

Electron microscopic and immunohistochemical analysis of neuroendocrine features in lung tumors

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Introduction. Pulmonary tumors display several interesting aspects, especially the relationship between the cell biology of lung carcinoma and the response of these neoplasms to therapeutic intervention. The clinical significance of neuroendocrine differentiation in lung carcinomas remains unclear and is widely discussed.

Material and methods. Immunohistochemical and electron microscopic evaluations were performed to assess the correlation between the immunohistochemical and ultrastructural evidence of neuroendocrine differentiation in pulmonary tumors with neuroendocrine features at the histological level. Forty eight surgically treated lung tumors were investigated. All tumors were classified histologically (depending on hematoxylin and eosin staining) as: carcinoid (5 cases), small cell lung carcinoma (2 cases), large cell neuroendocrine carcinoma (4 cases), squamous cell carcinoma (18 cases) and adenocarcinoma (16 cases). These tumors were immunostained with a panel of neuroendocrine markers (chromogranin A, synaptophysin, NSE) and cytokeratin. All of them were ultrastructurally investigated for the presence of neuroendocrine dense core granules.

Results. The presence of neurosecretory granules was directly correlated with immunodetection of neuroendocrine markers: chromogranin A and synaptophysin. Immunoreactivity for NSE was not significant and this marker does not appear to be useful for detecting neuroendocrine features in pulmonary tumors. The results of this study indicate, however, that carcinomas without histologic features of neuroendocrine differentiation may show positive immunoreactivity to investigated markers. Our findings also demonstrate that 32% of adenocarcinomas were positive for chromogranin A and 62% – for synaptophysin; in squamous cell carcinomas 33% were positive for synaptophysin. We did not find any neuroendocrine features at ultrastructural level in this tumors.

Summary. Positive immunoreactivity for selected neuroendocrine markers does not always correlate with light and electron microscopy characteristics of lung tumors.

Mikroskopowo-elektronowa i immunohistochemiczna ocena neuroendokrynności w nowotworach płuc

Wprowadzenie. Celem pracy była ocena mikroskopowo-elektronowa i immunohistochemiczna nowotworów płuc ze szczególnym uwzględnieniem korelacji ultrastruktury guza i reakcji immunohistochemicznych w nowotworach płuca, wykazujących histologiczne cechy neuroendokrynności.

Materiał i metody. Zbadano 48 przypadków nowotworów płuca leczonych chirurgicznie w Centrum Onkologii w latach 1997-1999, w tym 5 przypadków rakowiaka, 2 – raka drobnokomórkowego, 4 – raka wielkomórkowego neuroendokrynnego oraz 18 przypadków raka płaskonabłonkowego i 16 przypadków gruczolakoraka. Wykonano odczyn immunohistochemiczne przeciwciałami skierowanymi przeciwko wybranym markerom neuroendokrynności (chromogranina A, synaptofizyna i NSE) oraz cytokeratynie. Wszystkie guzy poddano analizie w mikroskopie elektronowym pod kątem obecności charakterystycznych ziarnistości neuroendokrynnych w komórkach nowotworowych.

Wyniki. We wszystkich przypadkach, w których w obrazie ultrastrukturalnym stwierdzono obecność ziarnistości neuroendokrynnych, badanie immunohistochemiczne na obecność chromograniny A i/lub synaptofizyny wykazało dodatni odczyn. Wykazano niską swoistość odczynu na NSE. W przypadkach, gdzie obraz histologiczny przemawiał za neuroendokrynnym charakterem guza, stwierdzono ziarnistości neuroendokrynne w obrazie mikroskopowo-elektronowym. Nie stwierdzono na-

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tomiast ultrastrukturalnych wykładników neuroendokrynności w rakach płaskonabłonkowych i gruczołowych, choć część z nich wykazywała dodatni odczyn w reakcjach immunohistochemicznych.

Podsumowanie. W guzach płuc dodatni odczyn immunohistochemiczny na markery neuroendokrynne nie zawsze odpowiada histologicznemu i ultrastrukturalnemu charakterowi tych nowotworów.

Key words: lung carcinoma, neuroendocrine tumors, electron microscopy, immunohistochemistry, chromogranin A, synaptophysin, NSE

Słowa kluczowe: rak płuca, nowotwory neuroendokrynne, mikroskopia elektronowa, immunohistochemia, chromogranina A, synaptofizyna, NSE

Introduction

Lung carcinoma is one of special interest for epidemiologists, genetics, pathologists and clinicians as the most common malignancy observed in developed countries. In spite of generally unfavorable prognosis there are significant differences in biology and responses to treatment depending on histologic type of tumors. The simplest and most widely used method of classification recognizes four major histologic types of lung carcinoma: squamous cell carcinoma, small cell carcinoma, adenocarcinoma and large cell undifferentiated carcinoma [1]. Considering the biology of these tumors, their staging and their response to implement treatment lung carcinomas can be divided into small cell carcinoma and non-small cell carcinomas. This classification is justified in view of genetics, histogenesis, morphology and especially response to treatment. Small cell carcinoma belongs to the group of neuroendocrine tumors which, in the lung, originate from Kulchitzky cells. These cells are normally found in the bronchial and bronchiolar epithelium and mucosa glands [2]. The Kulchitzky cells, like other neuroendocrine cells, are capable of decarboxylation of biogenic amines and of the collection and secretion of endocrine products (NSE, NCAM, synaptophysin and other). Ultrastructural features of these cells are characterized by a presence of dense-core granules in the cytoplasm. These granules measure from 50–450 nm.

Introduction of electron microscopy and immunohistochemistry increased interest and recognition of neuroendocrine neoplasms. For many years only two major categories of pulmonary neuroendocrine tumors were recognized: carcinoid and small cell carcinoma. At present four categories of these tumors are recognized: carcinoid, atypical carcinoid, large cell carcinoma and small cell carcinoma, which comprise a spectrum of malignancies. This classification of lung tumors was included into the new version of the WHO classification, which had been published recently [3, 4]. Three grades of malignancies of pulmonary neuroendocrine neoplasms are recognized: low-grade – carcinoid, intermediate grade – atypical carcinoid and high grade – large cell carcinoma and small cell carcinoma.

To diagnose tumor as belonging to the neuroendocrine family certain microscopic features should be found. They include a specific type of chromatin structure (so called „salt and pepper”), and cell arrangement (rosette and/or palisades). The diagnosis of neuroendocrine character of a tumor should be confirmed by ultrastructural evaluation (the presence of neuroendocrine granules)

and/or positive immunoreactivity for chromogranin A and synaptophysin. Other markers, such as neuroendocrine [5], hormonal and epithelial [6], molecular [7] or so-called „lymphoidal” [8] can be also implemented.

Objective

The aim of the study was to perform electron microscopic and immunohistochemical evaluation of lung tumors in order to assess the correlation between the ultrastructural and immunohistochemical evidence of neuroendocrine differentiation in pulmonary tumors with neuroendocrine features at the histological level in H&E stain slides.

Material

Forty eight tumors of the lung from patients treated surgically at the Department of the Lung and Chest Tumors of the Institute of Oncology in Warsaw, between the years 1997 and 1999 were investigated. Only cases of primary surgical treatment were selected, cases pretreated with chemotherapy or radiotherapy were not included in the study.

Methods

Light microscopy. For light microscopy analysis all tissue specimens were formalin fixed and embedded in paraffin. The sections were routinely stained with hematoxylin and eosin and then examined for histologic diagnosis. The tumors were classified according to the WHO system [1, 4] and the specimens for immunohistochemical investigation were selected.

Immunohistochemistry. The identification of chosen markers was determined by immunohistochemical staining with the monoclonal, affinity-purified antibodies (DAKO, Denmark): anti-chromogranin A, DAK-A3 clone (kat.no M0869), anti-NSE, BBS/NC/VI-H14 clone (kat.no. M0873), anti-cytokeratin, MNF 116 clone (kat.no. M0821) and poliklonal: anti-synaptophysin (kat.no. A0010). After deparaffinization and rehydration, the slides underwent pretreatment microwave boiling in 0,01 M citric acid buffer, pH 6,0 (600 W for 12 min.). Endogenous peroxidase activity was blocked with 3% H₂O₂. Incubation with specific antibodies diluted 1:50 in TRIS-buffered saline (TBS) pH 7,6 supplemented with 1% bovine albumin (fraction V, Sigma, kat.no A-9647) was performed for 60 min. at room temperature in a moist chamber. The reactivity of primary antibodies was detected with streptavidin-biotin complex peroxidase technique with LSAB-kit (DAKO, LSAB Universal Plus KIT/HRP, kat.no K0690). The reaction product was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAKO, DAB+ Substrate Chromogen, kat.no K3468) as chromogen. Finally, the sections were lightly counterstained with hematoxylin.

For evaluation an immunohistochemical staining 4-steps scale from (-) to (+++) was used; (-) was categorized as nega-

tive; (+++) categorized as strongly positive staining of tumor cells.

Electron microscopy. For ultrastructural investigations small samples of tissue taken from resected pulmonary tumors were immersed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 and postfixed in 1% osmium tetroxide with 0.8% potassium ferrocyanide (Sigma, kat.no P-3289), dehydrated in a graded series of ethanol and embedded in Epon 812. Semithin 1 μ m sections were stained with toluidine blue and viewed under Olympus BX60 light microscope. Ultrathin sections were contrasted with uranyl acetate and Reynold's lead citrate and examined under Philips CM 120 BioTWIN transmission electron microscope.

Results

Histopathology

Basing on H&E stained slides histological diagnosis had been established: squamous cell carcinoma – 18 cases, adenocarcinoma – 16 cases, small cell carcinoma – 2 cases, carcinoid – 5 cases and large cell carcinoma with histologic neuroendocrine features – 5 cases. Three tumors have presented mixed structures.

In 2 cases adenocarcinoma and squamous cell carcinoma structures have been found and 1 case had presented mixed subtype of small cell carcinoma (small cell and adenocarcinoma, Fig. 1a). Based on light microscopic features 2 of 5 carcinoids were classified as typical carcinoids. The tumor cells had presented regular tissue arrangement; no mitotic figures or necrosis were present (Fig. 1b). The remaining 3 tumors were diagnosed as atypical carcinoids, with tumor cells presenting some polymorphism, mitotic figures and small area of necrosis (punctate necrosis). In 4 cases of non-small cell carcinoma microscopic features presented organoid arrangement of tumor cells with extensive necrosis and palisade arrangement of tumor cells at the periphery of solid areas of tumor growth. These tumors were classified as non-small cell

carcinoma with neuroendocrine features. The number of tumors in each histologic category is shown in Table I.

Male constituted 61 % of the patients. The mean age of patients was 60 years.

In the majority of patients (65%) their symptoms were related to respiratory tract and in 2 patients symptoms were related to brain metastases (headache and dizziness). In 1 case of carcinoid the patient presented Cushing's syndrome due to ectopic secretion of ACTH. Eleven patients were asymptomatic.

Immunohistochemistry

Strong positive or positive staining for cytokeratin has been found in 44 out of the 48 analyzed cases. Strongly positive staining for NSE has been seen in 29 of 48 cases, weakly positive in 12 and negative in 7 cases. It has been found that 77% of squamous cell carcinomas and 81% of adenocarcinomas were positive for this neuroendocrine marker (Fig. 1c). It should be stressed that all carcinoids and small cell carcinomas has been positive for NSE.

Immunoreactivity for chromogranin A has been strongly positive in 5 cases, weak in 9. All others were either negative or doubtful. A strongly positive reaction for this marker was found in 2 typical carcinoids, 2 atypical carcinoids and 1 adenocarcinoma. Weak reaction has been found in 1 atypical carcinoid, 1 small cell carcinoma, 4 adenocarcinomas and 3 non-small cell carcinomas with neuroendocrine features. One small cell carcinoma and one non-small cell carcinoma has been negative for chromogranin A.

Immunoreactivity for synaptophysin has been considered as strongly positive in 16 cases. The strong reaction for this marker has been observed in all carcinoids but also in 3 squamous cell carcinoma and 6 adenocarcinomas (Fig. 1d). Weakly positive reaction for synaptophysin was found in 1 atypical carcinoid, 1 small cell car-

Tab. I. Pulmonary carcinomas. Immunoreactivity for selected neuroendocrine markers does not always correlate with light and electron microscopy characteristics of lung tumors

Histological type of tumor	No. of cases	No. of cases positive for (weak/very strong)			CK	Presence of NE granules in the cytoplasm
		ChrA	Syn	NSE		
Squamous cell carcinoma	18	0/0	3/3	3/11	4/13	0
Adenocarcinoma	16	4/1	4/6	4/9	3/12	0
Typical carcinoid	2	0/2	0/2	0/2	0/2	2
Atypical carcinoid	3	1/2	1/2	0/3	1/2	3
Small cell carcinoma	2	1/0	1/1	0/2	0/2	2
Large cell neuroendocrine carcinoma	4	3/0	3/1	4/0	1/2	4
Combined small cell carcinoma	2	0/0	0/0	0/2	1/0	0
Clear cell carcinoma	1	0/0	0/1	1/0	1/0	0
Total no. of cases	48	9/5	12/16	12/29	11/33	11

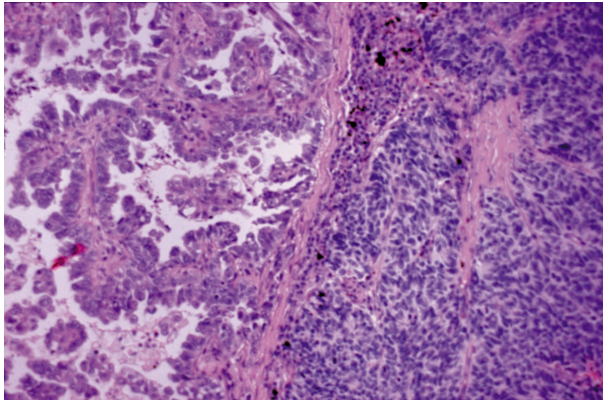


Fig.1a. Combined small cell carcinoma. A mixture of small cell carcinoma and adenocarcinoma may be seen (H&E)

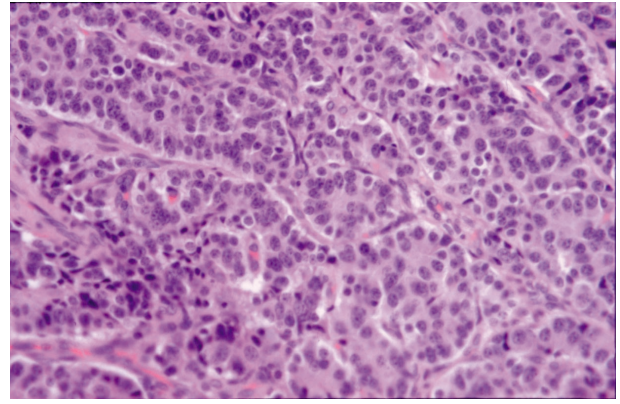


Fig. 1b. Typical carcinoid. Please note the organoid arrangement of tumor cells (H&E)

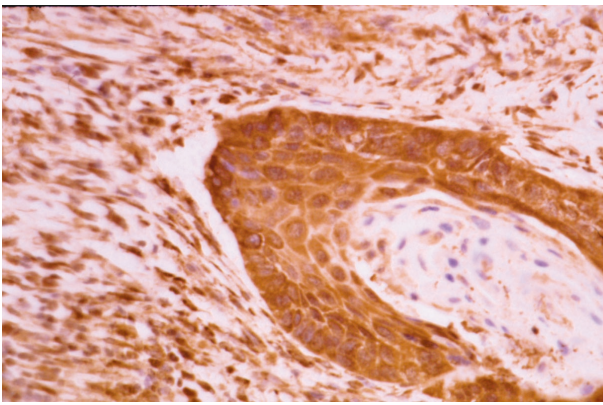


Fig. 1c. Positive reaction for NSE in squamous cell carcinoma

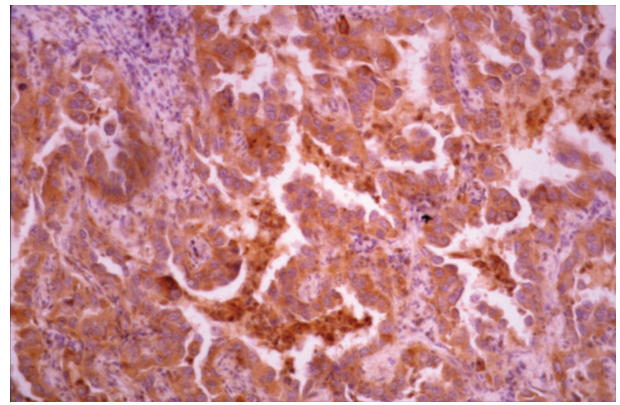


Fig. 1d. Positive reaction for synaptophysin in adenocarcinoma

cinoma, and 3 non- small cell carcinomas with histologic neuroendocrine features.

Electron microscopy

On the ultrastructural level typical neuroendocrine granules have been found in small cell carcinomas, all carcinoids (typical and atypical) and in all non-small cell carcinomas with neuroendocrine features.

Cells of small cell carcinomas contained neuroendocrine dense-core granules. These granules were present in small number and occasionally aggregated in one pole of a cell. They were round or oval and ranged between 120–250 nm in size. Electron-density of these granules were relatively variable in our material. It was often difficult to distinguish them from primary lysosomes. The cells has scanty cytoplasm poor in intracytoplasmic organelles, confined to a few mitochondria and small cisternae of cytoplasmic reticulum. Occasionally, a few small desmosomes with thin bundles of tonofilaments were observed in neighboring cells.

Neuroendocrine granules were numerous in large cell carcinoma (Fig. 2a). They were round, uniform, but usually smaller (approximately 120–150 nm in diameter) and their limiting membranes were separated by a narrow halo from the dense core. Their morphology was different from primary lysosomes; they were rather numerous in this carcinoma. Sometimes tonofilament bundles close

to dense core granules were found (Fig. 2b). Cells of large cell carcinoma were polymorphic and large in size. They possessed cytoplasm rich in organelles, well-developed Golgi system, numerous mitochondria and lysosomes. Intracytoplasmic lumina lined by small microvilli and less numerous desmosomal attachments have been found.

Cells of carcinoids, both typical and atypical (Fig. 2c) contained dispersed, numerous neuroendocrine granules that vary considerably in shape (from round to oval) and size (from 100 to 350 nm). The density of the cores was variable, but most of them had high electron density. Sometimes, in one cell two kinds of dense-core granules have been found. In some cells of typical carcinoid the cytoplasm was filled with mitochondria which were closely packed and possessed oncocytic features (Fig. 2d). Some desmosomes and few tonofilament bundles had been found. Occasionally, nests of tumor cells were enclosed by a basal lamina. Cytoplasm of carcinoid tumor cells contained well-developed rough endoplasmic reticulum. Some cells had shown intermediate filaments and intracytoplasmic lumina with smooth surface. Cells of atypical carcinoids presented greater nuclear polymorphism. Neuroendocrine granules were fewer in number than in typical carcinoid cells but a similar spectrum of granule size has been seen.

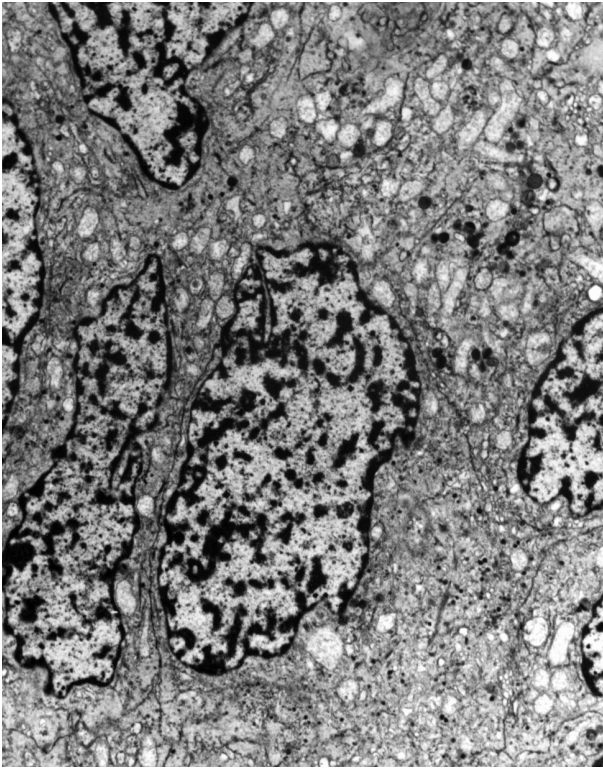


Fig. 2a. Non-small cell carcinoma with neuroendocrine morphology (4600x)

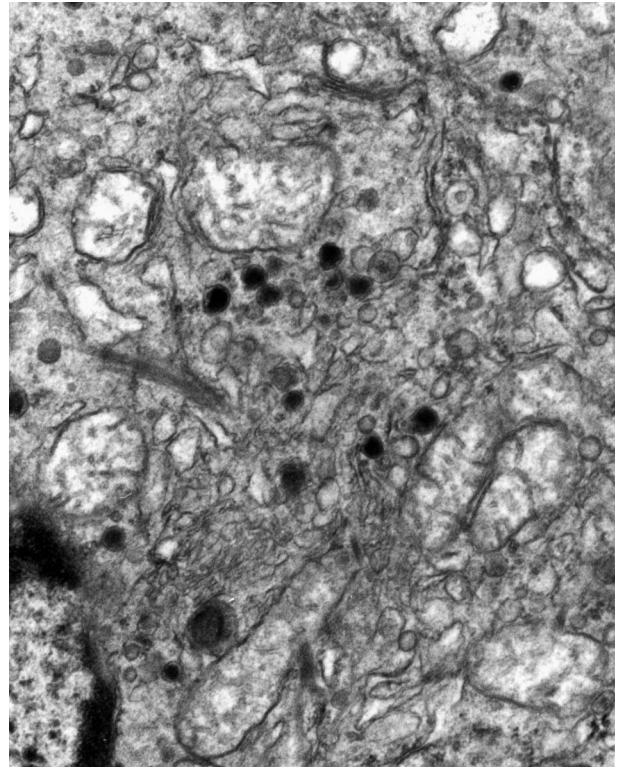


Fig. 2b. Neuroendocrine granules and tonofilaments in a cell of non-small cell carcinoma with neuroendocrine morphology (22000x)

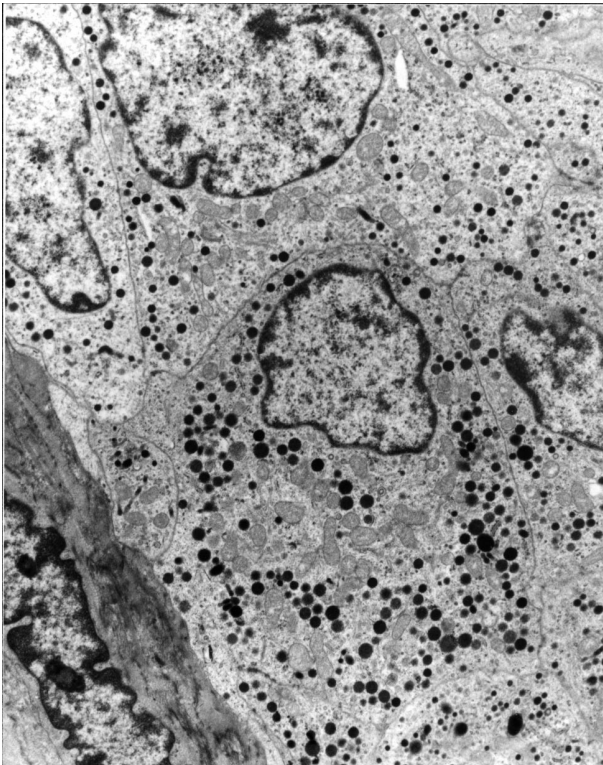


Fig. 2c. Typical carcinoid. Numerous neuroendocrine granules may be seen in the cytoplasm of tumor cells (6200x)

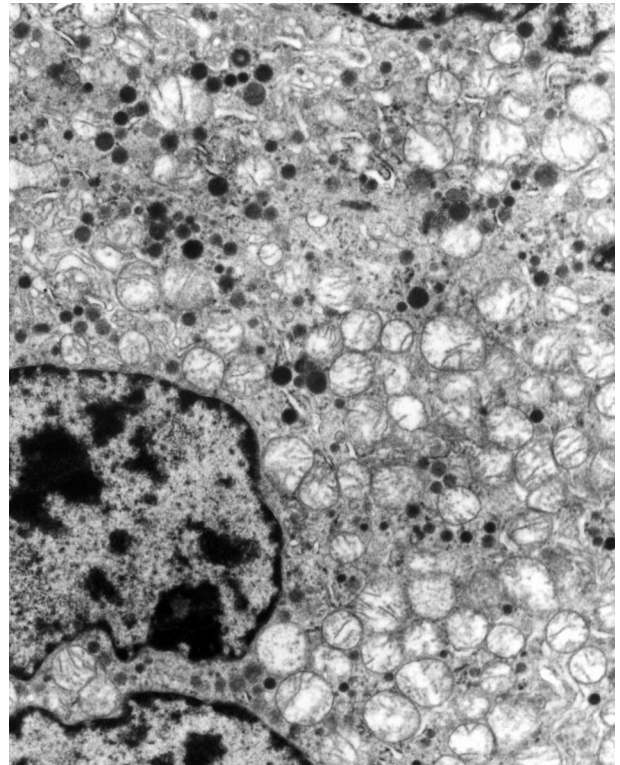


Fig. 2d. Cell of atypical carcinoid with oncoctic features (9000x)

Among 48 cases of lung tumours investigated by electron microscopy ultrastructural features of neuroendocrine differentiation have been found in 11 cases. In our material neuroendocrine granules in squamous cell

carcinomas and adenocarcinomas were not identified, even in tumors with immunohistochemical positivity for one or more markers used to detect neuroendocrine differentiation in lung carcinomas.

Discussion

Our results indicate that positive immunoreactivity for selected neuroendocrine markers does not always correlate with light and electron microscopy characteristics of lung tumors. We have found that all cases with neuroendocrine features on light and electron microscopy levels were supported by immunoreactivity for some neuroendocrine markers. However, it should be stressed that a substantial percentage of tumors with positive neuroendocrine reactivity have been found to be non-neuroendocrine by histological standards. Among adenocarcinomas 32% of the tumours had been positive for chromogranin A, and 33% of squamous cell carcinoma while 62% of adenocarcinomas were positive for synaptophysin. Similar results were reported in literature. Loy and co-workers [5] have found positive immunoreactivity in 79% of cases, for at least one marker (including NSE) in carcinomas which did not show neuroendocrine features on light or ultrastructural levels. Addis and co-workers [9] have reported up to 35 % positive immunoreactivity in non-small cell carcinomas, mostly squamous cell carcinoma.

Use of special techniques like electron microscopy, immunohistochemistry and molecular biology techniques is useful in the evaluation of lung neuroendocrine tumours, but still it may not be the basis for their classification [10]. Considering immunohistochemistry the most reliable neuroendocrine markers are chromogranin A, synaptophysin, Leu-7 and PGP 9.5. Therefore the use of a panel of these markers is recommended [11]. Based on literature data and our results NSE seems to be a marker of low value in differential diagnosis. This marker is positive in almost all neuroendocrine tumours, but also positive in a high percentage of non-neuroendocrine neoplasms [12, 13].

In general, commercially available antibodies used as neuroendocrine markers are not specific enough to diagnose pulmonary neuroendocrine carcinomas [5]. It should be taken into consideration in differential diagnosis in cases when it may lead to therapeutical decisions.

The present classification of lung tumors is based on histological characteristic found in H&E stained material [1, 14]. The microscopic features of carcinoid, small cell carcinoma, and to some extent of atypical carcinoid are very characteristic. The tumor cells present typically a fine and coarse granular chromatin pattern (salt & pepper), delicate nuclear membrane and scanty cytoplasm. Presence of cellular atypia, numerous mitotic figures and prominent necrosis allows for an easy differentiation of small cell carcinoma from carcinoid. The diagnostic problems do exist in low differentiated tumors, in which neuroendocrine differentiation indicates a different biology of a neoplasm with more aggressive outcome.

Most of small cell carcinomas of the lung show neuroendocrine differentiation on immunohistochemistry. However, it has been noted that even 25–30% of these tumors may be negative for neuroendocrine markers even when using panel of antibodies [15]. On the other hand some 10–15 % of non-small cell carcinomas without histo-

logical features of neuroendocrine differentiation are positive for neuroendocrine immunohistochemical markers [6]. These data indicate a critical approach to the immunohistochemical tests and warrant to apply ultrastructural evaluation in difficult and doubtful cases. If a low differentiated tumor requires three or more antibodies to determine the diagnosis, electron microscopy seems to be a less expensive modality [16]. Different authors present, however, the absence of neuroendocrine granules on ultrastructural evaluation from 5–35% of small cell carcinomas [17]. On the other hand, neuroendocrine granules have been found in all typical and atypical carcinoids [10].

It is suggested that the presence of neuroendocrine differentiation may have therapeutical and prognostic value. These opinions are not shared by all authors [18]. On immunohistochemical evaluation neuroendocrine differentiation may be found in more than 30% of non-small cell carcinomas of the lung. In those tumors positive correlation has been found between the presence of neuroendocrine markers and the response to chemotherapy. It is suggested that this observation should be considered while undertaking therapeutical decisions [12]. Schleusener and co-workers [19] have noticed that the presence of one or two neuroendocrine markers correlated with a longer survival time in the patients treated by chemotherapy even in high clinical stage (IIIA, IIIB, IV).

From the practical point of view for the appropriate treatment (and staging) of patients with lung tumors the separation of small cell carcinoma from non-small carcinoma is of great significance, especially considering the clinical consequences of neuroendocrine differentiation in tumours without microscopic neuroendocrine features. Further studies are needed to determine whether these markers have any value in predicting tumour behavior or response to therapy. In their conclusions, Hua and co-workers [20] have suggested to search for new markers, e.g. so called "lymphoidal" markers.

Conclusions

1. Immunoreactivity to cytokeratin and NSE is positive in a vast majority of lung tumours. NSE reactivity is not specific enough to differentiate between neuroendocrine and non-neuroendocrine lung carcinomas.
2. Immunoreactivity for chromogranin A and synaptophysin allows to confirm the neuroendocrine character of the tumor suggested in light microscopy after H&E stain.
3. Immunoreactivity for chromogranin A and synaptophysin was positive in all cases in which neuroendocrine granules on electron microscopic evaluation were found.
4. In those cases in which light microscopy features are characteristic for neuroendocrine morphology electron microscopy the study revealed the presence of neuroendocrine granules.
5. In all evaluated cases electron microscopy investigation does not reveal neuroendocrine granules in adenocarcinomas and squamous cell carcinomas, even in tu-

mors positive for neuroendocrine markers in immunohistochemistry.

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