DISSERTATION ON ROLE OF IMMUNOHISTOCHEMICAL STUDIES
IN NEUROENDOCRINE TUMOURS OF GASTROINTESTINAL TRACT

Dissertation submitted to

Tamil Nadu Dr. M.G.R Medical University
Chennai

for

MD (PATHOLOGY)
March 2008

Govt. Stanley Medical College
Chennai

The TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY
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CERTIFICATE

This is to certify that this dissertation entitled "**Role of Immunohistochemical Studies in Neuroendocrine tumors of Gastrointestinal tract**" is the bonafide work done by **Dr. K. Lavanya** in partial fulfillment of the requirement for **MD (Branch III) PATHOLOGY** examination of the **TAMIL NADU Dr.M.G.R. Medical University Chennai** - Tamil Nadu to be held in **March 2008**

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DECLARATION

I, **Dr. K. Lavanya,** solemnly declare that the dissertation titled **"ROLE OF IMMUNOHISTOCHEMICAL STUDIES IN NEUROENDOCRINE TUMOURS OF GASTROINTESTINAL TRACT"** is the bonafide work done by me at Govt. Stanley Medical College and Hospital during the period August 2005 to September 2007 under the expert guidance and supervision of **Prof.A.Sundaram MD, Head of the Department,** Department of Pathology.

The dissertation is submitted to the **Tamil Nadu Dr. MGR Medical University** towards partial fulfillment of requirement for the award of **MD Degree (Branch III) in Pathology.**

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ACKNOWLEDGEMENT

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<u>INTRODUCTION</u>

Neuroendocrine tumors of the gastrointestinal tract are not a rare entity. Neuroendocrine tumors were initially called APUDOMAs and were believed to be of neural crest origin. Later the endodermal origin was established. Due to the neurotransmitter like substance production the name neuroendocrine tumors have remained.

These tumors occur anywhere in the gut with a higher incidence in the large intestine.

Many attempts have been made to classify these tumors. The first classification was based on topography, then histological pattern, recently on immunohistochemical marker expression. Amongst the many neuroendocrine markers Chromogranin A, Synaptophysin are the most useful.

Most cases of well differentiated neuroendocrine tumors the diagnosis is easily made from the histological appearance. But in less well differentiated types the interpretation poses a challenge. A correct diagnosis is required because of the prognostic implication and variation in treatment modalities.

This work aims at confirming histologically diagnosed cases of neuroendocrine tumors and also detects neuroendocrine differentiation in otherwise exocrine and poorly differentiated tumors with the help of immunohistochemical markers Chromogranin A and Synaptophysin.

The association between neuroendocrine marker expression and their prognostic implication has also been studied.

AIMS AND OBJECTIVES

- To detect and confirm neuroendocrine tumors of gastrointestinal tract by immunohistochemistry, especially in cases where other malignant tumors cannot be excluded based on routine Hematoxylin and eosin stained sections alone

- To assess the extent of neuroendocrine differentiation in adenocarcinomas of gastrointestinal tract

- To determine the prognostic significance of neuroendocrine marker expression in the above categories

REVIEW OF LITERATURE

Neuroendocrine system is a dispersed system of cells with an endocrine and paracrine function. They are embryologically diverse but share some common functional characteristics that define them physiologically and diagnostically when they form tumors. They are also clinically fascinating because of their hormonal effects and syndromic effects.

Gastrointestinal system has the largest population of neuroendocrine cells. [1]

Neuroendocrine system – it consists of a variety of cells present in the CNS, PNS and in many organs including classic endocrine organs

- Heidenhain in 1980 described chromaffin cells in the GIT and suggested endocrine function
- Oberndofer in 1907 introduced the term carcinoid , an ileal tumor with a different behaviour from colon carcinoma
- Feyer in 1938 described clear cells in the GIT and suggested that they formed a part of the dispersed epithelial endocrine system and that some cells could have a paracrine effect [2].
- These cells have the ability to take up amine precursors such as DOPA AND 5-hydroxy tryptophan and subsequently decarboxylase them – these were termed APUD cells [3].
- There are 14 endocrine cell types in the gut and along with the pancreas producing at least 33 hormones and biogenic amines [4].

Components of diffuse neuroendocrine system

- GIT, bronchopulmonary and urothelial tract endocrine cells.

- Peptide and amine producing cells – C cells, islet cells of pancreas, pituitary and parathyroid cells.

- Chromaffin cells of adrenal medulla, carotid body and other paraganglia and Merkel cells of the skin

- Excludes hormone producing cells of the adrenal cortex, testis, ovary and thyroid follicular cells.

Why neuroendocrine?

- Pearse was the first to suggest that the diffuse endocrine system was of neural crest origin because of the similarity between APUD cells [amine precursor uptake and decarboxylation] and neurons. Similarities being production of bioactive substances that serve transmitter functions, contain secretory granules and similar cellular antigens [5].

- Le Doufarin refuted the neural crest origin based on embryological data

- GIT and bronchopulmonary carcinoids are definitely of endodermal origin.

- Only cells of proven neural crest origin are – cells of adrenal medulla, paraganglia and sympathetic ganglia.

- These cells produce substances similar to neurotransmitters/

neurohormones such as cholinesterases, peptides, hormones and substances for paracrine regulation – somatostatin.

- The term carcinoid literally means carcinoma-like was coined in 1907 to describe the histological similarity of these tumors to carcinomas on one hand and their general indolent behavior on the other [6].

- 1963 William and Sandler classified carcinoid tumors on the basis of embryogenesis into foregut, midgut and hindgut carcinoids

Neuroendocrine tumors comprise approximately 2% of all malignant tumors of the gastrointestinal system. They have been classified into two types – the carcinoid and neuroendocrine carcinomas. The incidence of all non – carcinoid neuroendocrine tumors is approximately one half of that of all carcinoids [7]. Non-carcinoid neuroendocrine tumors have been reported to occur in 0.4 – 1.5/100,000 of the population [8].

Site of occurrence of neuroendocrine tumors:

Neuroendocrine tumors are most common in the large intestine (especially in the descending colon and recto sigmoid) [9] closely followed by the appendix and the small intestine.

Carcinoids in GIT are more prevalent in the appendix and small intestine followed by the rectum and it is least common in stomach [10].

Origin (or) **tumor hypothesis:**

Jejuno-ileal tumors arise from IECH [intraepithelial endocrine hyperplasia]. The progenitor cells are intraepithelial and these tumors arise from an area that has been diffusely primed for their development – field effect.

Appendicular carcinoids – arise from sub-epithelial complex not associated with IECH [11].

Gastric carcinoids – ECL hyperplasia and tumor development associated with hypergastrinemic states due to unregulated hormone (gastrin) production. Increased incidence found in case of autoimmune chronic atrophic gastritis, Zollinger Ellison syndrome and MEN-TYPE 1 syndrome.

Net morphology:

- Pattern – nests, cords, rosettes, islands, small glands, sinusoidal stroma.

- Cytology – central or eccentric nuclei, stippled chromatin [salt and pepper nuclei]

Histochemistry of neuroendocrine tumours

- Chromaffin reaction – histological staining affinity for chromium salts and silver salts.

- Argyrophilia – staining with silver salts in the presence of a reducing agent

- Argentaffinity - histological staining affinity for silver salts.

- Formalin induced fluorescence

- Neurosecretory granules.

Initial markers of neuroendocrine cells were argentaffin and argyrophilic reaction. Carcinomas with numerous argentaffin or argyrophilic reactions were termed atypical carcinoids or neuroendocrine carcinoma [12].

Based on these reactions, gastric cancers with endocrine cells have been classified by Tahara E et al as:

1. Classical carcinoids
2. Endocrine cell carcinoma showing poorly differentiated adenocarcinoma
3. Endocrine cell cloning due to differentiation of carcinoma cell
4. Scirrhous argyrophilic cell carcinoma [13].

Markers of neuroendocrine differentiation

Hormonal markers:

Calcitonin, gastrin, somatostatin, vasointestinal peptide, serotonin, pancreastatin – almost 3 dozen products. Many of these substances when produced in excess produce specific clinical syndromes [14].

Non-hormonal markers:
1. Chromogranin - acidic proteins in secretory granules [TYPE A , B and Sg II]
2. Synaptophysin
3. Pro-Convertases
4. Bombesin , Lei 7 And Grp
5. neuron specific enolase

Chromogranin A considered as a realistic marker of neuroendocrine cells as it is a specific matrix component of endocrine granules.

A Blood level of chromogranin is also one of the best non-specific markers except in cases of insulinoma where chromogranin B can be used [14].

Synaptophysin is localized within a small capsule membranes related to the secretion granules, its specificity and sensitivity less than chromogranin. NSE has poor specificity and widely distributed in all tissues [15, 16].

Classification of Gastro-entero-pancreatic neurondocrine tumors
WHO CLASSIFICATION - 2000

Neoplasm categories

- well differentiated neuroendocrine tumor

  - benign

  - uncertain behaviour

- well differentiated neuroendocrine carcinoma

- poorly differentiated neuroendocrine carcinoma

- mixed exocrine – endocrine carcinoma

- tumor like lesions [17,18].

Classification of Gastro-entero-pancreatic neurondocrine tumors

WHO CLASSIFICATION - 2000

Classification of neuroendocrine neoplasms

(Based on neuroendocrine neoplasms of lung proposed by **TRAVIS** [19] and was subsequently used to classify neuroendocrine neoplasms of gastrointestinal tract [45])

- Typical carcinoids
- Atypical carcinoids
- Large cell neuroendocrine carcinoma
- Small cell carcinoma

Grading of immunohistochemical neuroendocrine marker distribution [19]

- 0 – none
- 1 – < 10% of tumor cells
- 2 - 10% to 50% of tumor cells
- 3 - > 50% of tumor cells

Staining intensity

- 1+ = mild
- 2+ = moderate
- 3+ = marked

Neuroendocrine tumors were more than 50% positive for neuroendocrine markers with a 2+ or 3+ staining intensity.

Typical carcinoids

Growth pattern – organoid, others solid, spindle cell, glandular, pallisading, oxyphilic, papillary and follicular

Cells – uniform size with moderate n: c ratio

Nuclei – fine granular to stippled chromatin

Mitoses – rare

Necrosis – not seen

Vascular invasion – may be seen

Site: Carcinoids tumors of gastrointestinal tract are most common in the appendix followed by small intestine, the rectum, and the stomach. Carcinoids are rare in the Ampulla of Vater [20] occurring in lower mean age than those with Adenocarcinoma of the region. Carcinoids of Ampulla of Vater carry a good prognosis [21]

Atypical carcinoids

Growth pattern – organoid, others solid, spindle cell, pseudo glandular, pallisading, oxyphilic.

Cells – uniform size with moderate n : c ratio

Nuclei – fine to slightly coarse nuclear chromatin and faint nucleoli. Nuclear pleomorphism present

Mitoses – rare 4 to < 10 mitoses per HPF

Necrosis – foci of necrosis

Vascular invasion and interstitial pattern of spread of pattern seen

Large cell neuroendocrine carcinoma [17]

Neuroendocrine carcinomas are heterogeneous and potentially aggressive when compared with classic carcinoids [22].

Neuroendocrine by appearance by light microscopy [organoid, pallisading or rosette like pattern]

Cytological features of large cells [most cells greater than nuclear diameter of 3 resting lymphocytes, low nuclear cytoplasmic ratio, polygonal shape, fine granular eosinophilic cytoplasm with an eosinophilic hue , coarse nuclear chromatin, and frequent prominent nucleoli]

Mitoses greater than 10/10 high power field.

Necrosis present consists of large infarct like areas.

Hematoxylin staining of DNA encrustation of vessel walls seen

Neuroendocrine features either by IHC or EM or both.

Mitotic figures were formed at a magnification of x400 counting 3 sets of 10 high power fields for each tumor. Areas with highest number of mitoses were counted.

Small cell carcinoma

Growth pattern – nesting, solid, pallisading and spindle pattern

Cells – small [less than nuclear diameter of three small resting lymphocytes], round to fusiform cells

Nucleus – high n: c ratio, hyperchromatic nuclei with fine granular chromatin and absent or inconspicuous nucleoli

Necrosis – present

Mitosis – high

Hematoxylin decoration of DNA encrustation of vascular walls seen.

Electron microscopy

Typical carcinoids – numerous granules, 90-450 nm variable round to oval

Atypical carcinoids – moderate numbers, diffuse distribution, 100-200 nm less variation

Large cell neuroendocrine carcinoma – 100-270 nm focal or patchy distribution, minimal variation.

PROGNOSIS

Tumor type

Typical carcinoids – indolent tumors, carry an excellent prognosis.

Atypical carcinoids – 70 % metastasis, 30% died; mean survival 27 months, mean survival – 25 months, 21 months [23]

Peripheral Small cell neuroendocrine carcinoma
    Low grade – mean survival 21 months,
    High grade – 19.1 months [23].

Large cell neuroendocrine carcinomas carry a bad prognosis [24].

Size of the tumor – > 2cm carries a bad prognosis [10]. However size alone is not a good independent predictor.

Adenocarcinomas with neuroendocrine differentiation

Incidence of neuroendocrine differentiation is common in carcinomas arising in organs that normally contain neuroendocrine cells such as the gastrointestinal tract [25]. Neuroendocrine cell expression in large intestinal adenocarcinomas was found to be more when compared with adenocarcinomas at other gastrointestinal sites (stomach) and extraintestinal sites like prostate and breast. The number of hormone products was also more in gastrointestinal tumors with neuroendocrine differentiation, upto five different hormone products have been noted in these tumors [26]. neuroendocrine cells were observed at the metastatic sites of these tumors. Chromogranin A was the most reliable marker to detect neuroendocrine expression in adenocarcinomas [25]

No exact correlation was found between the carcinoma differentiation and neuroendocrine cell expression. Adenocarcinomas with neuroendocrine differentiation showed better prognosis than those without neuroendocrine differentiation [26, 27].however in a study by Ooi et al endocrine differentiated tumor cells was seen to occur more frequently in gastric carcinomas of advanced stage [25].

IMMUNOHISTOCHEMISTRY

Immunohistochemistry involves two disciplines – immunology and histology.

Immunohistochemistry is used to not only to determine if a tissue expresses a particular antigen [or does not express] a particular antigen, but also determine the antigenic status of particular cells within that tissue and the micro anatomic location of the antigen.

Immunohistochemistry uses antibodies to distinguish the antigenic differences between cells.

These differences can specifically identify the lineage of cell population, define biologically distinct populations of cells within the same lineage, identify functional differences between cells and can be used to identify specific infections. [28]

IHC started in 1940 when Coons developed an immunofluorescence technique to detect corresponding antigens in frozen sections [29].

IHC found wide application only since 1990 following series of technical developments such as enzyme label methods (horse radish peroxidase) developed by Avrameas and colleagues [30]

The sensitivity of the technique increased when simple one step procedure was converted to multistep detection procedure such as Peroxidase-Antiperoxidase, avidin-biotin conjugate and biotin streptavidin methods, together with amplification methods and highly sensitive polymer based labeling systems. [31]

Hybridoma technique facilitated the development of IHC and manufacture of abundant, highly specific antibodies many of which found early application in staining of tissues. Brown revolution happened when IHC was applicable to paraffin embedded tissue sections.

Taylor and colleagues in 1974 showed it was possible to demonstrate antigens in routinely processed tissue [32]

Huang and colleagues introduced enzyme digestion as a pre-treatment process to unmask antigens in formalin fixed tissues [33]

Leong and colleagues showed that enzyme digestion did not improve IHC staining [34]; the optimal digestion conditions were different for each antibody and difficult control.

Antigen retrieval technique is a simple method that involves heating paraffin processed sections at high temperatures before IHC staining. The technique was introduced by Shi and associates in 1991[35]. Antigen retrieval technique improved Immunohistochemical staining which was shown by numerous published articles [36].

PRINCIPLES OF IHC

The basic principle of IHC is a sharp localization of target components in the cell and tissue based on satisfactory signal to noise ratio. Amplifying the signal and reducing the non-specific background staining (noise) achieves a practical and useful result.

IHC technique is a valuable adjunct that expands the variety of tissue components that can be demonstrated.

Antigen –is a protein, carbohydrate or lipid molecule which bears on its surface one or more antibody-binding sites. These are highly specific topographic regions composed of small number of amino acids or monosaccharide units and are known as antigenic determinant groups or epitopes [37].

Antibodies – belong to a class of serum proteins known as immunoglobulins. Antibody molecule has the property of combining specifically with the second molecule, termed as antigen.

Antigen-antibody binding – the amino acid side chains of the variable domain of an antibody form a cavity which is geometrically and chemically complementary to a single type of antigen epitope.

The analogy of lock (antibody) and key (antigen) has been used and the precise fit explains the high degree of antibody-antigen specificity seen.

The associated antibody-antigen complex is held together by a combination of hydrogen bonds, electrostatic forces and van der Waal's forces.

The use of an antibody in IHC depends on the sensitivity and specificity of the antigen-antibody reaction. The Hybridoma technique provides limitless source of highly specific antibodies.

Monoclonal antibodies cannot guarantee antigen specificity as different antigens share similar reactive epitopes but practical specificity is high.

Polyclonal antibodies – an antiserum which contains several antibodies with different affinities and specificities. It carries the disadvantage of producing more non-specific background staining than monoclonal antibodies, but detects antigens which cannot be detected by monoclonal antibodies.

Polyclonal antibodies are more sensitive but less specific than monoclonal antibodies, as polyclonal antibodies may recognize several different epitopes on a single antigen whereas a monoclonal antibody recognizes only a single epitope. AR techniques with amplification systems have minimized this difference.

The specificity of the reaction can be assessed depending on the pattern of staining in control tissue sections. Correlation of the staining result with literature references for antigen distribution and comparing the staining of the test antibody with a second antibody known to bind to bind to the same antigen but different antibody [38].

Blocking non-specific background staining

Background staining is due to either non-specific antibody binding or presence of endogenous enzymes.

Non-specific binding seen with polyclonal primary antibody is minimized with pre-incubating sections with serum from same species on optimal working dilution.

Endogenous enzymes such as peroxidase seen in normal and neoplastic tissues abolished by peroxidase blocking or by using alternate systems such as immunogold or glucose technique.

Methods suggested to overcome endogenous activity include incubation in methanol containing 0.5% hydrogen peroxide for 10 minutes at room temperature (almost complete abolition of endogenous peroxidase activity)[39], alkaline phosphatase blocked with 1mM concentration of levamisole in the final incubation medium. Enzyme labels glucose-oxidase and bacterial beta-2-galactosidase are non problematic.

Detection systems

Antibodies are labeled or flagged by some method to permit visualization; these include fluorescent substances, enzymes forming colored reaction with suitable substrate (Light microscopy) or heavy metals (Electron microscopy). These systems also enhance sensitivity through signal amplification .

Different methods of IHC

DIRECT METHOD

- ***Direct conjugate-labeled antibody method***

Antibody is attached with a label by chemical means and then directly applied to tissue sections. It is a rapid and easy procedure, and is more useful with primary monoclonal antibodies.

Disadvantages being difficulty in preparing final labeled reagent without free label molecules, detection of multiple antigens require separate incubation with the respective antibodies and high concentration of antibodies.

INDIRECT METHOD

- ***Indirect or sandwich procedure***

Primary antibody that has specificity against desired antigen added, labeled secondary antibody which has specificity against an antigenic determinant is then added; it serves to localize the primary antibody and hence the site of antigen. This is the technique employed in this study.

The advantages include increased versatility, conjugation applied to secondary antibody, higher working dilution of primary antibody, secondary antibodies against primary antibodies of a different species easy to prepare and omission of primary antibodies during the process can act as a negative control.

- ***Unlabeled antibody methods***

The original immune enzyme bridge method using enzyme specific antibody became rapidly superseded by the improved technique using a soluble peroxidase – antiperoxidase complex (PAP). Sternberger and colleagues introduced this technique for detecting treponemal antibodies [40]. These complexes are formed from 3 peroxidase molecules and 2 antiperoxidase antibody molecules and are used as a third layer in the staining method. They are added to the unconjugated primary antibody e.g. rabbit anti-human IgG by a second layer of bridging antibody that is usually swine antirabbit applied in excess so that one of its two identical binding sites binds to primary antibody and the other to rabbit PAP complex.

Alkaline phosphatase antibodies raised in mouse by the same principle can be used to form the alkaline phosphatase-antialkline phosphatase complexes (APAAP). For unknown reasons this form of amplification APAAP is not as successful as the PAP technique which may be ascribed to the excessive background staining

- ***New indirect technique:***
(Dextran polymer conjugate two step visualization system)

The primary antibody in enhanced polymer one step method is replaced with a secondary antibody. Available in either as anti-rabbit or an anti-mouse format it offers greater sensitivity than the traditional indirect systems, is less time consuming than the 3 stage Avidin-biotin system and does not react with endogenous biotin.

- ***Avidin-biotin techniques***

This procedure uses the high affinity binding between biotin and Avidin. Biotin is chemically linked to primary antibody and Avidin chemically conjugated to enzyme. The Avidin binds to biotinylated antibody thus localizing the peroxidase moiety at the site of antigen.

Disadvantages include different affinities of different batches of biotin and Avidin, endogenous biotin producing non-specific background staining.

Avidin biotin conjugate procedure

A modification of Avidin biotin method where the primary antibody is added followed by a biotinylated secondary antibody and next by preformed complexes of Avidin and biotin horse radish peroxidase conjugate . This is a more sensitive method [41].

- ***Biotin streptavidin systems***

Streptavidin is used in place of Avidin. This is more sensitive than Avidin biotin conjugate procedure and streptavidin enzyme complexes are more stable so they can be prepared well ahead.

- ***Hapten labeling techniques***

Bridging techniques using haptens such as dinitrophenol and arsenilic acid have been advocated [42]. The Hapten is linked to 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*

This is used in ultrastructural immunolocalisation. The advantages were highlighted by Holgate et al in 1983 [43]. Gold particles are enhanced by addition of several layers of metallic silver. The low forming metallic silver has tolerance for natural light. This is more sensitive than the PAP technique but fine silver deposits in the background create confusion when small amounts of antigen are identified.

Tissue fixation, processing and antigen retrieval techniques:
Tissues for IHC undergo fixation, dehydration and paraffin embedding

Fixation

This is a critical step as the morphological preservation is essential for IHC interpretation. 10% buffered neutral formalin commonly used because of the following advantages:
1   Good morphological preservation
2. Cheap
3. Sterilizes tissues
4. Carbohydrate antigens are better preserved [44]
5. Many antigens are preserved during the process of cross linking.

The disadvantage of masking of antigens during fixation can be overcome by antigen retrieval technique. Coagulant fixatives (ethanol) can also be used and are known to produce lesser changes in IHC changes.

Subsequent treatment with absolute ethanol during dehydration serves as double fixation.

Antigen retrieval (AR)

Process involves unmasking of antigens by one of these four techniques:

1. proteolytic enzyme digestion
2. microwave antigen retrieval
3. microwave and trypsin antigen retrieval technique
4. pressure cooker antigen retrieval.

Enzyme digestion is difficult to control and produces inconsistent results. In the AR technique involving heat, the result is influenced by heating condition (temperature and time of heating) and pH of the AR solution. High temperature being the most important factor. Most antigens show no significant variation between pH 1.0 to 10. AR technique enhances immunostaining and standardizes routine IHC [35].

Microwave AR technique is a new technique. Heating is done in plastic coplin jars. Drying of sections can take place and hence careful monitoring required. Pressure cookers do not require close inspection and do not suffer from inconsistent results. In any of the conditions wherein heat is employed slides are coated with silane to prevent loss of sections.

MATERIALS AND METHODS

Source of data

A total of 152 resected gastrointestinal specimens were received in the department of pathology, Stanley Medical College from general surgical and surgical gastroenterology departments during the period August 2005 to September 2007. Among these 26 specimens satisfied the criteria for the study.

Inclusion criteria

Tumors which on histopathological examination showed focal or diffuse neuroendocrine pattern.

Criteria for neuroendocrine differentiation

1. Architectural clues :
   i. organoid architecture manifested by solid nests, sheets and broad trabeculae with peripheral pallisading
   ii. Rosette formation which is common to neuroendocrine tumors of various organs
2. Cytological clues
   i. Nuclear features – fine to coarsely granular chromatin, evenly distributed smooth nuclear membrane
   ii. Cytoplasmic features – uniform, polygonal and cuboidal cell shapes, slightly eosinophilic and finely granular cytoplasm, indistinct cytoplasm membrane, and ill defined cellular boundaries [45]

Exclusion criteria

Tumors with no evidence of neuroendocrine pattern.

Method of data collection

The material consisted of 26 resected gastrointestinal specimens which included

2 gastrectomy specimens,

2 duodenojejunal resections

2 ileal resections

9 Whipple's procedure specimens,

5 Right hemicolectomy specimens and

6 Abdominoperineal resection specimens.

Method of Tissue Preparation For IHC

10% buffered formalin has been used for fixation of specimens, the tissues were processed in various grades of alcohol and xylol. Paraffin blocks were prepared and stained with hematoxylin and eosin. Suitable sections were chosen for IHC.

Slides coated with chrome alum were used[49]. Sections subjected to AR using the microwave technique with citrate buffer solution. Slides then treated by HRP polymer technique.

Steps involved in the HRP polymer technique

1. Treatment with peroxidase block – for inhibiting endogenous peroxidases in the tissue for 20 minutes. Wash in TRIS buffer for 5 minutes.

2. Application of power block – to block non-specific antigen antibody reactions for 20 minutes. The excess of power block is blot dried.

3. Application of primary antibody – murine antibodies for 60 minutes. Wash in TRIS buffer for 5 minutes.

4. Application of super enhancer for 30 minutes which increases the sensitivity of antigen antibody reaction thereby enhancing the final reaction product.

5. Application of SS label – secondary antibody from goat with the tagged horse radish peroxidase enzyme for 30 minutes. Wash in TRIS buffer.

6. Application of DAB (diaminobenzidine) chromogen for 5 minutes – which is cleaved by the enzyme to give the coloured product at antigen sites. Wash in distilled water for 5 minutes.

7. The slides are counterstained with hematoxylin. Slides are air dried and mounted with DPX.

The above polymer technique is found to be superior than the Avidin biotin system as it is more sensitive and can be used for a variety of primary antibodies (murine and rabbit).

Neuroendocrine markers used – chromogranin A and synaptophysin.

Sections negative for neuroendocrine markers were treated for cytokeratin, lymphoma markers (CD 45, CD20, CD 3), HMB-45, c-KIT depending on the tissue morphology of the sections.

Grading of immunohistochemical neuroendocrine marker distribution [TRAVIS] [19].

- 0 – none
- 1 – < 10% of tumor cells
- 2 - 10% to 50% of tumor cells
- 3 - > 50% of tumor cells

Staining intensity

- 1+ = mild
- 2+ = moderate
- 3+ = marked

Criteria for neuroendocrine tumor-Neuroendocrine tumors were more than 50% positive for neuroendocrine markers with a 2+ or 3+ staining intensity.

OBSERVATION AND RESULTS

Based on the pattern and extent of neuroendocrine differentiation the 26 tumors were grouped under 4 categories

1. **Category A** - tumors with predominantly neuroendocrine pattern. These cases showed rare mitoses, with no necrosis – HPE diagnosis – neuroendocrine tumors

2. **Category B** - tumors with predominantly glandular pattern with focal areas of neuroendocrine pattern. Variable mitosis, foci of necrosis was observed – HPE diagnosis adenocarcinoma with endocrine differentiation

3. **Category C** – tumors with predominantly solid sheets of cells with focal areas of neuroendocrine pattern. Numerous mitoses, large areas of necrosis noted – HPE diagnosis poorly differentiated carcinomas [adenocarcinomas/neuroendocrine carcinomas]

4. **Category D** – tumors with small round cells or spindle cells or with focal areas of neuroendocrine pattern. Variable mitosis, foci of necrosis was observed – HPE diagnosis small cell carcinomas, lymphomas, GIST and signet ring carcinomas

Table no:1 – case distribution among the 4 categories

Site distribution

Of the 26 resected specimens taken for study majority belong to the periampullary region (9 cases) followed by rectum (6cases), caecum (4 cases), the stomach (2 cases), duodenum (2 cases), ileum (2cases) and ascending colon (1case).

Table no:2
Site distribution of cases

Site	Frequency	Percentage
Ascending colon	1	3.8
Caecum	4	15.4
Duodenum	2	7.7
Ileum	2	7.7
Periampullary region	9	34.6
Rectum	6	23.1
Stomach	2	7.7
Total	26	100.0

Age distribution

The age distribution of the cases studied showed a range varying from 18 -68yrs.

The majority of patients were in the 40 - 49 yrs age group (12 cases) followed by 50 – 59 yrs age group (6cases), 60 – 69 yrs age group (5 cases). The age groups 10 – 19 yrs, 20 – 29 yrs and 30 – 39 yrs had one case each.

Table no:3

Age distribution of cases

Age group	No. of cases	Percentage
10 – 19	1	4
20 – 29	1	4
30 – 39	1	4
40 – 49	12	46
50 – 59	6	23
60 – 69	5	19

Statistical analysis of inter-category variations

Table no:4
Statistical analysis of age distribution

No. of cases	26
Mean	47.73
Std. Deviation	10.891
Minimum	18
Maximum	68

Table no:5

Percentage distribution of cases based on age

Age category	No. of patients	Percentage
<40	5	19.2
41-50	12	46.2
>50	9	34.6
Total	26	100.0

The mean age of occurrence was 47 years. When the total number of patients was subdivided into 3 groups as <40, 41-50, >50 the majority of the cases belonged to the 41-50 group (46.2%).

Sex distribution

Of the cases 15 were male and 11 were female patients. No statistical significance was found between tumor occurrence and sex distribution.

Table no:6

Sex distribution of cases

		sex				Chi-square test
		Male		Female		
		n	%	n	%	
age	<40	4	80.0%	1	20.0%	$\chi2=2.57$ P=0.58 Not significant
	41-50	5	41.7%	7	58.3%	
	>50	6	66.7%	3	33.3%	
Group Total		15	57.7%	11	42.3%	

Table no:7

Percentage distribution of cases amongst the categories

Category A

8 cases were included in this category.

Site of occurrence - 4 in periampullary region, 1 duodenum, 2 caecum, 1 rectum.

Age – ranged from 18 years to 47 years.

Sex distribution – 5 were males and 3 females.

Histopathological diagnosis – all the 8 cases were diagnosed as well differentiated neuroendocrine tumors

Muscle invasion – was observed in 1 case alone, all the others were confined to the sub mucosal region.

Lymphnode or distant spread-none showed evidence of lymphnode or distant organ involvement

Immunohistochemistry – all the cases were >50% positive for Neuroendocrine markers with +3 intensity.

Follow-up – all the 8 cases were available for follow-up from 6 months to 24 months. These patients were alive during this period.

Table no:8

Category A – neuroendocrine expression and extent of spread

Serial no.	Biopsy no.	Neuroendocrine marker		Muscle invasion	Lymphnode and distant spread
		% of positivity	Intensity of positivity		
1	1012/05	>50%	+3	_	_
2	3384/05	>50%	+3	_	_
3	336/06	>50%	+3	_	_
4	1129/06	>50%	+3	_	_
5	3830/06	>50%	+3	_	_
6	4238/06	>50%	+3	_	_
7	1420/07	>50%	+3		_
8	2655/07	>50%	+3	+	_

CATEGORY B

7 cases were included in this category.

Site- 4 from rectum 1 each from caecum, ascending colon and periampullary region.

Age-ranged from 44years to 68 years.

Sex- 3 were males and 4 were female patients.

Histopathological diagnosis-adenocarcinoma with neuroendocrine carcinoma.

Muscle invasion- muscle invasion was observed in 4 cases, of these Lymphnode involvement was seen in 2 cases and one showed liver metastasis.

Table no:9

Category B-extent of neuroendocrine positivity

Serial no	Biopsy no.	Neuroendocrine marker - % of positive cells	Intensity of positivity
1	506/07	1-10%	2+
2	609/07	1-10%	2+
3	802/07	10-50%	3+
4	813/07	1-10%	2+
5	881/07	10-50%	2+
6	1627/07	10-50%	2+
7	2013/07	10-50%	2+

3 cases showed 1-10% positive cells with an intensity of +3. the remainder 4 cases were positive for 10-50% cells with 3 of them belonging to +2 intensity and 1 case with +3 intensity.

All the 8 cases were alive during the period of follow up. 3 cases with 1-10% positivity and intensity of +2 were positive for muscle invasion and lymphnode involvement and 1 showed liver metastases.

Of the 5 cases which showed 10-50% positivity and +2 to +3 intensity, only one presented with muscle invasion none of them involved the lymphnodes or distant organs.

CATEGORY C
5 cases were included in this category
Sex- 3 were males and 2 females
 Age- ranged from 38 years to 54 years.
 Tumor Site - 4 cases were from periampullary region and 1 from stomach.
 All the 5 cases showed muscle and lymphnode involvement with one showing liver metastases.
 IHC -All he 5 cases were treated with neuroendocrine markers (chromogranin A and synaptophysin) and cytokeratin.

Table no:11
Category C-neuroendocrine and cytokeratin expression

Serial no	Biopsy no	Neuroendocrine markers		Cytokeratin
		% of positivity	Intensity of positivity	
1	3842/05	1-10%	2+	+
2	1121/05	>50%	3+	+
3	2486/05	1-10%	2+	+
4	4222/06	0	-	+
5	2275/07	1-10%	2+	+

Follow-up

Period of follow-up ranged from 6months to 18 months.2 patients died at 12 months, 1 at 18 months, the remaining 2 were alive for a period of 18 and 6 months respectively.

Table no:12
Category C-extent of neuroendocrine expression and survival

S.no	Biopsy no	Neuroendocrine marker		Period of follow-up	outcome
		% of positivity	Intensity of positivity		
1	3842/05	1-10%	2+	12 m	Died
2	1121/06	>50%	3+	12 m	Died
3	2486/06	1-10%	2+	18 m	Died
4	4222/06	0	_	18 m	Alive
5	2275/06	1-10%	2+	6 m	Alive

CATEGORY D

6 cases were included in this category
Age- ranged from 24years to 60 years
Tumor site- 2 cases from ileum, 1 each from stomach, caecum and rectum.

Histopathological diagnosis

- 4 cases showed solid sheet like pattern with focal neuroendocrine arrangement and consisted of round to polygonal cells – DD lymphoma/neuroendocrine carcinoma/poorly differentiated carcinoma

- 2 cases showed sheet and focal neuroendocrine pattern composed of spindle shaped to round cells – DD GIST/neuroendocrinecarcinoma/malignant melanoma poorly differentiated carcinoma

Muscle invasion – noted in the 2 cases.
Lymphnode involvement – 3 cases showed lymphnode involvement.

IHC – in addition to neuroendocrine markers cytokeratin, c-kit, lymphoma markers and HMB-45 were applied.

Table no:13
Category D-IHC marker expression

S..no	Biopsy no.	Neuroendocrine markers	Cytokeratin	c-kit	HMB-45	Lymphoma markers		Diagnosis
						CD 20	CD45	
1	210/07	–	–	NA	NA	+	+	lymphoma
2	777/07	–	–	–	+	NA	NA	Amelanotic melanoma
3	2219/07	–	–	NA	NA	+	+	lymphoma
4	2241/07	–	–	+	–	NA	NA	GIST
5	2402/07	–	–	NA	NA	+	+	lymphoma
6	2517/07	–	+	–	–	–	–	Poorly differentiated carcinoma

2 cases which showed mucle involvement were GIST and poorly differentiated carcinoma.

Lymphnode involvement which was observed in 3 cases included 2 cases of lymphomas and 1 case of poorly differentiated carcinoma.

Follow up-cases were followed up from a minimum of 6 months all the patients were alive during this period.

STATISTICAL ANALYSIS AMONG THE 4 CATEGORIES

Table no:14

Analysis of extent of neuroendocrine marker positivity in the 4 categories

Neuroendocrine marker		Category								Chi-square test
		A		B		C		D		
		n	%	n	%	n	%	n	%	
positivity	0	0	0%	0	0%	1	14.3%	6	85.7%	χ2=48.94 P=0.001 significant
	1-10 %	0	0%	3	50.0%	3	50.0%	0	0%	
	10-50 %	0	0%	4	100.0%		0%	0	0%	
	>50%	8	88.9 %	0	0%	1	11.1%	0	0%	
Group Total		8	30.8 %	7	26.9%	5	19.2%	6	23.1%	

Table no:15

Extent Of Neuroendocrine Positivity Of The 26 Cases

Neuroendocrine marker positivity	No Of cases	Diagnosis
0	7	Lymphoma – 3 Poorly differentiated adenocarcinoma – 2 Amelanotic melanoma – 1 GIST – 1
1-10%	6	Adenocarcinoma with neuroendocrine differentiation – 4
10-50%	4	Poorly differentiated adenocarcinoma – 3 Adenocarcinoma with neuroendocrine differentiation – 3
>50%	9	Well differentiated Neuroendocrine tumors – 8 Large cell Neuroendocrine carcinoma – 1

Table no:16

Intensity of neuroendocrine marker positivity of the cases

		intensity				
		2		3		Chi-square test
		n	%	n	%	
positivity	1-10%	6	100.0%			$\chi 2=15.99$ P=0.001 significant
	10-50%	3	75.0%	1	25.0%	
	>50%			9	100.0%	
Group Total		9	47.4%	10	52.6%	

Extent of neuroendocrine positivity:

>50% positivity was seen in 9 of the 26 cases which includes 8 cases from category A and 1 from category B (8 cases of well differentiated neuroendocrine neoplasms and 1 case of large cell neuroendocrine carcinoma).

Intensity of neuroendocrine positivity:

19 cases were positive for neuroendocrine markers. Of these 19 cases, 9 showed +2 intensity and 10 were of +3 intensity. These 9 cases showed <50% staining (6 with 1-10% and 3 with 10-50% positivity) and of the remaining 10, 9 were >50% positive with 1 case being 10-50% positive. A significant association was found between the extent of staining and intensity of staining.

Frequency of positivity of other markers

Table no:17

Lymphoma Markers-frequency of positivity

Lymphoma markers	Frequency	Percentage
_	2	7.7
+	3	11.5
NA	21	80.8
Total	26	100.0

Table no:18

C- Kit-frequency of positivity

c-kit	Frequency	Percentage
_	2	7.7
+	1	3.8
NA	23	88.5
Total	26	100.0

Table no:19

Cytokeratin – frequency of positivity

Cytokeratin	Frequency	Percentage
No	13	50.0
Yes	13	50.0
Total	26	100.0

Table no:20

HMB -45-frequencyof positivity

HMB-45	Frequency	Percentage
-	2	7.7
+	1	3.8
NA	23	88.5
Total	26	100.0

Muscle invasion in the 4 different categories

The Overall frequency of muscle invasion was 42.3% (11 cases). Category D showed muscle involvement in all the 5 cases (100%).

Table no:21

Frequency of muscle invasion

Muscle invasion	Frequency	Percentage
no	15	57.7%
yes	11	42.3%
Total	26	100.0

Table no:22

Frequency of muscle invasion in the 4 categories

Category	Muscle Invasion			
	no		yes	
	n	**%**	**n**	**%**
A	7	87.5%	1	12.5%
B	3	42.9%	4	57.1%
C			5	100.0%
D	4	83.3%	2	16.7%
Total	15	57.7%	11	42.3%

Lymphnode involvement in the 4 categories

The overall lymphnode positivity was 34.6% (9 cases). Category D showed 100% positivity with all the case showing lymphnode involvement.

Table no:23

Frequency of lymphnode involvement

Lymphnode involvement	Frequency	Percentage
no	17	65.4
yes	9	34.6
Total	26	100.0

Table no:24

Frequency of lymphnode involvement in the 4 categories

Category	Lymph node positivity			
	no		yes	
	n	%	n	%
A	8	100.0%		
B	4	57.1%	3	42.9%
C			5	100.0%
D	3	50.0%	3	50.0%
Total	15	57.6%	11	42.3%

Distant metastasis in the 4 categories

Distant metastasis was seen in 2 cases (1case from category B and 1 from category D).

Table no:25

Frequency of distant metastasis

Distant metastasis	Frequency	Percentage
no	25	92.4
yes	2	7.6
Total	26	100.0

Table no:26

Frequency of distant metastasis in the 4 categories

Category	Distant metastasis			
	no		yes	
	n	%	n	%
A	8	100.0%		
B	6	85.8%	1	14.2%
C	4	80.0%	1	20.0%
D	6	100.0%		
Total	25	96.2%	1	3.8%

The final diagnosis based on the various IHC positivity patterns

Table no:27

Final diagnosis of the 26 cases

Final diagnosis	Frequency	Percentage
Adenocarcinoma with neuroendocrine Differentiation	7	26.9
Amelanotic melanoma	1	3.8
GIST	1	3.8
Large cell Neuroendocrine Carcinoma	1	3.8
Lymphoma	3	11.5
Neuroendocrine tumor	8	30.8
Poorly Differentiated adenocarcinoma	5	19.2
Total	26	100.0

Prognosis

Category C showed a fall in the survival rate after 12 and 18 months. The other categories had a similar survival rate (all the patients survived during the period of follow up with no deaths)
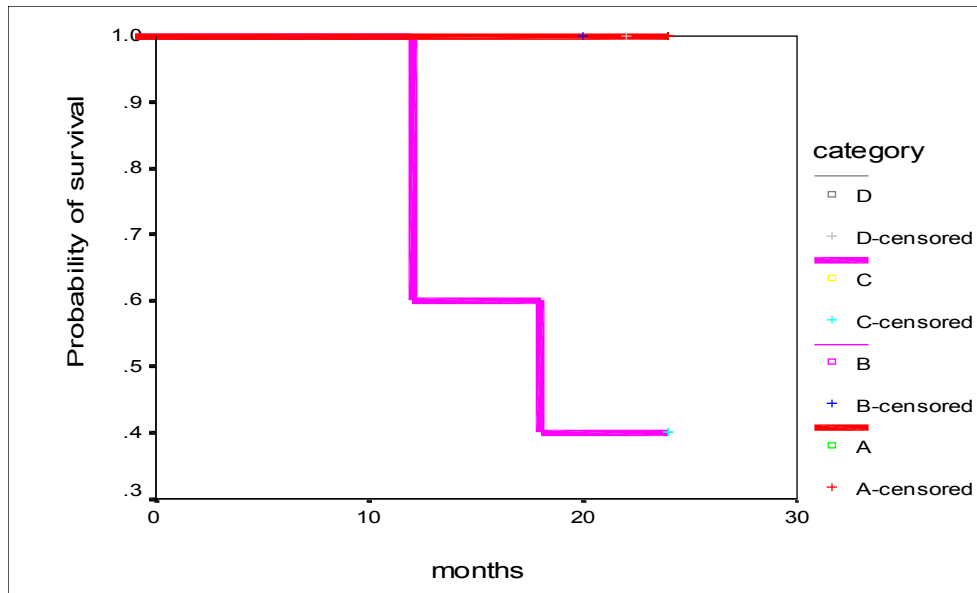
Kaplan Meier curve for comparison of survival of patients

Table no:28
Frequency of survival in the 4 categories

Category	status				Chi-square test
	Alive		Died		
	n	%	n	%	
A	8	100.0%			
B	7	100.0%			χ2=14.23 P=0.003 significant
C	2	40.0%	3	60.0%	
D	6	100.0%			
Total	23	88.5%	3	11.5%	

Category D showed the highest percentage of deaths 60% accounting for 3 cases.

DISCUSSION

Neuroendocrine differentiation can be seen in a wide range of gastrointestinal neoplasms which greatly differ amongst themselves both in morphological and behavioral pattern. Although admixtures and overlaps occur, most of the gastrointestinal tumors with neuroendocrine differentiation have been placed into following categories –

well differentiated neuroendocrine tumors

neuroendocrine tumors with atypical morphological features,

small cell carcinomas

adenocarcinomas with neuroendocrine cells

In the present study over a period of two years, 26 cases have been selected among a total of 152 gastrointestinal resected specimens which on routine histopathological examination with hematoxylin and eosin sections showed variable degree of neuroendocrine differentiation.

The selected cases have been placed under 4 categories

1.   Category A - tumors with predominantly neuroendocrine pattern. These cases showed rare mitoses, with no necrosis – HPE diagnosis – neuroendocrine tumors

2.   Category B - tumors with predominantly glandular pattern with focal areas of neuroendocrine pattern. Variable mitosis, foci of necrosis was observed – HPE diagnosis adenocarcinoma with endocrine differentiation.

2.   Category C - tumors with predominantly solid sheets of cells with focal areas of neuroendocrine pattern. Numerous mitoses, large

areas of necrosis noted – HPE diagnosis poorly differentiated carcinomas [poorly differentiated adenocarcinomas/ neuroendocrine carcinomas].

4. Category D - tumors with small round cells or spindle cells with focal areas of neuroendocrine pattern. Variable mitosis, foci of necrosis was observed. – HPE diagnosis small cell carcinomas, lymphomas, GIST and signet ring carcinomas.

Category A includes 8 cases. All these 8 cases showed a predominantly neuroendocrine pattern (> 50%) on HPE. Histological diagnosis of well differentiated neuroendocrine tumors was given (carcinoids). Consistent with the criteria proposed by Travis all the 8 cases were highly (> 50%) positive for neuroendocrine markers with +3 intensity. The average age of occurrence in the present study is 40.5 years, with the youngest patient being 18 years old.

Well differentiated neuroendocrine tumors are reported to occur predominantly in adults but have also been known to occur in children [46]. Predominant site of occurrence was in the periampullary region.

Previous literature cites that 60% of these well differentiated neuroendocrine tumors occurring within the gastrointestinal tract have their location in the appendix followed by small intestine, rectum and stomach. However Yukata Noda et al and Mark Hartel et al in separate studies concluded that periampullary carcinoids are more common than initially thought [24, 47]. All but 1 case were confined to the submucosa.

The 8 patients survived for follow up period ranging from 6 months to 24 months and showed no evidence of recurrence or metastasis. Well differentiated neuroendocrine tumors are considered to be indolent tumors and the 5 year

survival rate was found to be more than 90% [47]. This study also reflects a similar observation within the period of study.

Category B includes 7 cases. The neuroendocrine marker positivity ranged from 1-10% in 3 cases and 10-50% in 4 cases. The predominant tumor site in this group was rectum (4 cases) followed by 1 case in periampullary region, caecum and ascending colon. All these tumors were also positive for cytokeratin. Taking into consideration diffuse positivity for cytokeratin and < 50% positivity for neuroendocrine markers these tumors were designated as adenocarcinomas with neuroendocrine differentiation. Among adenocarcinomas with neuroendocrine cells, large intestinal adenocarcinomas come first [26]. In this study 6 out of the 7 cases were from large intestine. These cases were followed up for a period of 6 to 12 months. All the 7 cases survived during this period. However on comparing the degree of neuroendocrine positivity and extent of tumor spread it has been found that in 4 cases with 10%-50% positivity and +2 to +3 intensity none had lymphnode or distant organ involvement, on the other hand 3 cases with < 10% positivity and +2 intensity presented with advanced disease (all 3 with evidence of muscle invasion and lymphnode involvement and 1 with liver metastasis). Overall intensity of staining in adenocarcinomas is less than neuroendocrine tumors [19]. The staining intensity in this category consisting of adenocarcinomas with neuroendocrine differentiation the intensity is +2 in 7 cases with +3 in 1 case only. Regarding neuroendocrine differentiation in gastrointestinal adenocarcinomas conflicting reports have been published. Akishi Ooi et al has claimed that increased neuroendocrine differentiation in adenocarcinomas of stomach was associated with advanced disease [25]. Radi et al in gastric adenocarcinomas and Gen-You et al in large intestinal adenocarcinomas had showed that neuroendocrine immunoreactivity was associated with less advanced disease and carry a good prognosis [26, 27]. Similar to the latter our study showed greater neuroendocrine immunoreactivity to be associated with limited disease.

Category C includes 5 cases. These were histologically classified as poorly differentiated carcinomas. Of these, 1 case which was from periampullary region showed > 50% positivity for neuroendocrine marker with grade 3 intensity. This tumor was therefore diagnosed as large cell neuroendocrine carcinoma.

Large cell neuroendocrine carcinoma is a recently introduced entity in the gastrointestinal neuroendocrine neoplasms and has been reported in various sites with periampullary region being a rare site [47, 48]. The other 4 cases showed 0 to 10% immunoreactivity for neuroendocrine markers and diffuse positivity for cytokeratin. Based on the criteria recommended by Travis these tumors were designated as poorly differentiated adenocarcinomas (with some neuroendocrine positive cells).

The prognosis of large cell neuroendocrine carcinomas was found to be significantly worse than adenocarcinomas; this was observed by Sheryl R. Simon et al in their study of neuroendocrine carcinomas of the colon and Shi-Xu Jiang et al in their study of gastric large cell neuroendocrine carcinomas [22, 45]. These tumors behave aggressively and require radical surgery and chemotherapy in comparison with well differentiated neuroendocrine tumors [45].

In accordance with the above reports, this study shows that patient with large cell neuroendocrine carcinoma survived for 12 months only. Among the poorly differentiated adenocarcinomas 2 out of the 4 died, at 12 months and 18 months respectively, this poor prognosis is similar to the earlier observations of decreased survival in high grade carcinomas with or without neuroendocrine positivity and that it is not significantly different from large cell neuroendocrine carcinomas [45].

Category D consists of 6 cases. This category includes tumors which posed diagnostic difficulties due to a combination of varied morphological appearances.

None of the cases were positive for neuroendocrine markers, therefore neuroendocrine tumors or neuroendocrine differentiation was ruled out. 3 cases were for positive lymphoma markers, 1 case positive for c-kit, 1 case positive HMB-45, 1 case for cytokeratin, hence diagnosed as lymphoma, GIST (Gastrointestinal stromal tumor), amelanotic melanoma and adenocarcinoma respectively. The follow-up period was 6 months and all of them survived during this time.

Neuroendocrine markers have helped confirm neuroendocrine nature in category A, endocrine differentiation in otherwise exocrine tumors in category B, to differentiate large cell neuroendocrine carcinoma from other high grade carcinomas in category C and rule out neuroendocrine carcinomas in tumors with varied morphological appearances in category D.

The prognostic implication varies among the 4 groups. Category A - well differentiated neuroendocrine tumors carries an excellent prognosis. Category B - adenocarcinomas with neuroendocrine differentiation, shows less advanced disease associated with more extensive neuroendocrine positivity. Category C – large cell neuroendocrine carcinoma and other poorly differentiated carcinomas associated with poorer prognosis. Category D- prognosis varies depending on the histological nature of these tumors. Among the 4 categories, category C carries the worst prognosis. However the available period of review has not been consistent in all the cases, varying from 6 months to 24 months, hence a longer period of follow-up in these cases will through more light on the behavior of these tumors.

SUMMARY AND CONCLUSION

During the period of study between August 2005 and September 2007 the 26 gastrointestinal resected specimens with evidence of neuroendocrine differentiation were taken up to confirm and detect neuroendocrine immunoreactivity.

Based on morphological features and extent of neuroendocrine differentiation division of these 26 cases into 4 categories was done.

IHC has helped confirm histological diagnosis of neuroendocrine tumors, to detect neuroendocrine expression in adenocarcinomas and make a definitive diagnosis in tumors with varied morphology. This is essential as the prognosis and treatment modalities vary amongst these tumours.

Poorly differentiated carcinomas and large cell neuroendocrine carcinoma were found to be associated with grim prognosis. In case of adenocarcinomas with neuroendocrine differentiation, tumours with greater neuroendocrine reactivity were less advanced than adenocarcinomas with minimal or no neuroendocrine reactivity. Since the period of follow-up is not uniform in all the cases and not more than 24 months, a longer period of review is necessary to arrive at a definitive prognosis.

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