Aldona Kasprzak[1], Maciej Zabel[1], Wiesława Biczysko[2]

Selected Markers (Chromogranin A, Neuron-Specific Enolase, Synaptophysin, Protein Gene Product 9.5) in Diagnosis and Prognosis of Neuroendocrine Pulmonary Tumours

[1]Department of Histology and Embryology, Poznań University of Medical Sciences, Poznań, Poland
[2]Department of Clinical Pathomorphology, Poznań University of Medical Sciences, Poznań, Poland

Neuroendocrine tumours of lungs represent a subgroup of pulmonary tumours with typical morphofunctional traits. In light microscopy, the four principal types of the tumours (typical and atypical carcinoids, small cell lung cancer, large cell neuroendocrine carcinoma) demonstrate typical arrangement of cells (organoid nesting, palisading, a trabecular pattern, and rosette-like structures), variable number of mitoses, presence or absence of necrosis. In ultrastructure, neuroendocrine tumours manifest groups of cells with cytoplasmic granules (and the so called dense-core neurosecretory granules in particular). Neuroendocrine cells release hormones to circulation or in a paracrine manner. Some pulmonary tumours exhibit no neuroendocrine morphology at the level of light microscopy but demonstrate ultrastructural and/or immunohistochemical traits of neuroendocrine differentiation. Proteins the presence of which confirms neuroendocrine origin of the tumours have been found relatively early to include neuron-specific enolase (NSE), the group of chromogranins and synaptophysin.

Present study aimed at summing up results of investigations conducted in, approximately, recent 30 years pertaining expression and/or serum concentrations of four neuroendocrine markers (chromogranin A, neuron-specific enolase, synaptophysin, protein gene product 9.5) and at an attempt to evaluate the role of such studies in extension of diagnostic and prognostic potential as related to neuroendocrine pulmonary tumours.

Until now, the most sensitive and specific marker or marker combination for early detection of neuroendocrine subtypes of lung tumours has not been identified. All of the markers examined in present study were detected both in the typical neuroendocrine pulmonary tumours and in a certain proportion of non-endocrine tumours. In the case of chromogranin A improved sensitivity and specificity of immunocytochemical studies was obtained using a panel of antibodies directed to various epitopes of the protein. Both in endocrine and non-endocrine tumours, neuron-specific enolase (NSE) is thought to represent mainly a prognostic index, and only quantitation of serum concentrations of the protein or of the fraction of immunopositive cells may permit to differentiate between subtypes of the tumours. Synaptophysin is regarded to represent one of the most specific markers of neuroendocrine differentiation, manifesting a much higher sensitivity than chromogranin A and NSE. With increasing frequency, PGP 9.5 is regarded to provide a prognostic marker in diagnosis of non-small cell lung carcinomas rather than of typical neuroendocrine tumours.

Introduction

According to the current WHO classification of lung tumours, neuroendocrine tumours are included to the group of malignant epithelial tumours [95, 96]. Neuroendocrine pulmonary tumours include a range of tumours, from low-grade typical carcinoids (TC), intermediate-grade atypical carcinoids, (AC), high-grade small cell lung cancers (SCLC) to large cell neuroendocrine carcinomas (LCNEC) [53, 97, 98]. Although in general the four mentioned above types of neuroendocrine pulmonary tumours are distinguished, also some non-small cell carcinomas (approximately 10–20% squamous cell carcinomas, adenocarcinomas and large cell carcinomas) are included into the group of so called non-small cell lung cancers with neuroendocrine differentiation (NSCLC-ND) [14, 95]. Histological heterogeneity of lung carcinomas (mixed forms, e.g, neuroendocrine and squamous cells pulmonary carcinomas) may involve SCLC and LCNEC but never pulmonary carcinoids [43, 96]. Most of therapeutic

problems accompany atypical carcinoids and LCNEC, while surgery remains the method of choice in treatment of TC, similarly as chemotherapy in cases of SCLC. Chemotherapy and radiotherapy are used also in advanced forms of AC, particularly those with presence of inoperable metastases [5, 106].

The search for neuroendocrine origin of pulmonary tumours reflects first of all the hypothesis that tumours of the type are linked to poor prognosis but they manifest higher sensitivity to chemotherapy [19]. However, results of studies based on the premise remain controversial: some authors demonstrated that neuroendocrine origin affect survival of patients and is linked to better therapeutic effects while other authors deny prognostic significance of such a differentiation of cancers [18, 19, 65, 83]. In addition, interpretation of such divergent opinions is complicated due to the absence of a gold standard which would define neuroendocrine differentiation. The idea of a neuroendocrine cell continues to evolve: first of all the approach to histogenesis of neuroendocrine cells has changed (they do not always develop from neuroectoderm) and definition of neuroendo-
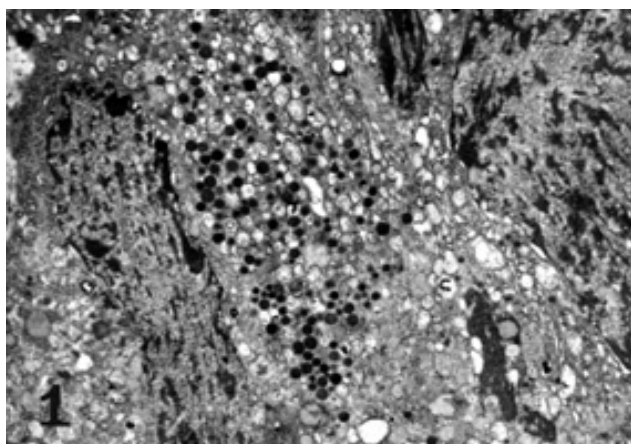

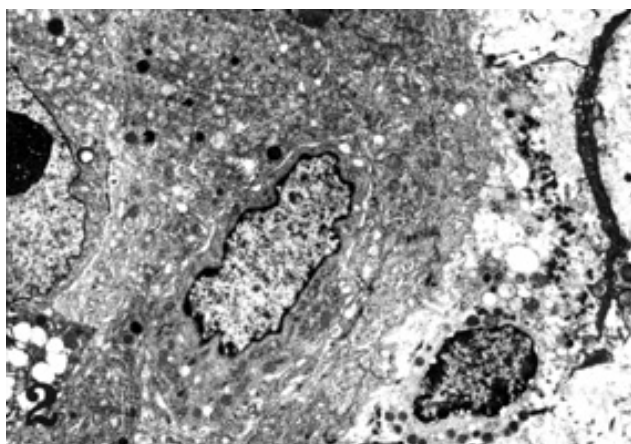Fig. 1. Small cell lung carcinoma. Numerous neurosecretory granules are present in cytoplasm of one cell. TEM ×14 000.

Fig. 2. Combined small cell lung carcinoma. The cells contain a few dense core granules. TEM ×10 000.

crine phenotype has been proposed to be supplemented by aspects of genetic determination. According to Langley, the neuroendocrine phenotype is manifested by a cell which produces active substances of neurotransmitter, neuromodulator type or neuropeptide hormones, and forms no axons and/or synapses. Ultrastructurally, the most distinctive feature of all neuroendocrine cells is the dense-cored, membrane-bound, neuroendocrine granule, which is also called neurosecretory granule [57]. The number, structure, and size of these granules may vary considerably (Fig. 1, Fig. 2). In all tumors, containing cells with neurosecretory granules, the variability of these cells was noted [14].

Studies continue on defining a neuroendocrine marker of perfect sensitivity and specificity for evaluation of malignancy, for early detection and differentiation, for evaluation of therapeutic potential and prognosis in this type of lung tumours [34, 36, 93, 111].

Selected aspects of histogenesis of neuroendocrine pulmonary tumours

Malignant tumours with neuroendocrine traits (e.g, thyroid medullary carcinoma and small cell lung carcinoma) are prototypes of tumours developing from peripheral neuroendocrine cells. Such a tumour was diagnosed for the first time in 1928 [17]. From this time on our knowledge on biology of the tumours continued to grow [95, 112]. Mechanisms which control neuroendocrine phenotype have not been fully recognised. Most probably, the central role in the process of neuroendocrine differentiation is played by the basic helix-loop-helix (bHLH) enhancer, which is a homologue of such protein in *Drosophila* and in humans it is termed the human achaete-scute homologue-1 (hASH1). The group of bHLH proteins plays particular role in control of synthesis and secretion of polypeptide hormones, e.g, of calcitonin [8]. In rodents, homologues of the proteins involve Mash1 and Mash2 (Mammalian achaete-scute homologue) [7]. hASH1 acts as a transcription factor at early stages of development of nervous and neuroendocrine cell progenitors in several organs and systems, including central and autonomous nervous systems, medulla of adrenal glands, thyroid, lungs, prostate gland [7, 16]. Its over-expression was detected in cases of small cell lung carcinoma, medullary thyroid carcinoma and phaeochromocytoma of adrenal medulla. Studies *in vitro* demonstrated expression of hASH1 in a line of neuroendocrine tumour cells and the expression was controlled at the level of transcription. Moreover, restriction of hASH1 expression to neoplastic neuroendocrine cells depends upon two tissue-specific silencers, present in the proximal and distal, 5'flanking regions [20]. Studies on expression of mRNA for hASH1 *in vivo* demonstrated presence of the transcript in atypical pul-

monary carcinoids, adenocarcinomas, in SCLC and LCNEC but its transcription could be shown in squamous pulmonary carcinomas or large-cell carcinomas. The transcript seldom could be detected in typical pulmonary carcinoids (4/30 studied tumours). Moreover, in cases of SCLC and LCNEC strict correlations were noted between expression of mRNA for hASH1 and expression of chromogranin A, gastrin releasing peptide (GRP) and of calcitonin. No correlation could be noted between hASH1 and neural cell adhesion molecule (NCAM), suggesting that expression of hASH1, at least in cases of pulmonary carcinomas showed a much closer relationship to endocrine phenotype (in a broad understanding of the term) than to neuroendocrine differentiation. In addition, correlations were detected between expression of hASH1 and a significantly abbreviated survival of patients with SCLC [44]. Application of immunocytochemical techniques in normal pulmonary neuroendocrine cells (PNEC) demonstrated Mash1 expression while non-neuroendocrine cells (non- PNEC), such as Clara cells, were immunopositive to Hes1 protein, one of factors which silence bHLHs expression [40]. The studies suggest role of hASH1 in neuroendocrine differentiation of small cell lung carcinomas and role of Hes1 and the *Notch* signalling pathway in development of non-small cell lung carcinomas (NSCLC) [40]. In the model of transgenic mice, activation of *ras* oncogene in the common precursor cells was shown to lead to differentiation and proliferation of both neuroendocrine cells and non-neuroendocrine cells. Thus, the observations probably point to common histogenesis of various cell types in epithelium of respiratory tract [89]. Phenotype and biology of SCLC may reflect the developmental stage at which the tumour remained. Humans represent the only species in which this type of pulmonary carcinoma develops. While carcinoids develop from highly differentiated PNEC, only a low number of SCLC manifest markers of mature PNEC [32, 35, 87], suggesting that SCLC cells may originate from the epithelial stem cell [89]. Studies of Ishida et al. on pulmonary carcinoids indicate that the tumours may develop from a multipotential stem cell of both neuroendocrine and epithelial nature [39]. Other authors backed up the hypothesis that all pulmonary carcinomas originate from pluripotential reserve cell of bronchial mucosa, which may proliferate and differentiate into one or several cell lines [26, 30]. Studies are also available which suggest that all pulmonary carcinomas develop from a common stem cell of endodermal origin [101]. Studies using various forms of microarrays permitted to compare gene expression in SCLC, in the normal bronchial epithelium and in pulmonary carcinoids. The studies demonstrated that, in respect to their origin, small cell lung carcinomas are strictly related to bronchial epithelium cells

while lung carcinoids are related to tumours originating from neural crest [3].

Neuroendocrine markers of pulmonary tumours

Tumours originating from neuroendocrine cells, in addition to typical hormones, frequently release additional polypeptide hormones, not always of a typical amino acid composition. They frequently hamper attempts to detect synthesized hormones and documentation of neuroendocrine character of cells. Therefore, in tumour pathology proteins pointing to neuroendocrine origin are diagnostically more important than polypeptide hormones [113]. The proteins confirming neuroendocrine origin relatively early were found to include neuron-specific enolase (NSE), group of chromogranins and synaptophysin [33, 66, 102, 105]. Subsequently, some hope was linked to markers such as, i.a, protein gene product 9.5 (PGP 9.5), GRP (gastrin releasing hormone), proteins of small vesicles (SV2 and p65), Leu-7 protein (a marker of NK cells) or NCAM (CD56) [46, 51, 54, 66, 90, 113]. In daily pathological practice, detection of neuroendocrine markers was recommended, co-expressed with various cellular structures, e.g. NSE localized in cytosol, chromogranin A in secretory granules and synaptophysin in membranes of small vesicles [113]. At present, a tumour is accepted to be of neuroendocrine character when presence of neuroendocrine granules can be demonstrated in its ultrastructure or tissue expression of at least two neuroendocrine markers, other than NSE, is detected [90, 97].

A dynamic development of immunocytochemical techniques for detection of antigens (markers) in lung carcinomas took place at the beginning of 1980-ties. Already at that time several authors demonstrated that cells of lung tumours may demonstrate expression of both markers typical of nerve cells and those of epithelium [45, 61, 78]. Markers such as neurofilament protein, bombesin (in mammals gastrin releasing peptide, GRP), chromogranin and synaptophysin were found to be specific for neuroendocrine tumours (including SCLC) but the markers lacked sufficient specificity and sensitivity [78]. Eleven % to 30% cases of non-small cell lung cancers (NSCLC) also demonstrated expression of neuroendocrine markers despite the absence of neuroendocrine tumour morphological traits [1, 6, 14].

It should be mentioned that detectability of neuroendocrine markers at the cellular level depends also on the type of material fixation, the applied techniques and reagents used, including specific antibodies or genetic probes [1, 13, 113]. When serum concentrations are tested, the potential for falsely positive NSE test results in samples showing haemolysis should be borne in mind (this does not pertain tests for chromogranin A) [31, 90]. Therefore, some of the published test results should be approached with criticism.

Chromogranin A

Chromogranin A (secretory protein I) (CgA) has been best recognised and is most prevalent in neuroendocrine cells of humans and rodents. It belongs to the so called granins or the group of anionic (acidic) soluble glycoproteins which accompany cellular secretion in secretory granules in cells of APUD system [109]. CgA is thought to represent a panendocrine marker. The glycoprotein of 49 kD size is present in dense secretory granules in most of endocrine and several neuroendocrine cells [99]. Due to their potential for release to systemic circulation it is supposed to provide a clinical marker of the tumour [24]. Biological functions of CgA have not been fully recognised. It has been even suggested that it represents a precursor protein, similar to proopiomelanocortin (POMC), for a family of biologically active peptides [22]. At present, CgA is regarded to provide a very sensitive and specific serum marker of tumours of neuroendocrine origin [9]. Role of tests for the marker is stressed in diagnosis of hormonally inactive tumours [28, 63, 84]. Apart of synaptophysin, the marker manifests the most extensive correlation with ultrastructural traits of neuroendocrine differentiation [19, 99]. Also a pronounced correlation has been noted between size of the tumour and elevated serum levels of CgA. As a general neuroendocrine marker, CgA cannot assist in differentiation of tumour subtypes and, in addition, its serum levels rise relatively late in evolution of tumours [63].

In cases of lung tumours, CgA is one of the most frequent markers employed for confirmation of neuroendocrine origin of the tumours. For diagnostic and prognostic purposes the studies pertain both serum levels of the protein and immunocytochemical techniques performed on tissue material [24, 31, 70, 85]. CgA is thought to provide a useful marker, more sensitive in detection of typical pulmonary carcinoids as compared to atypical forms of the tumour and to small cell lung carcinoma. It serves for demonstration of the neuroendocrine phenotype, both at the level of mRNA and at the level of protein in the tumour and also in non-small cell lung carcinomas [1]. A decreased expression of CgA was observed in cases of more malignant forms of cancer. On the other hand, elevated concentrations of CgA in serum used to be demonstrated mainly in small cell lung carcinomas, as compared to patients with NSCLs, patients with other chronic pulmonary diseases and healthy individuals. In more pronounced neoplastic lesions higher concentrations of the protein were disclosed as compared to more restricted forms of the lesions [24, 63, 84]. Some *in vitro* results suggest a potential for common origin of small cell and non-small cell lung cancers since CgA and other neuroendocrine markers have been detected in both types of the tumours [12, 67]. Studies of other authors on 11 cell lines of

non-small cell lung carcinomas failed to confirm expression of CgA, which was described earlier in 7 other lines of the pulmonary tumour [42]. Subsequent comparative studies related to expression of various neuroendocrine markers in pulmonary carcinomas indicated lower role of CgA as a specific marker [1, 81]. In every day pathological diagnosis, detection of CgA was suggested to take advantage of polyclonal antibodies, detecting all proteins of chromogranin family (subtypes A, B and C) [113]. Recent years have brought interesting results stemming from expression analysis of specific CgA epitopes (i.a: vasostatin, chromostatin, chromacin, mid-parastatin) as related to histological subtype of neuroendocrine pulmonary tumour. Out of six specific epitopes of CgA molecule only the expression of the so called chromacin (176–195 AA) was noted in all studied cases of neuroendocrine tumours [68]. Application of an antibody panel for detection of various CgA epitopes and, in addition, of synaptic vesicle protein (SV2) permitted to increase CgA specificity in diagnosis of neuroendocrine and non-neuroendocrine cancers [69]. Moreover, application of *in situ* hybridisation augmented sensitivity of CgA detection in lung tumours [35].

In electron microscopy, chromogranin A is localized in the matrix of secretory granules of various type neuroendocrine cells [82, 99, 100, 105]. In cases of pulmonary tumours, significantly more numerous CgA-positive granules were demonstrated in pulmonary carcinoids, as compared to small cell lung cancers, in which in addition a correlation was noted between CgA immunoreactivity with density of neuroendocrine granules [21, 74]. Application of *in situ* hybridization with ImmunoMax amplification permitted to demonstrate mRNA for the protein in all studied cases of SCLC, amplifying the related diagnostic potential [88].

In light microscopy a variable intensity expression of the protein involved cytoplasm of tumour cells. A particu-
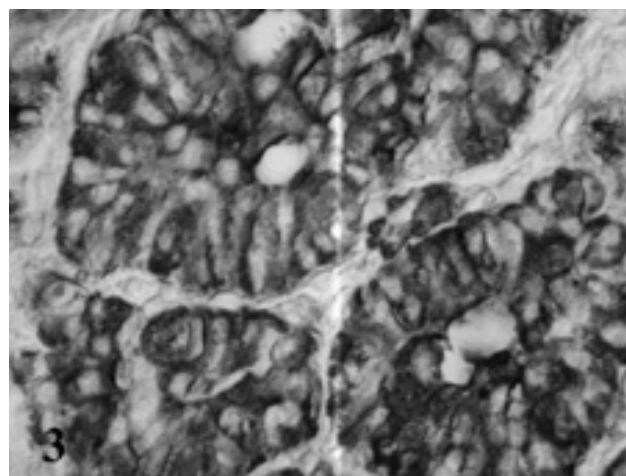

Fig. 3. Immunocytochemical localization of chromogranin A (CgA) in typical lung carcinoid. ABC technique. Nomarski optics. Magn. × 600.

larly intense expression of the protein was noted in typical pulmonary carcinoids (Fig. 3).

Neuron-specific enolase (NSE)

NSE involves a soluble cerebral protein, described for the first time by Moore and McGregor in 1965 [50]. It is a specific isoenzyme of the glycolytic enzyme, enolase (EC 4.2.1.11), currently termed the neuron-specific enolase. It provides a marker not only to neurons of any type but also to all neuroendocrine and paraneuronal cells [50, 113]. Nervous and neuroendocrine cells as well as tumours stemming from the cells provide source of serum NSE, producing the gamma-gamma (γγ) form of enolase (only this form is defined as NSE). Erythrocytes and blood platelets contain the alfa-gamma (α–γ) form of enolase, which may induce falsely positive tests for NSE [48]. Enolase is present also in non-neuroendocrine cells (e.g, in smooth muscle cells, myoepithelial cells, some epithelial cells of kidney, type II pneumocytes) as well as in tumours originating from the cells (e.g, renal carcinomas, some sarcomas). Diagnostic significance of NSE in neuroendocrine tumours remains beyond question even if the NSE-positive tumours have not been fully recognized. Appearance of NSE at late stages of neuronal differentiation caused that it provides a useful marker of neuron differentiation. Moreover, demonstration of NSE in various tumours of nervous system and in tumours of neuroendocrine origin stimulated the research line on usefulness of NSE as another neoplastic marker [31, 70].

Among numerous neuroendocrine markers detected in tissues only NSE is broadly used also as a serum marker [31, 73, 90]. Some authors regard the marker to be most sensitive in the panel of markers used in diagnosis of SCLC [48, 49, 64, 77], although the opposite opinions are also available [52, 80]. Detectability of NSE in SCLC ranges from 50% to 100%, and in cases of non-small cell lung carcinomas no NSE can be detected or the proportion of positive cases may involve a few tens of percents [1, 13, 50, 74, 79]. Studies on NSE in serum and tissue in pulmonary tumours indicate that the protein is important more as a prognostic than a diagnostic marker of neuroendocrine lung carcinomas [29, 38, 76, 80, 97]. According to some authors, increase in NSE following treatment of SCLC may point to relapse of the disease [62]. Elevated serum levels of NSE are noted also in 23-60% patients with non-small cell lung carcinoma. Even if the tumours manifest better prognosis in treatment with cytostatic agents, NSE-positive NSCLC are linked to worse prognosis [11, 29, 73, 90]. In general, elevated NSE concentrations in serum represent an unfavourable prognostic factor, reflecting the size of NSCLC [47]. High serum NSE concentrations in patients with squamous pulmonary carcinomas significantly correlated with aneuploidy, histological

grade 3 and S-phase proportions [73]. However, the positive correlation between higher NSE and the more advanced clinical and histological stage of NSCLC has not always been detected [60]. In general, originally elevated NSE level is thought to represent a negative prognostic index for survival both in SCLC and NSCLC patients [29, 38] but also positive correlations have been demonstrated between serum concentration of NSE and survival of patients with SCLC, subjected to total resection of the primary tumour [56]. Measurements of serum NSE concentration are regarded by many authors to be important in differentiation between NSCLC and SCLC. The role of NSE determinations is stressed in monitoring of results of treatment in small cell lung carcinomas [31, 38, 49, 62, 80]. Lack of normalization of NSE concentration during treatment is linked to poor prognosis irrespectively of clinical response. Comparison of serum concentrations with tissue expression of NSE has demonstrated, as a rule, positive correlations, particularly in cases of carcinoids and small cell lung carcinomas [79]. In significantly lower concentrations, as compared to small cell lung carcinomas, and in parallel with the absence of NSE expression in tissue the marker was detected in sera of patients with other types of lung tumours [79]. Subsequent analyses did not demonstrate significant correlations between histological immunopositivity index for NSE and serum concentrations of the marker. In parallel, the studies confirmed that serum concentrations of NSE and not tissue expression of the marker represented the more significant prognostic index in SCLC [48]. Studies with use of immunocytochemical techniques in various types of pulmonary tumours did not confirm in general extensive specificity and sensitivity of NSE since the marker could be detected independently of neuroendocrine origin of the tumour [2]. According to some authors, proportion of cells immunopositive for NSE in small cell lung carcinoma was definitely higher than in NSCLC (approximately 60–100% and 10–57%, respectively) [13, 55]. In line with the current decisions related to classification of neuroendocrine pulmonary carcinomas and SCLC/NSCLC differential diagnosis, tissue markers other than NSE should be examined [97]. In cytological tests (serous fluid, bronchial biopsies) a similar proportion of NSE positivity is detected in preparations of small cell carcinoma, accompanied by the absence of the marker in NSCLC [86]. In turn, other authors have demonstrated correlations between NSE concentrations in serum and in bronchoalveolar lavage fluid but have concluded that NSE levels should be tested only for research purposes due to their low sensitivity (60%) and low specificity (40%) [52].

In *in vitro* studies significant differences were detected in NSE concentrations in supernatants of cultured cell lines,

SCLC (30.8±22.4 ng/ml) and NSCLC (9.2±8.7 ng/ml), pointing to usefulness of NSE testing in differentiation of neuroendocrine character in pulmonary tumours [56]. Interestingly, positive NSE expression at the tissue levels in small cell lung carcinomas seems to indicate longer survival of the patient [4, 18, 26].

Employing biochemical and immunocytochemical techniques, NSE was localized in cytosol of nervous and endocrine cells and in tumours originating from the cells [66]. Immunocytochemical studies on the level of electron microscope demonstrated presence of NSE in cells of all tumours which contained dense-core neurosecretory granules [74, 100, 104, 108]. At the level of light microscopy, NSE was localized in cytoplasm of tumour cells. A particularly diffuse intense immunocytochemical reaction pertained typical lung carcinoids (Fig. 4).
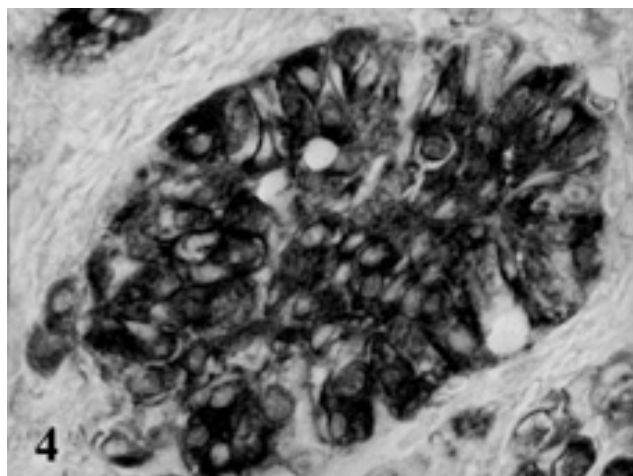

Fig. 4. Immunocytochemical localization of NSE in typical lung carcinoid. ABC technique. Nomarski optics. Magn. × 600.

Synaptophysin

Synaptophysin involves a glycoprotein isolated for the first time from nervous tissue (ox neurons), present in presynaptic vesicles of almost all nerve cells. It represents an integral transmembrane protein of 38 kD molecular weight, described and named first by Wiedenmann et al. in neurons of brain, spinal cord, retina and in neuro-muscular junctions [42, 102]. It has been demonstrated also in adrenal medulla, cells of pancreatic islets and in numerous neuroendocrine tumours [58, 103, 104]. Synaptophysin represents a more specific marker of nervous structure than NSE [103]. At first, the role of the protein has been reduced to formation of synaptic vesicles and to exocytosis. Subsequently, the protein has been shown to behave as calcium-binding protein in membranes of synaptic vesicles [71]. The protein probably produces also canals in membranes of synaptic vesicles and may play role in release of

neurotransmitters. According to some opinions, in neuroendocrine cells synaptophysin may be present not in vesicles but in membranes of secretory granules. Disputes continue on accurate location of the marker at the subcellular level [102]. Employing immunocytochemical techniques, expression of synaptophysin has been demonstrated in several human neuroendocrine cells and in numerous tumours of both nervous and epithelial types. Its expression has been independent from expression of other neuronal markers [33]. Expression of synaptophysin was demonstrated in lung neuroendocrine cells of mouse, rabbit and newborns and in human foetuses [58]. The marker was noted to undergo co-expression with neurofilament protein in nerve plexuses and bronchial nerves while in isolated neuroendocrine cells and neuroepithelial bodies (NEB) of bronchial mucosa it was co-expressed with cytokeratins. Studies showed that continued production of synaptophysin was typical also of neoplastic transformation of several cells even if synthesis *de novo* of the marker in the course of malignant transformation could not be excluded [102, 104]. At present, synaptophysin is thought to represent one of the most specific markers of neuroendocrine cells.

In pulmonary tumours, beginning at carcinoids of low malignant type up to highly malignant metastases-forming neuroendocrine tumours, expression of synaptophysin was not affected by co-expression of other markers, e.g. of serotonin, desmoplakin and was independent of clinical neuroendocrine signs in the patients [58]. Subsequent immunocytochemical investigations confirmed the much higher sensitivity of synaptophysin, as compared to CgA and NSE in pulmonary tumours and their cell lines [42, 81], even of the protein was also detected in 8% to approximately 30% non-small cell lung carcinomas [1, 18, 55]. Among all the examined neuroendocrine markers, the highest detectability of synaptophysin was detected in large cell lung carcinomas with neuroendocrine differentiation (LCNEC) [65]. Studies on synaptophysin expression in cell lines using techniques of molecular biology (RT-PCR) confirmed its production both in cases of SCLC and in NSCLC, but with a distinct intensity [67].

Using biochemical and immunocytochemical techniques, synaptophysin was identified as a component of membrane in pre-synaptic vesicles. Using SY 38 antibodies it was demonstrated on the cytoplasmic side of the vesicles, suggesting that it is an integral protein of the membrane [103]. In electron microscope, synaptophysin was immunolocalized in small, transparent-looking neurosecretory vesicles [105]. A more pronounced tissue expression of synaptophysin in light microscopy using immunocytochemical techniques pertained cytoplasm of tumour cells in both pulmonary carcinoids, SCLC and LCNEC, as compared to NSCLC (our own observations). A definitely more
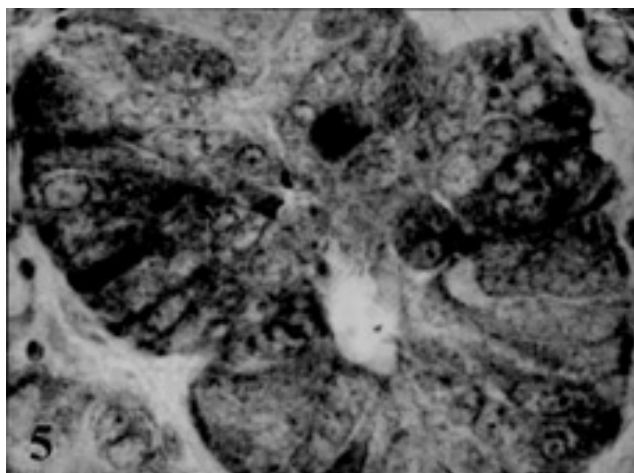
Fig. 5. Immunocytochemical localization of synaptophysin in pulmonary large cell carcinoma. ABC-ImmunoMax technique. Nomarski optics. Counterstained with hematoxylin. Magn. × 600.

pronounced immunocytochemical reaction in detection of the protein can be obtained using amplification with biotinylated tyramine (the ImmunoMax technique) (Fig. 5).

PGP (Protein gene product) 9.5

PGP 9.5 involves a protein isolated at first from tissue extracts of various human organs. It represents 1–2% of soluble cerebral proteins [41]. Standard immunocytochemical techniques at the levels of electron or light microscopy locate the protein in neurons, nerve fibres at all levels of the central and peripheral nerve systems and in numerous neuroendocrine cells [59, 94]. It involves a cytoplasmic polypeptide, 24–27 kD in molecular weight [25, 94]. Even if PGP 9.5 differs in structure and in immunological properties from NSE, it remains frequently co-localized with the enzyme in normal neuroendocrine cells. The two markers can also be interchangeably detected [27, 72]. The respective protein as well as sequencing of cDNA for human PGP 9.5 identified the polypeptide to be identical to L1 (UCH-L1; ubiquitin C-terminal hydrolase) isoenzyme of ubiquitin hydrolase [107]. PGP 9.5 as a neuron-specific peptide removes ubiquitin from ubiquitinated proteins, in this way preventing their degradation in proteasomes [37]. More pronounced deubiquitinization of cyclins by PGP 9.5 may lead to an uncontrolled growth of somatic cells [110]. Expression of PGP 9.5 was demonstrated both in tumours of nervous and/or neuroendocrine origin and in non-neuro-endocrine tumours [72, 75, 91, 92].

A significant expression of the polypeptide is noted in primary lung tumours and cell lines originating from them while its over-expression characterizes more advanced stages of the disease. Thus, PGP 9.5 may be regarded to play some role in carcinogenesis [15, 37, 72]. Interaction with other proteins, JAB1, Jun was demonstrated, mediated by

activation domain, which binds to p27 (Kip1) protein for its degradation. The two proteins (PGP and JAB1) may form p27-containing complexes in cell nuclei of lung tumours. PGP 9.5 may participate in degradation of p27 (Kip1) through interaction and nuclear translocation with JAB1. According to several authors, PGP 9.5 represents a potential oncogene in lung carcinomas since it shares several conservative oncogenic domains with other UCH proteins. In the normal lung in principle no PGP 9.5 gene expression is noted and it begins to be activated in the course of neoplastic transformation in approximately 50% primary lung carcinomas and in almost all cell lines originating from pulmonary tumours [15, 37, 75]. In cases of lung tumours activation of gene expression has not been linked to methylation of PGP 9.5 promoter region, as it was noted in HeLa cell line [15]. Studies on expression of the protein in cell lines of either small cell carcinomas or NSCLC were conducted also as related to the *hASH1* status. Expression of mRNA for PGP 9.5 and of respective protein was noted in both types of pulmonary carcinomas, independently of *hASH1* gene expression or independently of neuroendocrine differentiation [37]. A relationship was demonstrated between augmented expression of PGP 9.5 and more advanced clinical stages of non-small cell lung carcinomas (NSCLC) [37], even if other studies failed to confirm a correlation between PGP 9.5 expression and duration of survival of the patients, their age, sex and histological subtypes of NSCLC [75]. Combined application of immunocytochemistry and RT-PCR demonstrated expression of protein and mRNA for PGP 9.5 in approximately 13% NSCLC, with no indications of preference to squamocellular carcinomas. A higher proportion of cells with PGP 9.5 expression (over 80%) was detected in cytological studies on large cell lung carcinomas as compared to approximately 40% atypical lung carcinoids and 30% SCLC [51].
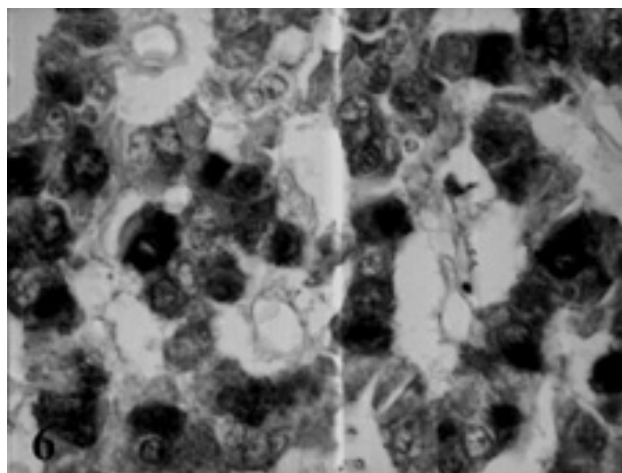

Fig. 6. Immunocytochemical localization of PGP 9.5 in typical lung carcinoid. ABC technique. Nomarski optics. Magn. × 600.

Both at the level of ultrastructure and in light microscopy using immunocytochemical techniques, PGP 9.5 can be demonstrated in general in the cytoplasm of nervous cells and in axons, neuroendocrine cells as well as in tumours originating from the cells [27, 72, 59]. In the cytoplasm of Schwann cells expression of PGP 9.5 was noted only following injury to nervous fibre [59]. Similar to NSE, the protein was detected also in individual cell nuclei in pulmonary tumours [72]. The pronounced, mostly cytoplasmic immunocytochemical reaction can be noted in Fig. 6.

Summary and Conclusions

A significant difficulty in diagnosis of the so called neuroendocrine pulmonary carcinomas reflects the fact that approximately 10–30% non-small cell lung carcinomas demonstrate neuroendocrine phenotype and, thus, expression of neuroendocrine markers at least in a proportion of the cells [14, 19]. Most probably, as pointed by some authors, this reflects common histogenesis of neuroendocrine and non-neuroendocrine tumours [12, 67, 89].

In cases of a tumour suspected in every day pathological practice for its neuroendocrine character, application of immunocytochemical tests is recommended to detect general markers of various subcellular localization and, in turn, application of studies aimed at detection of specific hormones for closer classification of the tumour. Markers most commonly employed in differential morphological diagnosis of pulmonary tumours include: chromogranins, neuron-specific enolase and synaptophysin [113].

Chromogranin A, the so called panendocrine marker, represents one of best recognised proteins of the chromogranin group and one of the most frequently applied markers in diagnosis of neuroendocrine tumours, including lung tumours. Sensitivity and specificity of the marker can be augmented in immunocytochemical studies using a panel of antibodies against various epitopes of the protein [68]. CgA may be released to systemic circulation and elevated serum levels of the protein are observed mainly in small cell lung carcinomas.

Results of studies on neuron-specific enolase (NSE) as a marker of neuroendocrine lung tumours remain controversial. In general, NSE is thought to provide mainly prognostic index both in neuroendocrine and non-neuroendocrine pulmonary tumours. In line with current recommendation of WHO, confirmation of neuroendocrine origin of a pulmonary tumour requires demonstration of tissue expression of at least two neuroendocrine markers distinct than NSE [98]. However, several authors stress the fact that quantitation of serum concentration of the marker and/or of proportion of

immunopositive cells may be helpful in differential diagnosis of pulmonary tumours and may serve for monitoring of treatment in small cell lung carcinomas [76]. In recent studies on practical application of NSE in diagnosis of pulmonary tumours the marker failed to gain approval [52].

Synaptophysin is currently regarded to provide one of the most specific markers of neuroendocrine origin, manifesting a much higher sensitivity than chromogranin A and NSE in diagnosis of lung tumours. Nevertheless, also this protein cannot be accepted as an ideal marker [42, 81].

PGP 9.5 undergoes broad expression in nervous tissue at all stages of nervous differentiation as well as in numerous neuroendocrine cells and was studied as a marker of neuroendocrine differentiation also in oncology [107]. The polypeptide differs in structure and in immunological properties from NSE but the two markers co-localize in several types of normal and neoplastic nerve cells and neuroendocrine cells. Expression of the peptide could not be demonstrated in normal epithelium of respiratory pathways. Activation of respective gene expression was observed in the course of neoplastic transformation both *in vivo* and in cell lines of human lung tumours [15, 37]. Over-expression of PGP 9.5 was shown both in typical neuroendocrine lung carcinomas and in NSCLC, independently of *hASH1* status [37]. PGP 9.5 is regarded to represent a potential oncogene which could participate in uncontrolled proliferation of somatic cells [75]. In recent years the protein is thought to provide a prognostic index, more useful in non-small cell lung carcinomas than in neuroendocrine tumours. Studies on diagnostic significance of the marker in various subtypes of lung tumours continue.

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Address for correspondence and reprints requests to:
Aldona Kasprzak
Department of Histology and Embryology
University of Medical Sciences
ul. Święcickiego 6
60–781 Poznań, Poland
Email: akasprza@amp.edu.pl