONCLUSIONS

1. Undifferentiated cancers in the head and neck represents 3.57% of all tumors diagnosed in this study.
2. Undifferentiated head and neck cancers found predominantly in the males (66%) and highest incidence after the 4<sup>th</sup> decade of life (68%).
3. Undifferentiated head and neck cancers most frequently occurs in the nasopharynx (23%), nose (14%) and paranasal sinuses(16%) in this study.
4. Immunohistochemical technique is sensitive enough to conclusively diagnose 80% of the undifferentiated head and neck cancer.
5. The most common tumor type diagnosed in this study is carcinoma(43%) followed by lymphoma(23%)
6. 100% conclusive diagnosis achieved in oral cavity, Nasopharynx and Sinonasal cancers

**This study supports the notion that the immunohistochemical technique has a fundamental role in the investigation and definition of undifferentiated tumor origin, thus determining correct guidance for treatment and possibly improving the prognosis for head and neck oncological patients.**

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