

Neuroendocrine differentiation in lung carcinoma

Since their first description, neuroendocrine cells in the lung (Feyrter cells, Froelich cells, or Kulchitsky cells depending on your allegiance) have fascinated lung biologists and pathologists. They are readily identified in fetal and infant lungs, both as single cells and neuroepithelial bodies, and appear to have a paracrine role in regulating lung development.¹ Hyperplasia of neuroendocrine cells with the formation of carcinoid tumourlets is seen most frequently in fibrotic areas of lung associated with bronchiectasis, but also occurs in other chronic lung diseases and can be induced experimentally.^{2,3} Carcinoid tumour is the most obvious and straightforward example of neuroendocrine cell neoplasia, but occasionally the distinction between hyperplasia and neoplasia becomes blurred and it is possible to demonstrate proliferation of neuroendocrine cells adjacent to the tumour and elsewhere in the lung.^{2,4}

At the ultrastructural level endocrine function is associated with the presence of dense core secretory granules, and the discovery of identical granules in small cell lung carcinoma, typically in very small numbers and confined to cell processes, was initially interpreted as evidence that this tumour also arose from neuroendocrine cells.⁵ Its neuroendocrine nature was supported by clinical evidence of hormone production, usually antidiuretic hormone or adrenocorticotrophic hormone (ACTH). However, the presence of neuroendocrine granules and hormone production in otherwise undifferentiated tumours does not necessarily reflect the cell of origin. A more likely explanation is that small cell lung carcinoma arises from a primitive "stem" cell with the capacity to differentiate in a number of different directions.⁶ The nature of the stem cell remains uncertain, and it is not clear why the neuroendocrine pathway should be favoured. It is tempting to think that this might be related to the observation that neuroendocrine cells are the first of the specialised bronchial epithelial cells to appear in the human embryo.⁷ However, the tendency for morphologically undifferentiated small cell tumours to show neuroendocrine differentiation is also seen in other sites such as the oesophagus, cervix, bladder, and prostate.

Neuroendocrine differentiation is not confined to lung carcinomas of small cell phenotype; it has also been seen in non-small cell lung carcinomas, particularly in "large cell" variants. Some of these are otherwise unclassifiable but others have morphological features suggesting their possible neuroendocrine nature.^{8,9} The terms "neuroendocrine carcinoma", "atypical carcinoid", or "malignant carcinoid" are applied loosely; some tumours closely resemble carcinoid tumours and have a large cell phenotype, whereas others have a carcinoid pattern but cytological features almost indistinguishable from small cell lung carcinoma. There is no clear continuum and proposed classifications for pulmonary neuroendocrine tumours are neither conceptually satisfactory nor easily applied.¹⁰ Some tumours have a combined phenotype in which an obvious neuroendocrine component is mixed with another non-small cell lung carcinoma component, providing evidence that divergent differentiation is possible in the same tumour. This is not surprising if we remember that the simple tubular epithelium of the fetal lung gives rise to all the specialised cells of the normal mature bronchial epithelium, including neuroendocrine cells. Neoplastic cells are unlikely to recapitulate exactly the normal pathways of differentiation and aberrations are to be expected, a feature of lung carcinomas first recognised by Willis.⁶ Dual glandular and squamous cell differentiation is commonly seen, particularly at the level of the electron microscope.¹¹

Allowing for a minor degree of heterogeneity it is apparent that, in everyday practice, using light microscopy, most non-small cell lung carcinomas are fairly readily classified into a small number of well defined categories. However, typical morphological features of squamous cell carcinoma or adenocarcinoma do not necessarily exclude an attempt at neuroendocrine differentiation. Dense core neurosecretory granules can be seen by electron microscopy in some tumours, indicating bidirectional differentiation in the same neoplastic cell. Identifying these tumours, where there are no morphological clues to neuroendocrine differentiation, poses a problem. Treatment often has to be based on very small biopsy samples, sometimes even on cytological evidence alone. Electron microscopy is not an option because of the very limited amount of material available, considerable sampling difficulties, and sub-optimal fixation. The search for reliable immunocytochemical markers of neuroendocrine differentiation therefore continues, and in this issue of *Thorax* (pages 116-120) Gosney and his associates¹² present their experience with a group of commercially available antibodies in assessing neuroendocrine differentiation in bronchoscopic tumour biopsies.

The range of antibodies available includes those directed against neural or neuroendocrine cell antigens such as neurone-specific enolase (NSE), a glycolytic enzyme; protein gene product (PGP) 9-5, a protein originally isolated from brain; the brain isoenzyme of creatine kinase (CK-BB); and the natural killer cell membrane antigen Leu-7 (CD 57 or HNK1). Antigens associated with the secretory apparatus include the chromogranins (chromogranins A and B and secretogranins), a family of proteins which forms a major component of the matrix of the dense core secretory vesicles, and synaptophysin, a membrane protein originally isolated from neuronal presynaptic vesicles.

Early hopes that NSE would provide a useful marker for neuroendocrine differentiation have not been realised; in a number of studies, including the present one, tumour reactivity seems to be randomly distributed among the different types.^{4,13,14} Defenders of NSE might still argue that available antibodies, which are raised against brain antigens, vary in their specificity. Biochemical estimation and quantitation of gamma enolase isoenzymes in tumour tissue seems to provide a more specific indicator but is clearly not a routine procedure.¹⁵ Similarly, the results of the present series and others suggest that PGP 9-5, whatever its merits as a neural marker in other tissues, is disappointingly non-specific in lung carcinomas.⁴ Serum levels of CK-BB are raised in patients with extensive malignant disease including small cell lung carcinoma, and small cell lung carcinoma cell lines can produce CK-BB in vitro.^{16,17} As a marker of neuroendocrine differentiation, however, current evidence indicates that it, too, is insufficiently specific.

Expression of chromogranin appears to correlate with the numbers of dense core granules and, since small cell lung carcinoma cells are poorly granulated, we would not expect to identify immunoreactivity in many tumours. However, there is considerable variation between different series. Some, predictably, identify weak and focal staining, but Gosney and his colleagues found eight of 21 small cell tumours to be positive, together with a small number of non-small cell tumours, and others report comparable findings.^{13,14,18,19} Such differences are difficult to explain. The presence of raised serum levels of chromogranin in patients with small cell lung carcinoma indicates that it may not always be confined to granules, but there is no

evidence that free immunoreactive chromogranin is present in tumour cells.²⁰

Most commercial antibodies to synaptophysin are derived from the SY38 clone and give inconsistent results in formalin fixed, paraffin embedded tissue. The use of fresh tissue or alternative fixatives has been recommended, but new antigen retrieval techniques should make this unnecessary.^{21 22} In the series reported by Gosney *et al*, in which routinely fixed and processed tissue was used without antigen retrieval, most small cell tumours and a small number of non-small cell tumours were positive. Similar results have been reported previously and synaptophysin is emerging as one of the most useful markers.²³

Normal and neoplastic lung cells synthesise several hormonally active peptides including gastrin releasing peptide (GRP), ACTH, and the pro-opiomelanocortin-related peptides, arginine vasopressin, calcitonin and calcitonin gene related peptide, ACTH, Leu-enkephalin, and substance P. At first sight it would seem attractive to use antibodies to these peptides to determine neuroendocrine differentiation in carcinomas. However, detection depends on a sufficient amount of the normal peptide being stored in the cell. In well differentiated endocrine tumours (carcinoid tumours) peptides are usually easily identifiable, but where neurosecretory granules are sparse, staining, if detectable at all, is focal and variable in intensity; none was identified by Gosney *et al*. Another possible reason for this is that most peptides are produced as larger precursor molecules and cleavage is required before the active peptide is available for storage or release. For example, GRP – which acts as an autocrine growth factor for small cell lung carcinoma – is produced as prepro-GRP.^{24 25} There is some evidence that part of this larger molecule, more specifically the N-terminal fragment, could be a more consistent marker of malignant neuroendocrine tumours than GRP itself.²⁵

If cell constituents and peptides are unreliable markers, a logical step is to identify the genetic material coding for the markers of neuroendocrine differentiation. Small cell lung carcinoma cells, which contain very little stored chromogranin, contain cytoplasmic chromogranin mRNAs that are readily detectable by *in situ* hybridisation.¹⁸ GRP mRNA is also highly expressed in small cell lung carcinoma despite the lack of immunoreactivity for the final product.²⁶ Hamid *et al* were unable to identify chromogranin mRNA in any non-small cell lung carcinoma, but the same authors found GRP mRNA in seven of 10 non-small cell lung tumours.^{18 26} Routinely processed small cell biopsy material does not at present lend itself readily to *in situ* hybridisation, but there are rapid technical advances and it seems certain that molecular biology will make a significant impact in this field.²⁷ It will also cause us to question our definition of neuroendocrine differentiation. Genetic material encoding for specific peptides may be present in most lung carcinoma cells, but is the presence of mRNA sufficient or should we require evidence of protein translation?

Another approach has been to produce monoclonal antibodies against different types of lung carcinoma. Analysis of the data from a series of three international workshops has identified 15 clusters of antibodies which recognise different antigens.²⁸ The best characterised of these is the cluster 1 small cell lung carcinoma membrane antigen, now identified as the neural cell adhesion molecule (NCAM), a group of closely related glycoproteins involved in cell-cell contact in neural tissues.²⁹ NCAM is present in small cell lung cancers, related neuroendocrine tumours, and some non-small cell lung tumours.^{29 30} Other potentially useful antigens/antibodies include cluster 10 which consists of three neuroendocrine-specific proteins localised in the endoplasmic reticulum.³¹ Unfortunately, the use of these antibodies is limited by their availability and because

they are not generally applicable to formalin fixed, paraffin embedded tissue. With current methods of antigen retrieval, using microwaves or pressure cookers, further assessment of this group is possible.

To clinicians, who are generally content with the distinction between small cell lung carcinoma and non-small cell lung carcinoma, it must seem that pathologists are placing undue emphasis on the recognition of neuroendocrine differentiation. For them the obvious questions must be: does it have clinical significance and does it have prognostic or therapeutic implications? Clearly a diagnosis of small cell lung carcinoma has all these things, but it is by no means certain that its aggressive clinical behaviour or its sensitivity to chemotherapy and radiotherapy are in any way directly related to its neuroendocrine features *per se*. In non-small cell lung carcinoma only very careful clinicopathological studies, with consistent classification and reliable markers, can determine whether neuroendocrine differentiation, as an independent variable, indicates a better or worse prognosis or should influence assessment and treatment. The evidence so far is unclear; if neuroendocrine differentiation is defined using conventional, commercially available antibodies, there is some evidence that it is associated with greater metastatic potential, in terms of nodal status and stage, and with increased response to chemotherapy, but it is doubtful whether this influences survival.^{14 32 33} The antibody 123C3 to NCAM identifies a group of non-small cell lung cancers (about 20%) with a shorter postoperative and disease-free survival than NCAM-negative tumours.³⁰

There is no “gold standard” for neuroendocrine differentiation. We do not have sufficient confidence in any of the antibodies available to say that it stains with antibody X therefore neuroendocrine differentiation must be present, regardless of its cell type. We have to begin by classifying the tumours by traditional morphological means and assessing immunoreactivity against this. The problem is compounded by differences in sensitivity and specificity for identical antibodies in different hands, reflecting many different variables in fixation, processing, staining techniques, and interpretation. When an antibody that appears to be consistently reactive with tumours diagnosed as small cell lung carcinoma also stains, for example, squamous cell carcinomas, this is, quite reasonably, interpreted as evidence of neuroendocrine differentiation in some tumours of overtly squamous cell phenotype.

On this basis several studies, including the current one, indicate that, of the commercially available antibodies, the most specific markers are synaptophysin and chromogranin A. Leu-7 would be a reasonable third choice, although it seems to stain more non-small cell lung carcinomas than the other two.^{10 15 19} The use of a panel of antibodies has been recommended, with the suggestion that reactivity with two or more should be required before neuroendocrine differentiation is accepted.^{15 32 33} The evidence is that greater confidence should be placed in these three antibodies than other markers, but they may shortly be complemented or displaced by antibodies to NCAM and other more specific tumour antigens. This is the best we can offer at present but, by concentrating on neuroendocrine differentiation, we are probably seeing only one facet of a more fundamental change that endows some lung carcinomas with more aggressive properties. It is likely that other characteristics, such as proto-oncogene expression, may eventually provide us with more insight into the nature of this change.

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