

Comparative Immunohistochemical Expression of Chromogranin A and Histidine Decarboxylase in Pulmonary Neuroendocrine Carcinomas

Pulmoner Nöroendokrin Karsinomalarda Kromogranin A ve Histidin Dekarboksilaz Salınımının İmmunohistokimyasal Kıyaslaması

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ABSTRACT

Objective: Neuroendocrine (NE) differentiation has been detected and suggested as an indicator of poor prognosis in a subgroup of a variety of carcinomas, including the pulmonary neuroendocrine carcinomas (PNECs) which express multiple NE cell markers. With the hypothesis that histidine decarboxylase (HDC) and its mechanism of releasing and regulating biogenic amines and peptides, may outline a significant link between NE differentiation of any malignancy, we studied the immunohistochemical expression of this marker, along with chromogranin A (CgA), in various subtypes of PNECs.

Material and Method: A cross-sectional descriptive study was carried out comprising 125 patients of PNECs, with the formalin fixed, paraffin embedded tissue blocks which, after H/E staining, were subjected to immunohistochemistry (IHC) with monoclonal anti-HDC and anti CgA antibodies. Positive staining was asserted following the criterion proposed in the previous literature.

Results: Our findings of the study revealed that HDC immunostaining in the PNECs demonstrated much better positivity, i.e 88%, as compared to CgA which was positive in only 57.6% cases (P<0.001). The association of positive HDC and CgA immunostaining with the histological grades of PNECs, showed a notably enhanced positivity by the latter in PD tumours, of which 89.3% were strongly positive as compared to CgA, which was positive in 54.3% cases (P<0.001).

Conclusion: HDC may be applied as a reasonably reliable marker for demonstrating NE differentiation in PNECs, regardless of their degree of differentiation as compared to the dependence of CgA positivity on the differentiation of a particular malignancy.

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Key words: Neuroendocrine, pulmonary neuroendocrine carcinomas, chromogranin A, histidine decarboxylase, immunohistochemistry

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ÖZET

Amaç: Nöroendokrin (NE) farklılaşma, pulmoner nöroendokrin karsinomaların (PNEK) da içerisinde bulunduğu ve çok sayıda NE hücre belirleyicilerinin salındığı, farklı karsinoma tiplerinde kötü prognoz belirleyicisi olarak tanımlanmaktadır. Biyojenik aminlerin ve peptidlerin salınımını düzenleyen histidin dekarboksilazın (HDK) malignitelere NE farklılaşmayı etkilediği hipotezi bulunmaktadır. Buradan yola çıkarak çalışmamızda farklı PNEK tiplerinde, HDK ve kromogranin A (KgA) İmmunohistokimyasal salınımını değerlendirdik.

Gereç ve Yöntem: 125 PNEK hastasında tanımlayıcı, çapraz kesitsel bir çalışma yapıldı. Hemotoksilen/eozin boyası sonrasında, formalin ile fiske edilip parafine gömülen hücre blokları monoklonal anti-HDK ve anti-KgA antikorları ile immunohistokimyasal değerlendirme yapılır. Pozitif boyanma daha önceki literatürlerde tanımlanan kriterlere uygun olarak değerlendirildi.

Bulgular: Sonuçlarımız PNEK hastalarında HDK immunohistokimyasal boyamasının %88 ile KgA (%57.6) ile kıyaslandığında daha yüksek oranda pozitif olduğunu göstermektedir (p<0.001). İmmunohistokimyasal olarak HDK ve KgA için pozitif olan karsinomalar PNEK'lerin histolojik evreleri ile korelasyon gösterir ve PD tümörler olarak tanımlanırlar. Bu grup tümörler %54.3 gibi bir oranla KgA için düşük oranda pozitif olmasına karşın her iki belirleyici için %89.3 gibi yüksek bir pozitifliğe sahiptir (p<0.001).

Sonuç: HDK, PNEK'lerin bazı gruplarında NE farklılaşmasını, farklılaşma derecesinden bağımsız olarak KgA pozitifliğine göre daha iyi gösteren, güvenilir bir belirleyici olabilir.

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Anahtar sözcükler: Nöroendokrin, pulmoner nöroendokrin karsinomalar, kromogranin A, histidin dekarboksilaz, immunohistokimya

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LIST OF ABBREVIATIONS

1. AC : Atypical carcinoids
2. c DNA : Complementary DNA
3. CgA : Chromogranin A
4. FNA : Fine needle aspiration
5. H&E : Hematoxylin and eosin
6. HDC : Histidine decarboxylase
7. IASLC : International Association for Study of Lung Cancer
8. IHC : Immunohistochemistry
9. LCLC : Large cell lung carcinoma
10. LCNEC : Large cell neuroendocrine carcinoma
11. MD : Moderately differentiated
12. NE : Neuroendocrine
13. NSE : Neuron-specific enolase
14. PD : Poorly differentiated
15. PET : Positron emittance tomography
16. PNECs : Pulmonary neuroendocrine carcinomas
17. ProGRP : Pro-gastrin-releasing peptide
18. SCLC : Small cell lung carcinomas
19. TC : Typical carcinoids
20. WD : Well differentiated
21. WHO : World Health Organization

INTRODUCTION

Neuroendocrine (NE) cells are widely distributed in various organs of the body, including the stomach, thyroid, adrenal gland, prostate [1], breast [2], colorectum [3], appendix [4] and lungs [5]. The term NE defines a specific group of cells based on their secretory products, distinct staining characteristics, ability to uptake and decarboxylate amine precursors [6] and distinguished ultrastructurally by the presence of variable numbers of dense core neurosecretory granules [7]. The presence of NE cells in the lungs was first described by Frölich in 1949 [8]. NE carcinomas in lungs, called pulmonary neuroendocrine carcinomas (PNECs), are derived from the Kulchitsky cells [9] and are clinically present with a wide range of pathologic entities. These entities oscillate from low-grade typical carcinoids (TC) intermediate-grade atypical carcinoids (AC) to high-grade large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinomas (SCLC) [10]. Morphologically, the comparison in grading between these tumours is, above all, with respect to the incidence of mitosis and necrosis [11]. Clinically, these tumours are significant because of the ectopic ACTH syndrome, especially produced by the relatively benign carcinoids and the highly aggressive SCLC [12]. A number of studies have attempted to evaluate the therapeutic and prognostic significance of the NE differentiation substantiated by the expression of NE markers with, an overall theoretical assumption that NE-differentiated tumours may be associated with an adverse prognosis, earlier dissemination [13] and greater chemosensitivity [14]. Supported by various studies [15], the prognosis varies between the different subgroups or clinical entities, and therefore such a classification system based on grading system is reproducible [16]. During the past decade, the primary malignant PNECs have been diagnosed by fine needle aspiration (FNA) or routine biopsy and immunohistoche-

mistry (IHC) [17]. However, problems exist, especially in diagnosing the moderately differentiated carcinomas (AC) and poorly to undifferentiated LCNEC and even SCLC [18]. Chromogranin A (CgA), Pro-gastrin-releasing peptide (ProGRP), Neuron-specific enolase (NSE), Synaptophysin and Leu 7 are known as immunohistochemical tissue markers selective or closely associated with NE differentiation in various carcinomas [19].

Chromogranins are matrical proteins that are associated with neurosecretory granules and as such they are absolutely specific for NE differentiation [20]. Cg A, isolated from chromaffin cells of the adrenal medulla, is an acidic glycoprotein with 439 amino acids [21] and is present in secretory dense core granules located within the cytoplasm of these NE cells [22]. However, CgA production seems to depend on tumor type or tumor differentiation and the sensitivity of CgA detection in tissue or plasma may be low in moderately or poorly differentiated NE carcinomas [23]. SCLC show variable staining for chromogranin A [24].

Histamine levels in cells and tissues are regulated by histidine decarboxylase (HDC), the only enzyme that catalyzes the formation of histamine from L-histidine [25]. In humans, HDC can be found in the histamine secreting enterochromaffin-like cells, in the mast cells in almost all tissues [26] as well as in a variety of other cell types including basophils, platelets, endothelial cells, NE epithelial cells, neurons and fetal liver [27]. It is a dimer of approximately 53- to 55-kDa subunits [28] and its complementary DNA (cDNA) has been cloned from human and mouse tissues [29]. Because of the growth factor activity of histamine and its elevated levels in rapidly proliferating tumor cells, there may be a possibility that HDC might be an early indicator of neoplasia. Highly increased histamine biosynthesis and content has been reported in different human and experimental neoplasias, such as in breast, gastric and colorectal carcinomas and various adenocarcinomas [30,31]. With the hypothesis that HDC and its mechanism of releasing and regulating biogenic amines and peptides secretion, may outline a significant link between NE differentiation of any malignancy, we studied the immunohistochemical expression of this marker, along with CgA, in various subtypes of PNECs.

MATERIALS and METHOD

This was a cross-sectional descriptive study comprising of one hundred and twenty five patients of PNECs selected for this study with unilateral, operable lung cancer who underwent resection of primary tumour at Gulab Devi Chest Hospital Lahore from January 2005 to January 2008 (mean age 45 years, age range 08-80 years) . All patients gave written informed consent. The formalin fixed, paraffin embedded tissue blocks were collected from the hospital. Cases with history of co-morbidity or taking medication /therapy for their malignancy, i-e follow up cases or, if any, necrotic tissue specimens, were excluded. Relevant clinical and laboratory data of these patients including age, sex, tumor location, and type of surgical procedure, were recorded in separate proformas. Gross observations took account

of size and location of tumors. Microscopic features tabulated included patterns of growth, nuclear features, mitoses, necrosis, and stromal reaction. One section from each tissue, 4-7µm for hematoxylin and eosin (H&E) staining and two sections of 3-6µm for immunohistochemistry were recut by rotary microtome and collected on poly-L-lysine-coated slides. One tissue section of each sample was stained using the conventional H&E stain following the method of Harris haematoxylin [32]. After confirmation of the diagnosis by microscopy, we were able to segregate the following subtypes of PNECs: 95 SCLC (76%), 15 TC (12%), 07 AC (8%) and 08 LCLC (4%). The histological grading of these tumour types was undertaken following the criterion by the World Health Organization [33]. To determine the sensitivity and specificity of HDC in distinguishing the carcinomas with NE differentiation only, 50 paraffin embedded tissue blocks of non small cell lung carcinomas (NSCLC) were also included in the study. The following subtypes of NSCLC were included; 25 (50%) squamous cell carcinomas (SCC), 15 (30%) adenocarcinomas (AC) and 10 (20%) large cell lung carcinomas (LCLC). After H/E staining, immunohistochemistry was performed using the standard 'Avidin Biotin Peroxidase' method. The primary antibody with a specified protocol employed [34] were mouse monoclonal IgG1 anti-CgA antibody, LK2H10 + PHE5], (Abcam; USA) at a concentration of 0.200 mg/ml with a dilution of 1:50-100 and concentrated polyclonal anti-HDC antibody (Abcam; USA) raised in rabbits against human recombinant HDC produced in Escherichia coli, with a working dilution of 10 µg/100 ml of the diluent buffer. A total of 30 controls, 20 positive and 10 negative, were included in the study. Positive controls included paraffin sections of 05 intestinal carcinoids, 03 small cell lung carcinomas, 03 benign adrenals, 02 adrenal pheochromocytomas, 02 benign duodenums, 02 benign stomach, 02 benign thyroid lesions and 01 medullary carcinoma thyroid. About 10 paraffin sections of lymph nodes (both neoplastic and non neoplastic) were taken as negative controls. For immunohistochemical staining, 04 positive and 02 negative controls were run with each batch of 25 and 10 histological sections of PNECs and NSCLCs. The entire slide was scanned for immunostaining

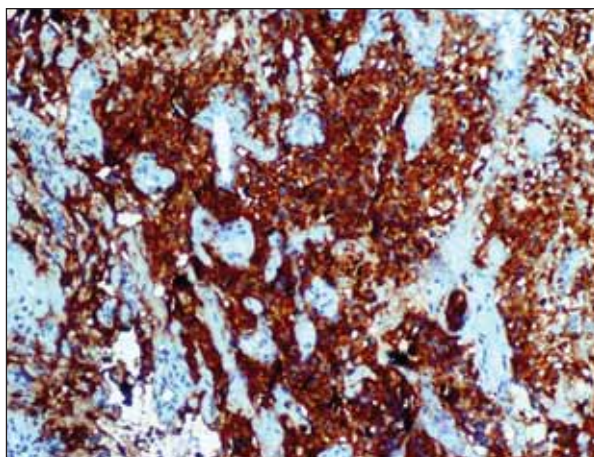


Figure 1. This figure shows the strong diffuse cytoplasmic staining of the tumour cells (SCLC) by HDC. (20X x 10X). SCLC: small cell lung carcinoma, HDC: histidine decarboxylase

and was scored on the basis of intensity of staining and the percentage of the cells that stained positively. When followed by a criterion proposed by Matsuki, staining for chromogranin was considered positive if > 5% of tumour cells were reactive, and was classified into two groups: [1] focally positive staining in < 10% of tumour cells, and [2] strongly positive in > 10% of tumour cells [42]. The pattern of staining for chromogranin is usually punctate [29]. Positive staining with HDC was asserted when at least >10% of the tumour cells showed moderate or strong, diffuse to granular cytoplasmic staining [35].

Statistical Analysis

The data was entered and analyzed using SPSS 17.0 and STATA 8.2. Mean±S.D. (standard deviation) were given for quantitative variables. Frequencies and percentages were given for qualitative variables. Pearson Chi Square and Fisher Exact test were applied to observe associations, if any, between the qualitative variables. Diagnostic statistics (sensitivity, specificity, positive / negative predictive values and diagnostic accuracy) were applied for both HDC and CgA. A p value of < 0.05 was considered as statistically significant.

RESULTS

The findings of our study revealed that the following subtypes of PNECs were included of which n=95 (76%) were SCLC, n=15 (12%) were TC, n=07 (8%) were AC and n=08 (4%) were LCLC. In keeping with the histological grades of these tumours, we segregated n=15 (12%) well differentiated (WD) TC, n=07 (8%) moderately differentiated (MD) AC and n=103 (80%) poorly differentiated (PD) SCLC & LCNEC. HDC immunohistochemical staining of the histological tissue sections demonstrated much better positivity in all PNECs i.e 88% (n=110) (Figure 1), as compared to CgA which was positive in only 57.6% (n=72) cases (P<0.001). However, the staining pattern of the tumour cells by both the markers was strong, focal to almost diffuse and strictly cytoplasmic, with a typical granular pattern seen only with CgA (Figure 2). The positive staining of different PNECs subtypes depicted a varying pattern (Table 1). The staining

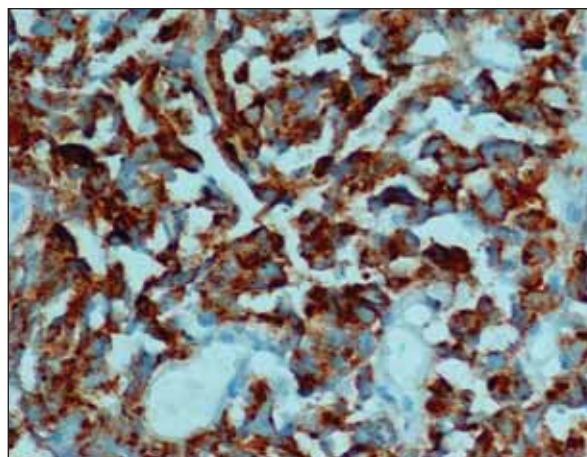


Figure 2. This figure shows the strong complete cytoplasmic staining of the tumour cells as in Fig:1. (SCLC) by CgA. Note the granular pattern of staining (40X x 10X). CgA: chromogranin A, SCLC: small cell lung carcinoma

pattern of the tumour cells by both the markers in NSCLCs however, depicted a poor outline as about 47 (94%) and 45 (90%) cases of these tumours demonstrated none to almost a few scattered cells being positive for HDC and CgA respectively. The 03 (6%) cases positive with HDC comprised of 02 (66.6%) ACs and 01 (66.6%) LCLC whereas of 05 (10%) positive NSCLCs with CgA, there were 03 (60%) ACs and 02 (40%) LCLCs. The staining pattern of these positive tumour cells by both the markers was moderately strong in intensity and focal (>10-30% of tumour cells) in ACs (Figure 3) and almost diffuse (>50% tumor cells) in LCLCs, whereas a typical cytoplasmic granular pattern was seen only with CgA. In view of these results, the sensitivity and specificity of HDC was 88% and 94% respectively, whereas for CgA, it was 57.6% and 90%. Similarly, the positive and negative predictive values for HDC and CgA were 97.35% & 93.5% and 75.8% & 45.9% respectively. The diagnostic accuracy was 89.7% and 66.8% for HDC and CgA respectively. The association of positive HDC and CgA immunostaining with the histological grades of PNEC's, showed that a significant difference in the percentage positivity with the WD (TC), MD (AC) (same as above) and the PD tumours (SCLC & LCNEC) was demonstrated with CgA only. However, when compared with HDC, a notably enhanced positivity by the latter was observed in PD tumours only, of which 89.3% (n=92) (Figure 4) were strongly positive as compared to CgA which was positive

in 54.3% (n=56) (P<0.001) (Figure 5). These results show that the sensitivity of CgA, not HDC, declined with the increasing grades of the PNECs. In the case of NSCLCs, no significant association between the tumour histological grades and the staining pattern of HDC and CgA was observed.

DISCUSSION

This study may add to the previous literature in terms of demonstrating the sensitive and specific IHC staining of the PNECs by HDC as compared to the conventional IHC marker, CgA. This also may be another effort not only in upholding the role of HDC as a presumably reliable marker for NE differentiation in lung carcinomas but also in classifying morphologically the epithelial PNECs and the NSCLCs according to the latest WHO classification in our part of world.

Table 1. Pattern of IHC positivity in various PNECs subtypes

Tumour Types (n)	Positive		p-value
	HDC n (%)	CgA (n %)	
1. TC (15)	13 (86.6)	12 (80)	0.6280
2. AC (07)	05 (71.4)	04 (57.1)	0.5767
3. SCLC (95)	87 (91.5)	54 (56.8)	<0.001
4. LCNEC (08)	05 (62.5)	02 (25)	0.1306
Total	110	72	

This table shows the pattern of immunohistochemical positivity of HDC and CgA in various subtypes of PNECs. Note the significantly enhanced sensitivity of HDC in SCLC and LCNEC as compared to CgA. HDC: histidine decarboxylase, CgA: chromogranin A, PNECs: pulmonary neuroendocrine carcinomas, TC: typical carcinoids, AC: atypical carcinoids, SCLC: small cell lung carcinomas, LCNEC: large cell neuroendocrine carcinomas

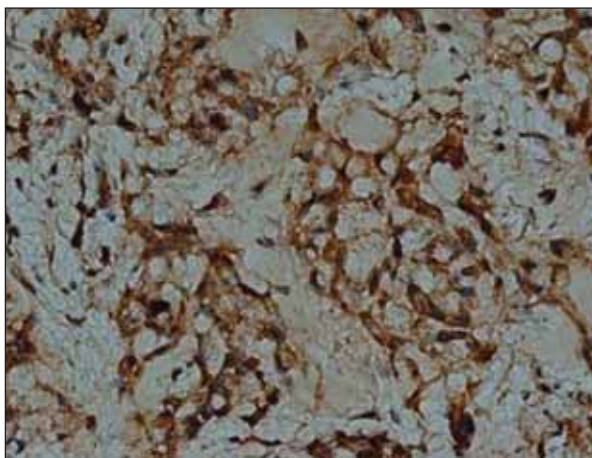


Figure 3. This figure shows the strong focal cytoplasmic staining of the poorly differentiated mucinous lung adenocarcinoma cells by HDC. (40X x 10X). HDC: histidine decarboxylase

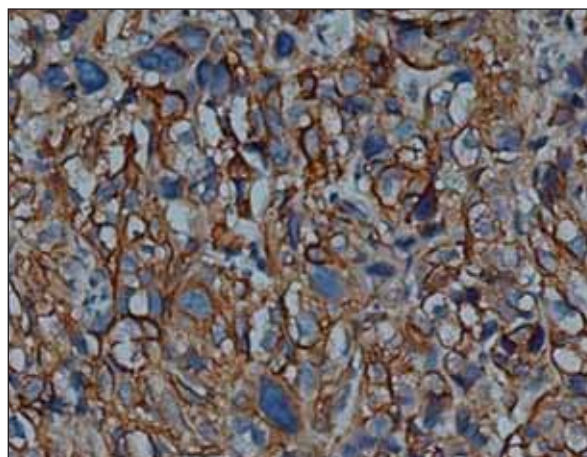


Figure 4. This figure shows the strong diffuse cytoplasmic staining of the poorly differentiated tumour cells (LCNEC) by HDC. (40X x 10X). HDC: histidine decarboxylase, LCNEC: large cell neuroendocrine carcinoma of lung

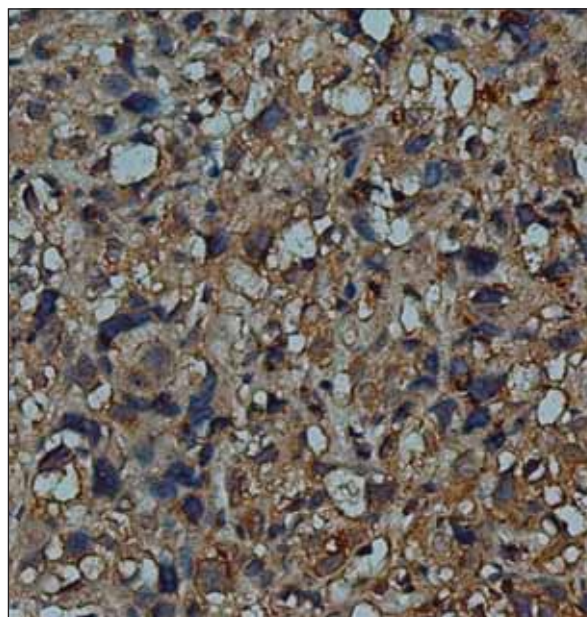


Figure 5. This figure shows the strong granular cytoplasmic staining of the poorly differentiated tumour cells (LCNEC) as in Figure 4 by CgA. (40X x 10X). CgA: chromogranin A, LCNEC: large cell neuroendocrine carcinoma of lung

The diagnosis of small cell carcinoma is sometimes difficult, due to crush artifact seen in bronchial biopsies [36] and LCLC, whenever diagnosed after conventional H & E staining, should always be confirmed for NE differentiation by applying NE specific markers as the microscopic features, especially the poor differentiation and nuclear morphology, closely resemble that of the LCLC of NSCLC series [37]. CgA was selected in this study as a NE marker for supporting the diagnosis of PNECs, especially the SCLC & LCNEC, and also to observe its association with HDC immunostaining. Previous studies performed on CgA staining in SCLC showed unsatisfactory results, such as Gosney et al, who justified the inclusion of CgA and synaptophysin in a panel of antibodies to demonstrate NE differentiation by proving them sufficiently specific and sensitive, but the staining pattern for SCLC with CgA depicted only 38.09% sensitivity [38]. Wilson and Matsuki demonstrated positive CgA staining in 40% and only 22% of the SCLC respectively [35,39]. In contrast to the above studies, the CgA positivity in SCLC included in our study showed positive IHC staining in 57% of the SCLC which is higher than the above reported results but lower than the results of Lyda et al, who have recently displayed positive CgA immunostaining in 84% of the SCLC cases [40]. On the other hand, HDC demonstrated excellent positivity (91.5%) in SCLC as compared to CgA ($P < 0.001$).

At present, it is unclear whether SCLC are derived from a NE precursor cell or from an undifferentiated epithelial cell [41]. A reasonable positivity by CgA and strong staining by HDC (91.5%) which in turn reflects the production of histamine by these PNECs, as also shown in this study, may add towards defining the origin as well as the biochemical characterization of SCLC since apart from mast cells, which are not NE in nature, no other histamine producing cell types have yet been detected in the lung [42], therefore, suggestive of NE nature of SCLC being solely responsible for the vigorous expression of the CgA and HDC in SCLC and the related PNECs.

The findings as regards NSCLCs included in this study, were the strong diffuse staining of HDC & CgA in 01 and 02 LCLCs cases respectively (Figure 3) which were diagnosed after H&E microscopy to be of LCLCs of simple NSCLC series based on their morphology. On the other hand, 03 and 02 AC cases showed strongly positive areas of NE differentiation (>10 and 10-30% of tumour cells) with CgA and HDC respectively which sub classified them as combined NE carcinomas or NSCLC with occult NE differentiation or simply NSCLC-NE.

A significant association was observed between positive CgA immunostaining and the histological grades of PNECs, ($P < 0.001$) with the sensitivity declining with increasing grades of malignancy. In contrast, no significant association was observed between HDC positive staining characteristics and the histological grades of PNECs, ($P = 0.218$) with the percentage being almost invariably similar between all grades. Consequently, HDC sensitivity (88 %) in PNECs, in contrast to CgA (57.6%), may not be dependent upon the presence of neurosecretory granules which are more abundant in well differentiated than the poorly differentiated NE carcinomas.

Using sensitive IHC methods may improve our understanding of the tumour biology and NE differentiation, representing an important diagnostic tool for future therapeutic modalities [43]. Corresponding to Matsuki et al., the presence of HDC in human PNECs indicate that other substrates of cellular enzymes e.g DOTA LAN, mepyramine and 11C tryptophan, histidine/histamine- derived tracers, in future, may well be used in similar attempts as tools to follow and characterize non invasively the PNECs and occult metastases in patients through Positron Emission Tomography (PET) or Scintigraphy [35]. These tracers may also be helpful in the screening of such carcinomas with NE derivation, in high risk groups in our population long before they reach an extensive stage. Moreover, histidine-histamine receptor (pulmonary NE cell specific) targeted therapies can be made available to the patients suffering from lung carcinomas with NE differentiation because of the fact that the process of histopathological differentiation, tumour progression and also metastasis of these carcinomas has strong biological connection with histidine/histamine, as demonstrated in other histamine dependent tumour models.

From the above results, we have observed that HDC may be applied as a reasonably reliable marker for demonstrating NE differentiation in PNECs, regardless of their degree of differentiation. According to the non clinical issues, such as the traditional use of a given laboratory or, most important, the cost of the kits in each particular country, NE specific markers through affordable techniques should be utilized before a final diagnosis of PNECs be established by the histopathologist. More prospective analysis relating to the prognostic significance of NE differentiation in PNECs and other NE malignancies with respect to HDC immunoreactivity, should be followed.

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Conflict of Interest

No conflict of interest is declared by authors.

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