1—Neuroendocrine differentiation in lung tumours

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Great advances in knowledge of neuroendocrine lung tumours have resulted in the past 20 years from the application of new techniques, including electron microscopy, cell culture, immunocytochemistry, radioimmunoassay, and more recently molecular biology. These techniques have added greatly to our understanding of these complicated and often fatal tumours but they also raise many further questions, to which we do not yet have answers. For pathologists this is reflected in the multiple and at times complicated classifications applied to these tumours, depending on the techniques used and the tissues studied. For clinicians this explosion in knowledge has provided many new avenues for investigation and the possibility of developing new therapeutic tools. The subject of neuroendocrine differentiation in lung tumours is only now being unravelled. These new findings may soon help to unlock some of the secrets of lung carcinogenesis and help patients by providing diagnostic, prognostic, and possibly therapeutic information.

Neuroendocrine cells in the lung

It was Feyrer in 1938 who first described clear cells in the respiratory epithelium of the human airway and included them in a “diffuse endocrine system” with other clear cells in the gastrointestinal tract and pancreas. Fröhlich found that these cells were argyrophilic (taking up silver salts) and with foresight (but no evidence) he considered them to be chemo-receptors capable of monitoring gas tensions in the airways and releasing hormones. Hamperl saw the similarity of these cells to the Kulchitsky or K cell of the gastrointestinal tract and suggested this name for them. Electron microscopy showed that the cells contained cytoplasmic dense core granules similar to granules found in endocrine cells elsewhere and in neurones. The cells were therefore called neuroendocrine cells to emphasise the association between the endocrine and the nervous system. Immunocytochemical and sensitive radioimmunoassay techniques confirmed the presence of several amines and peptides within these neuroendocrine cells, including serotonin, gastrin releasing peptide (GRP), the C flanking peptide of preproGRP, calcitonin, leu-enkephalin, calcitonin gene related peptide (CGRP), and endothelin. Many of these peptides are also found in neurones and nerves of the central and peripheral nervous system, again showing the close link between the endocrine and nervous systems.

This widespread collection of peptide containing cells and nerves is known as the diffuse neuroendocrine system. All these peptides have potent actions on airway smooth muscle, vasomotor tone, and airway secretion and have been proposed as important modulators of physiological changes in the lung. The peptides may carry out their effects in a classical endocrine manner via the bloodstream, via nerve transmission (neuroendocrine), or in a local (“paracrine”) fashion. Not surprisingly, given the extensive nature of this peptide containing system in the human respiratory tract, lung tumours can produce many of these neuroendocrine features.

Neuroendocrine lung tumour classification

The secretion of what were originally considered to be “ectopic” hormones by lung tumours has been known for some time. Adrenocortical overactivity leading to Cushing’s syndrome was described in a patient with lung carcinoma in 1928. Sensitive assay and cell culture techniques have permitted the detection of peptide hormone secretion by tumours, particularly lung tumours, in the absence of clinically overt syndromes. Overt endocrinological disturbance and biologically active peptide hormone production is common in small cell carcinomas and carcinoid tumours; but almost all lung cancers, irrespective of histological type, are capable of producing small amounts of peptide.

Both carcinoid tumours and small cell carcinomas can be recognised by light microscopy. Dense core neurosecretory granules in small cell carcinomas and carcinoid tumours added an ultrastructural feature to establish neuroendocrine differentiation and showed the close association between these tumours and normal pulmonary endocrine cells, which also contain dense core granules. Thus both carcinoid tumours and small cell carcinomas are considered neuroendocrine tumours of the lung and included in the World Health Organisation histological classification of lung tumours. Carcinoid tumours are considered to form the benign end of the spectrum and small cell carcinomas the highly malignant end. A distinct subgroup of carcinoid tumours, labelled atypical carcinoids, with histological and clinical evidence of malignancy and with a high rate of metastases, were described in 1972. These lung neuroendocrine tumours form a link between carcinoid tumours and small cell carcinomas. To reflect the overlap between these tumours it was suggested that...
they should be reclassified as Kulchitsky cell carcinomas to indicate their spectrum of aggressiveness and emphasise their origin from the pulmonary endocrine or Kulchitsky cell\(^*\) (table).

Immunochemical techniques using antibodies to general neuroendocrine markers, including neurone specific enolase,\(^{17}\) PGP 9-5, synaptophysin, and chromogranin,\(^{18,19}\) were applied to neuroendocrine lung tumours by ourselves and others. Sensitive radioimmunoassay techniques were also used to detect these markers in serum and monitor tumour progression and recurrence.\(^{20,21}\)

These new techniques clarified the neuroendocrine nature of the tumours, suspected from light microscopy, and produced some unexpected findings, which at first were difficult to explain. The presence of dense core granules, peptide or amine hormone production, and neuroendocrine immunochemical markers in undifferentiated large cell carcinomas, squamous cell carcinomas and adenocarci- nous tumours gave rise to the concept of atypical endocrine tumours,\(^{22,23}\) non-small cell carcinomas with neuroendocrine features,\(^{24}\) and large cell neuroendocrine tumours of the lung.\(^{25}\) 

These categories have caused much confusion among pathologists and clinicians concerning their behaviour, prognosis, and response to treatment.

The important question is whether these tumours with neuroendocrine features behave aggressively like small cell carcinomas and whether they therefore respond to chemotherapy. Alternatively, is neuroendocrine differentiation of no significance, merely reflecting the general heterogeneity of lung tumours? Another possibility is that neuroendocrine differentiation may indicate a more benign behaviour as typical carcinoid tumours, which display many neuroendocrine features, are usually indolent.

In an attempt to answer these questions Gould\(^{26}\) suggested a spectrum comprising several related but distinct types of neuroendocrine neoplasms in the lung (table). He retained the typical carcinoid tumour group but labelled atypical carcinoids as well differentiated neuroendocrine carcinomas to emphasise their malignant behaviour. Small cell carcinomas are categorised as neuroendocrine carcinoma of small cell type. In addition, he introduced a new category, which he called neuroendocrine carcinoma of intermediate cell type, and included many large cell undifferentiated carcinomas and tumours with squamous and glandular differentiation in this group (table). His clinical follow up of patients with these tumours shows a spectrum of behaviour, the intermediate cell type behaving in a fashion similar to that of the small cell type and being more aggressive than other large cell carcinoma:27 He considers that neuroendocrine differentiation in non-small cell carcinomas indicates aggressive behaviour and suggests that chemotherapy should be assessed for such tumours.

This classification is useful in delineating a spectrum of endocrine lung tumours with predictable behaviour but falls short on several points. Firstly, the description of the neuroendocrine carcinoma of intermediate cell type is too vague and non-specific to allow this to be separated from other undifferentiated tumours by light microscopy in my experience. Secondly, it is unfortunate that this category was named intermediate cell type as it is often confused with the "intermediate" small cell carcinoma subgroup of the WHO classification.\(^{14}\) Thirdly, the classification presumes a histogenetic relationship that has not yet been established. There is now sufficient evidence to regard carcinoid tumours as separate from small cell carcinomas and other lung tumours. Carcinoid tumours occur in a younger population and are not related to smoking habits or other risk factors for the more common forms of lung cancer. Furthermore, small cell carcinomas are rarely seen in association with carcinoid tumours. The initial response of a small cell carcinoma to combination chemotherapy and the poor long term prognosis contrast with the chemoresistance and good prognosis of most carcinoid tumours. These observations call into question the value of attempts to devise a terminology for a spectrum of neuroendocrine tumours and the classification of these tumours remains in a state of flux.

**Difficulties in the diagnosis of neuroendocrine lung tumours**

The more recent classifications of neuroendocrine lung tumours are difficult for pathologists to apply, particularly for the non-small cell types. Classification requires basic light microscopy plus electron microscopy and the use of immunochemical markers, which are expensive and time consuming. A patient with an unusual lung tumour suspected of being a neuroendocrine tumour should probably be referred to a specialist centre for appropriate investigations; formal follow up is essential to establish their behaviour and prognosis.

A second problem is that lung tumours show heterogeneity with both light microscopy and electron microscopy, which can lead to problems in interpreting neuroendocrine features. Up to 7% of lung tumours have a mixed morphology according to light microscopic appearances. Multiple sampling of a tumour by electron microscopy may reveal glandular, squamous, and neuroendocrine...
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Figure 1  Typical carcinoid tumour of lung with islands and ribbons (trabeculae) of cells showing uniformity of nuclei and cytoplasm. There is hyperchromatism of the nuclei at the periphery of the islands.

Figure 2  Dense granules in the cytoplasm of a carcinoid tumour cell. (Electron micrography by A Dewar, National Heart and Lung Institute.)

areas even within the same cell; the significance of large or small areas of neuroendocrine differentiation in a mixed tumour remains to be assessed. For definitive diagnosis large quantities of tissue may be required for special investigations. This may be a problem with bronchial biopsy, the only diagnostic technique used for many lung tumours as the patients are inoperable. Most neuroendocrine classifications rely heavily on the architecture of the cells within the tumour and this may not be seen easily in small biopsy specimens. Crush artefact, cellular distortion, and degeneration may make it difficult at times to separate carcinoid tumours from small cell carcinomas, a fact of great importance for treatment and prognosis. At electron microscopy the definition of a dense core neurosecretory granule, the hallmark of neuroendocrine differentiation, is not clear and several studies show a wide divergence in the size, shape, and distribution of these granules. Differentiating them from small lysosomes may be difficult.

Finally, the specificity of the general neuroendocrine markers has been questioned as these markers have been found in non-neuroendocrine tumours, such as breast or kidney tumours. The use of only one marker for immunocytochemical diagnosis of neuroendocrine differentiation is open to criticism and a panel of markers is now recommended (see below).
Carcinoid tumours of the lung

About 12% of all carcinoids arise in the lung but less than 1% of lung tumours are carcinoid.28 Hamperl29 was the first to separate carcinoids from other bronchial adenomas and point out their similarity to gastrointestinal carcinoids. The highest incidence occurs in the 31–40 year age group, with a female preponderance (62%). The tumour usually arises in a main bronchus (85%) but may be peripheral (15%).30 Multicentric growths scattered throughout the lung have been described11 and carcinoids can be associated with endocrine tumours elsewhere as part of a pluriglandular syndrome.32

Carcinoid tumours contain uniform cells with small round to oval nuclei, few nucleoli, and granular eosinophilic cytoplasm. The cells are most commonly in islands interweaving with one another in a mosaic pattern (fig 1), as interconnecting ribbons of cells in a trabecular pattern, or in an adenopapillary pattern; mixed forms are also seen.33 In a typical carcinoid nuclear pleomorphism and mitoses are rare.

At the ultrastructural level the main feature of all carcinoids is the large numbers of dense core neurosecretory granules in the cytoplasm of virtually all tumour cells (fig 2). Most carcinoid tumours are strongly immunoreactive for general neuroendocrine markers17 18 34 (fig 3), and immunocytochemical methods and radioimmunoassay show that bronchial carcinoids produce a wide range of amines and peptide hormones.12 26 Gould found that 5-hydroxytryptamine was the most frequently occurring immunoreactive substance in pulmonary carcinoids, followed in descending order of frequency by gastrin releasing peptide, vasoactive intestinal polypeptide, leu-encephalin, somatostatin, calcitonin, and adrenocorticotropic hormone. A single tumour may produce several peptides as well as 5-hydroxytryptamine. The latter has been put forward as an immunocytochemical marker of carcinoids but is found in only half of foregut carcinoids, which limits its diagnostic usefulness.35 S-100 positive sustentacular cells have been reported in pulmonary carcinoids36 and labelled as “paraganglioid” carcinoids. The feature that distinguishes these from the rare primary pulmonary paraganglioma is that paragangliomas rarely express keratin and are strongly immunoreactive for neurofilaments.37

Carcinoid tumours are generally easy to diagnose by light microscopy and do not require further investigations. They have an excellent prognosis generally. Five per cent of typical cases with no features of malignancy metastasise15 so the behaviour of individual tumours cannot be predicted. Deeply invasive tumours,38 tumours greater than 3.0 cm in diameter, and lymph node metastases predispose to recurrence.39

Atypical carcinoid tumours

Arrigoni40 first identified a group of tumours with a carcinoid architecture but with evidence of malignancy, characterised by nuclear pleomorphism, mitoses, and necrosis. The tumours were larger and more likely to be peripheral, and their behaviour was more aggressive than that of typical carcinoids, with regional or distant metastases in 70% of patients and a mortality of 30%.

In two studies that examined stage 1 resectable tumours diagnosed initially as small cell or large cell undifferentiated carcinomas 25%40 and 80%41 turned out to be atypical carcinoids or well differentiated neuroendocrine carcinomas. These tumours were associated with a very much better survival rate at two years (60–75%) than small cell carcinomas even when these were resectable (9%).40 The improved survival described for stage 1 resectable small cell carcinoma42 43 and the subsequent resurgence of interest in resecting small cell carcinomas must be looked at closely in view of these findings. Strict criteria must be used in labelling any peripheral lung lesion as a small cell carcinoma.

Dense core neurosecretory granules are far less numerous than in true carcinoids and they tend to be smaller (80–140 nm). Intertwined cytoplasmic processes are prominent and the granules are usually concentrated there. In a typical carcinoid more than half of the cells contain granules with at least five per cell whereas atypical carcinoids possessed granules

Figure 3 Positive immunostaining for chromogranin A in the cell cytoplasm of a carcinoid tumour.
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Their neuroendocrine nature. The granules are usually scanty and small (80–140 nm) and multiple sampling of a tumour is necessary to detect them. The use of electron microscopy has introduced new problems in the diagnosis of small cell carcinoma. Several authors report the absence of dense core granules in what appears at light microscopy to be classic small cell carcinoma. The presence of tonofilaments and desmosomes led Churg et al. to label them small cell squamous tumours whereas Nomori et al. labelled them undifferentiated carcinoma, small cell type. Patients with these tumours have been reported to survive longer than those with tumours with classical granules, but numbers of cases are small.

There has also been controversy about the prognostic significance of the subdivisions of small cell carcinomas. Some studies have led to claims that the small cell type has a better prognosis and is more responsive to chemotherapy, whereas others suggest that the intermediate cell type does best. Two further studies suggest no difference. This controversy, as well as the difficulty of separating the small cell from the intermediate cell type histologically, has led many pathologists and clinicians to abandon this subclassification.

The mixed small cell/large cell carcinoma was first proposed as a separate entity in 1982. These mixed tumours, originally thought to be rare, are being recognised more frequently especially in biopsy and necropsy specimens from patients treated intensively with radiation or chemotherapy. They represent 5–12% of all small cell carcinomas and have shown a poor response to chemotherapy with a shorter survival.

The International Association for the Study of Lung Cancer (IASLC) has recommended that small cell carcinoma should be divided into three groups: (1) small cell carcinoma, combining the classic small cell and intermediate cell types (90% of untreated small cell carcinomas are of this type); (2) small cell/large cell carcinoma, in which at least 24% of the cells have large granular or more open nuclei with prominent eosinophilic nucleoli (4–6% of untreated small cell carcinomas fall into this category); (3) carcinoma of mixed type similar to WHO group 3.

This classification is supported by experimental studies on small cell carcinoma. Many continuous cell lines and xenographs have been established. Most contain dense core granules, express neuroendocrine and other markers associated with small cell carcinoma, and are radiosensitive. The cytological features of these “classic” cell lines are of the small cell subtype of small cell lung carcinoma. Some small cell lines have a “variant” morphological appearance and resemble small/large cell carcinomas or large cell carcinomas. They lack dense core granules and other neuroendocrine features. They are sometimes associated with amplification of the c-myc oncogene and they grow more rapidly, are cloned more efficiently, and are radioresistant. These variant cell lines may develop from classic small cell carcinomas, especially if these have been treated with

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**Figure 4** Small cell carcinoma of the lung showing the small hyperchromatic nuclei, nuclear moulding, little cytoplasm, and high cell death rate.

**Figure 5** Small cell carcinoma of the lung giving a focal intense signal for chromogranin A messenger RNA. (From Hamid et al.; reproduced by courtesy of the Journal of Pathology.)

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chemotherapy, or from mixed small cell/large cell carcinomas. The development of large cell features in a small cell carcinoma after chemotherapy may be due to the treatment or to the survival of large cells already present in the tumour before treatment. There is wide support for the above classification, though some studies suggest no difference in prognosis.51 Small cell carcinomas are immunoreactive for general neuronal markers.17 18 34 Serum concentration of neuroendocrine markers, such as neurone specific enolase, have been used as tumour markers for small cell carcinoma and may be used to monitor treatment and predict relapse.20 21 Unfortunately from the diagnostic and prognostic point of view, in general the more malignant the neuroendocrine tumour is the less likely it appears to be to give positive immunocytochemical results for neuroendocrine antigens or peptide hormones.38

A surprising immunocytochemical finding is that gastrin releasing peptide, an established growth factor, is rarely expressed in small cell carcinomas. This may be due to the production of abnormal forms of the precursor preproGRP molecule, and the C terminal peptide in particular. Antibodies to C terminal peptide show strong immunostaining in 12% of typical carcinoids, in 56% of atypical carcinoids, and in 70% of small cell carcinomas,39 and these appear to be associated with a shorter survival than those without the immunoreactive peptide. The more malignant a tumour is the more likely it is to produce an abnormal peptide precursor. The mechanism underlying any of the paraneoplastic syndromes, such as neuro-pathies, encephalomyelitis, and myopathies, is unknown, though production of antigens by the tumour similar to neuronal antigens and the subsequent induction of antibodies by the body in response to the antigens are thought to explain many of the syndromes.37 This suggests that many more tumour products remain to be discovered.

Immunocytochemistry and radioimmunoassay provide useful data on stored and secreted peptide; information on gene expression and regulation requires molecular biology techniques, including Northern and Southern blotting, in situ hybridisation, and the polymerase chain reaction. When these techniques are applied the gene for gastrin releasing peptide has been found in all carcinoids and small cell carcinomas50 and in most large cell carcinomas. We have localised the neuroendocrine antigen chromogranin A gene in small cell carcinomas (fig 5).

These techniques have also helped to detect oncogenes and anti-oncogenes and abnormalities in their expression in neuroendocrine tumours. The major oncogenes implicated in neuroendocrine tumours are the myc and ras families. Myc amplification appears to be associated with tumour progression rather than pathogenesis. C-jun is also amplified in small cell lung cancer.39

Restriction fragment length polymorphism (RFLP) analysis indicates that deletions of chromosome arms 3p, 13q, and 17p are frequent in small cell lung cancer. Chromosome 13q is the site of the retinoblastoma gene and 17p of the p53 gene, and 3p is the site of an as yet unidentified anti-oncogene.40

**Non-small cell neuroendocrine carcinomas**

The first description of non-small cell neuroendocrine carcinomas, which by light microscopy had been diagnosed as large cell or squamous cell carcinomas or adenocarcinomas, came in 1981, when McDowell et al26 described seven cases in the periphery of the lung. Electron microscopy subsequently showed dense core neurosecretory granules, thus establishing them as neuroendocrine tumours (in 4% of 150 lung tumours). The tumours were argyrophilic and contained 5-hydroxytryptamine, confirming endocrine differentiation at the level of light microscopy. McDowell labelled them atypical endocrine tumours of the lung. Another report34 suggested that as many as 9% of non-small cell carcinomas in the lung contained neurosecretory granules, and that median survival and rates of response to treatment were in the ranges seen with non-small cell carcinoma. Others,24 25 however, claimed that they behaved more aggressively, like small cell carcinoma, and labelled them large cell neuroendocrine tumours or non-small cell carcinomas with neuroendocrine features. Gould38 included them as intermediate cell type in his spectrum of neuroendocrine carcinomas and suggested that cell nesting with peripheral palisading of nuclei would help to identify this tumour by light microscopy, a point emphasised by Mooi et al.24 Travis et al41 emphasised that the cells are large with prominent nuclei in the nuclei, a high mitotic rate, and a low nuclear:cytoplasmic ratio and have an organoid, trabecular, and palisading pattern. In a later study and follow up by Gould’s group the tumours behaved in a fashion similar to that of small cell carcinoma.29

The clinical relevance of a separate group of non-small cell neuroendocrine carcinomas remains to be determined; most studies are small and lack follow up.61

**Specificity of neuroendocrine markers**

Several workers applying neuroendocrine markers to lung tumours have found that a proportion of all tumour types give positive results.24 25 This may reflect the heterogeneity of lung tumours; others consider that the markers lack specificity for neuroendocrine tumours.40 We found that, although neuron specific enolase and PGP9.5 are positive in most neuroendocrine tumours,43 they are also present in 10–60% of non-neuroendocrine lung tumours and are not now considered as reliable markers of neuroendocrine differentiation. Chromogranin, Leu-7, and synaptophysin appear to be more useful markers but they stain fewer neuroendocrine tumours than neurone specific enolase and PGP9.5.24 43 Use of a panel of markers rather than a single marker is advised, with a combination of neurone specific enolase, PGP9.5, chromogranin, and synaptophysin.
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physin. A positive result with two of the four markers is recommended before a tumour can be labelled as neuroendocrine.

Conclusion

Neuroendocrine lung tumours are proving a fruitful area of research for understanding the basic mechanisms of carcinogenesis. Rapid advances in the molecular and cell biology of these tumours should help determine their spectrum of behaviour. New studies are essential, using light microscopy, electron microscopy, immunocytochemistry, and molecular biology techniques, for clarifying different subtypes and designing future treatments, particularly now that many growth factors are known to be produced by these tumours. A classification based on morphological and functional criteria may prove to be of therapeutic importance in the future.

1 Feyrer F. Ueber diffuse endobronchiale epitheliale Organe. Leipzig: Barth; 1938.
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