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ORIGINAL ARTICLE

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Immunohistochemical analysis of the proliferative activity of neuroendocrine tumors from various organs

Are there indications for a neuroendocrine tumor–carcinoma sequence?

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Abstract Small-cell neuroendocrine carcinomas (NECs) of the prostate are believed not to derive from benign orthotopic NE epithelial cells. Instead, an origin from a putative stem cell is actually the most favored concept. Whether this concept can also be applied to neuroendocrine tumors (NETs) of other organs, especially whether there are indications for well-differentiated NET–NEC sequence, is subject of the present study. A double-labeling technique for the proliferation marker MIB-1 and the NE markers chromogranin A (ChrA) and synaptophysin (SNP) was used for the immunohistochemical analysis of 45 well-differentiated NETs, 16 well-differentiated (low-grade) NECs, and 63 high-grade NECs of the esophagus, stomach, small intestine, appendix, colon, lung, prostate, and urinary bladder. The lowest proliferative activity was found in NETs (0.85% of tumor cells), and the highest activity was found in high-grade NECs (72.5%). The expression of ChrA was highest in NETs and lowest in high-grade NECs. None of the NETs and only sporadic cells in low-grade NECs showed double labeling (up to 0.05%). Up to 50% of the tumor cells in high-grade NECs were positive for MIB-1 and SNP. The percentage of double-labeled cells ranged between 0.9 and 39.6 (mean 9.7). No double-labeled cells were found in the normal epithelium adjacent to the tumors. Transitions from NET to NEC could not be observed. NETs and low-grade NECs differ in their proliferative activity from high-grade NECs, suggesting that they may arise from different precursor cell populations.

Keywords Neuroendocrine tumors · Neuroendocrine carcinoma · Low grade · High grade · Cell proliferation

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Introduction

Our knowledge about the histogenesis of neuroendocrine tumors (NETs) is incomplete and includes theories that vary from organ to organ [3, 6, 10, 12, 18, 19, 20, 22]. NETs and well-differentiated neuroendocrine carcinomas (low-grade NECs) are believed by some authors to arise from orthotopic NE cells of the epithelium of the respective organs [6, 17, 18]. High-grade NECs (poorly differentiated) are believed not to derive from benign orthotopic neuroendocrine cells. Instead, an origin from a putative stem cell is currently the most favored concept [5, 13, 14]. This concept was studied in detail in carcinomas of the prostate with focal NE differentiation and in small cell NECs of the prostate [13, 14]. Whether NETs or low-grade NECs are able to transform into high-grade NECs (NET–NEC sequence) is unknown. If tumor groups can be separated from each other on the basis of their proliferation activity, they most likely represent different categories. If, however, the tumors proliferative activity overlaps, a differentiation into different groups appears to be unjustified. In order to test our hypothesis that NETs and low-grade NECs are different from high-grade NECs and probably derive from different cell sources, we have studied the proliferative activity of NETs and low- and high-grade NECs using the proliferation marker KI67/MIB-1 in combination with immunostaining for the NE markers chromogranin A (ChrA) and synaptophysin (SNP) [2, 5, 7, 8].

Methods

We studied 124 NETs classified according to Solcia et al. [23]. The tumors were localized to the esophagus (n=7), stomach (n=13), duodenum/small intestine (n=17), appendix (n=15), colon/rectum (n=8), pancreas (n=3), lungs (n=31), prostate (n=18), and urinary bladder (n=12; Table 1). After fixation in 4% buffered formalin and paraffin embedding, 4-µm-thick tissue sections were cut and stained for hematoxylin and eosin (HE) and for immunohistochemistry (IHC) analysis.

For the demonstration of NE differentiation, the markers ChrA (Camon; Wiesbaden, Germany; 1:1) and SNP (Biogenex; Hamburg, Germany; 1:100) were used. The proliferative activity was

evaluated with the MIB-1 antibody (Dianova; Hamburg, Germany; 1:50). Incubation time for all primary antibodies was 60 min. Before the application of MIB-1, slides were pretreated by means of microwave antigen retrieval (three times 600 W for 5 min). All IHC reactions were developed with the avidin–biotin-enhanced immunoperoxidase technique. Tonsillar tissue for Ki-67/MIB-1 and intestinal tissue for ChrA and SNP served as positive controls. The primary antibody was omitted for negative controls. In order to simultaneously analyze the proliferative activity and the expression of NE markers in tumor cells, we performed double stainings for ChrA and Ki-67/MIB-1. Ki67/MIB-1 staining was performed as described above, and ChrA was detected in a second staining sequence using the avidin–biotin complex method and 3-amino-9-ethylcarbazole (AEC; Dako; Hamburg, Germany) as a second chromogen. A case of malignant melanoma served as a positive control for this double-labeling method (MIB-1 and HMB 45).

All IHC-stained sections were assessed by one of us (B.H.). We analyzed 2000 cells from different areas of two histological slides for each immunostaining (in an area of highest staining intensity). The nuclear Ki67/MIB-1 labeling index (LI) was expressed as percentage of labeled cells. The staining intensity of ChrA/SNP was classified into four groups: 1–25% (+), 25–50% (++); 50–75% (+++); and 75–100% (++++). The number of Ki67 and ChrA double-positive cells was expressed as percentage of (1) all MIB-1-labeled cells and (2) as percentage of all ChrA positive cells. The differences of percentages of expressed NETs and NECs low/high-grade tumors were analyzed using the Student's *t*-test.

Results

Well-differentiated NETs of the appendix measured less than 2 cm (range 0.8–2.6 cm, mean 1.4 cm) without extension into the mesoappendix. Two NETs measured 1.5 cm and 2.6 cm in diameter, with extension into the mesoappendix. The mean size of NETs from the other organs (stomach, ileum, and rectum) was 0.8 cm (range 0.1–1.0 cm) without angioinvasion. Low-grade NECs were predominantly localized in the ileum. Of 12 NECs, six measured 1.2–1.5 cm (mean 1.3 cm). The size of the other six tumors ranged from 2.9 cm to 9.0 cm (mean 3.95 cm). Of 12 low-grade NECs, five metastasized to the regional lymph nodes (n=3) and to the liver (n=2). High-grade NECs were diagnosed in needle biopsy tissue of the lung, prostate, urinary bladder, pancreas, and liver. Biopsy material was collected from the esophagus and stomach. Complete tumors measuring 5–6 cm were diagnosed after resection of the stomach, the colon, the rectum, and the ovaries. The survival time of patients with high-grade NECs of the lung, the prostate, and the urinary bladder ranged between 5.5 months and 5.7 months. The death rate [death on disease (dod)] in patients with those high-grade NECs ranged between 60% and 70% (mean 62.5%).

Orthotopic epithelial NE cells of the prostate, stomach, small intestine, and appendix

ChrA/SNP-positive cells of the normal epithelium of the stomach, small intestine, and appendix adjacent to the tumor and of benign prostatic glands adjacent to the tumor were negative for MIB-1.

Well-differentiated NETs (carcinoids)

All NETs were strongly positive for ChrA/SNP (75–100%). The MIB-1 LI was very low (range 0.5–0.95%, minimum 0.1, maximum 2.0%). There was no significant difference between the mean MIB-1 LI of the NETs from different organs. Double-labeled cells with ChrA and MIB-1 were not demonstrable (Fig. 1, Table 1, and Table 2).

Well-differentiated NECs (low-grade)

Relative to the expression pattern in NETs, low-grade NECs showed a less homogeneous ChrA expression pattern (Fig. 2a), whereas there was no difference in the in-

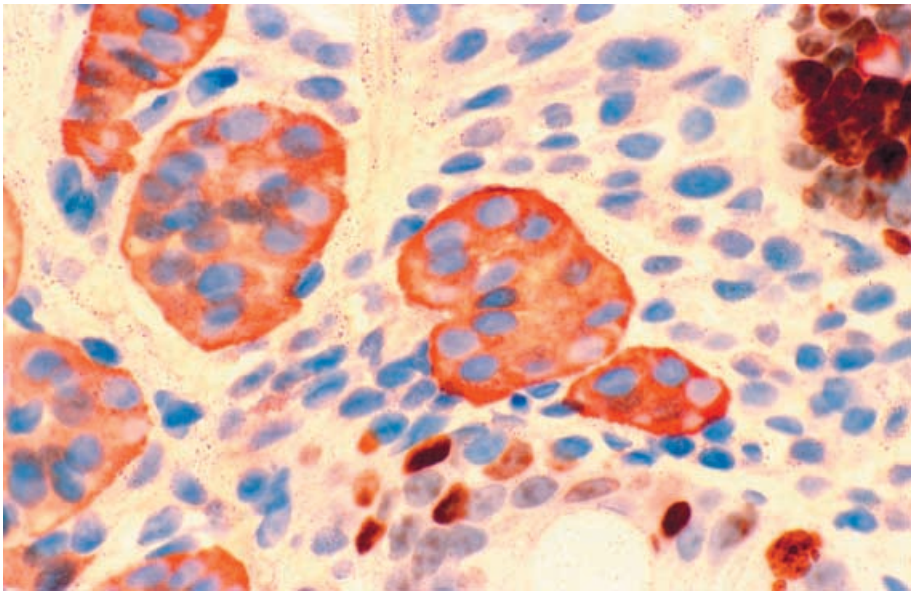

Fig. 1 Tumor cells of a well-differentiated neuroendocrine tumor (typical carcinoid) immunostained for chromogranin A (*red cytoplasm*) and MIB-1 (Ki67 antigen; *brown nuclei*). There are no double-labeled cells; 1:1000

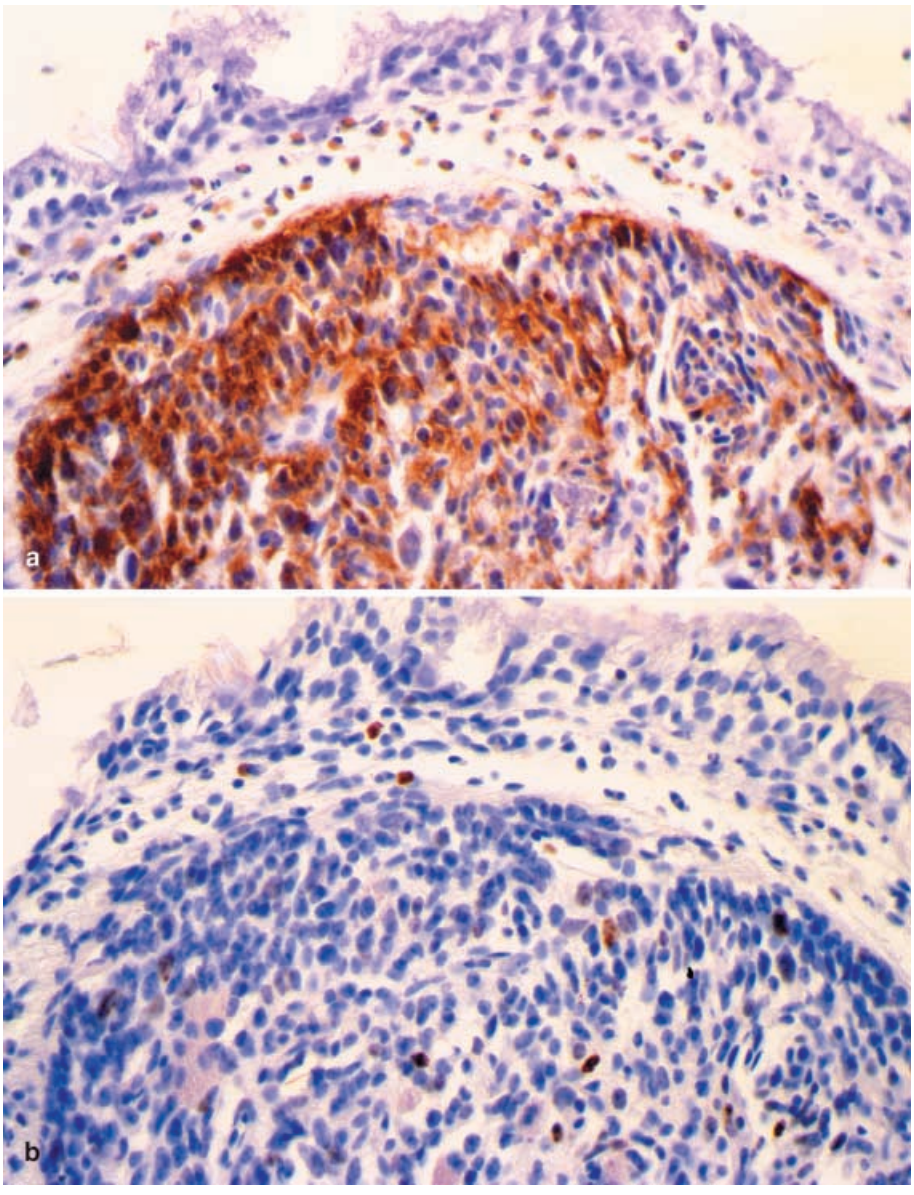

Fig. 2 Well-differentiated neuroendocrine carcinomas (NEC; atypical carcinoid) of the bronchus. **a** Extensive expression of chromogranin A; 1:400. **b** Very low labeling for MIB-1 (Ki67 antigen); 1:400

Table 1 Neuroendocrine marker expression and proliferative activity in well-differentiated neuroendocrine tumors (NETs), well-differentiated neuroendocrine carcinomas (low-grade NECs) and poorly differentiated carcinomas (high-grade NECs). *ChrA* chromogranin A; *SNP* synaptophysin; *MIB-1* Ki67 antigen

	ChrA			SNP			MIB-1 (%)		
	NET	NEC low	NEC high	NET	NEC low	NEC high	NET	NEC low	NEC high
Esophagus	3+	2+	1+	3+	3+	3+	0.8	6.4	71.5
Stomach	4+	2+	1+	4+	3+	3+	0.5	2.5	72.3
Small intestine	3+	2+	1+	3+	3+	3+	0.7	17.2	72.3
Appendix	4+	2+		4+	3+	0	0.9	11.8	0
Colon	3+	2+	1+	3+	3+	3+	0.8	3.3	67.0
Pancreas	3+	2+	1+	3+	3+	3+	0.5	7.2	87.9
Lungs	3+	2+	1+	3+	3+	3+	0.9	5.6	66.3
Prostate			1+	0	0	3+	0.2	1.7	83.5
Urinary bladder	0	0	1+	0	0	4+	0	0	78.2

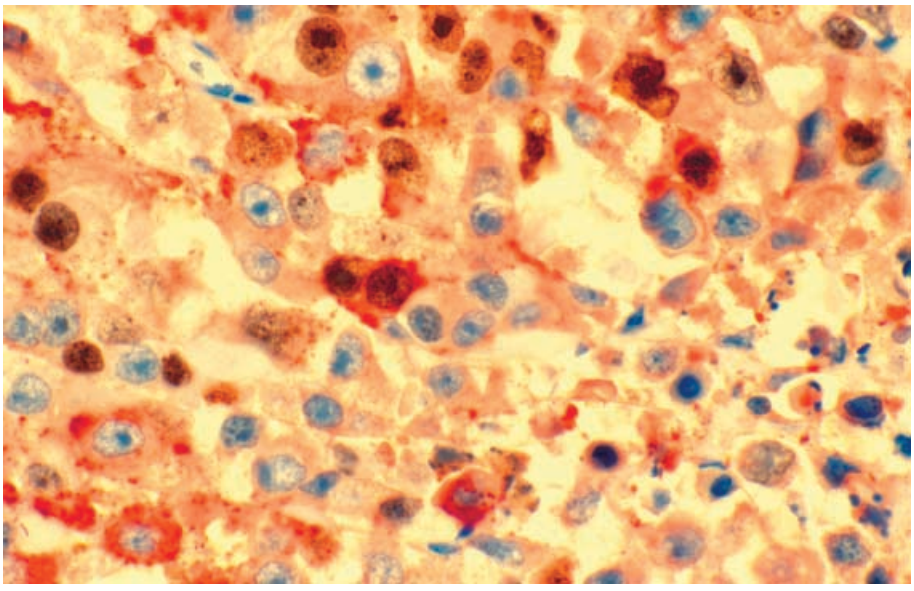

Fig. 3 Poorly differentiated neuroendocrine carcinoma (NEC) with double-labeled cells. Immunostaining for chromogranin A (*red cytoplasm*) and MIB-1 (Ki67 antigen; *brown nuclei*); 1:1000

Table 2 Percentage of tumor cells in low- and high-grade neuroendocrine carcinomas (NECs), double labeled either for MIB-1 (Ki67 antigen) and chromogranin A (ChrA) in relation to all MIB-1 positive cell nuclei or MIB-1 and ChrA in relation to all ChrA positive cells

	MIB-1/MIB-1 + ChrA (%)			ChrA/MIB-1 + ChrA (%)
	NET	NEC low	NEC high	NEC high
Esophagus	0	0	1.1	7.8
Stomach	0	0.01	0.9	12.7
Small intestine	0	0.05	1.6	9.8
Appendix	0	0	0	0
Colon	0	0	1.7	28.7
Pancreas	0	0	1.8	4.7
Lungs	0	0	2.3	39.6
Prostate	0	0	2.7	19.9
Urinary bladder	0	0	2.7	17.8

tensity of SNP expression. About 50–75% of the tumor cells were positive for ChrA and more than 75% were positive for SNP (Table 1). The decrease in ChrA staining was paralleled by an increase in the MIB-1 LI, which varied from organ to organ. The highest values were found in gastric low-grade NECs (mean 33.6%). In all other organs, the mean MIB-1 LI ranged between 1.7% and 17.2% (Fig. 2b and Table 1). Few cells of low-grade NECs of the stomach and small intestine coexpressed ChrA and MIB-1 (range of 0.01–0.05%, mean 0.03%; Table 2). The NE cells within the adjacent benign epithelium lacked double labeling.

High-grade NECs

High-grade NECs showed a variable ChrA expression pattern. Most cases stained only weakly for ChrA, especially in small cell carcinomas of the lung and the prostate. The SNP staining was more homogeneous and varied between 50% and 75% (Table 1). The MIB-1 LI was very high and ranged from 66.3% to 87.9% (Table 1). Many tumor cells in the lung, stomach, colon, prostate, and urinary bladder coexpressed ChrA and MIB-1 (range 0.9–39.6%, mean 9.7%; Fig. 3 and Table 2).

Discussion

This study shows that there is a significant difference in proliferative activity determined by MIB-1 LI between NETs and high-grade NECs [4, 9, 15]. The site of origin of the tumor had no influence. Differences were, however, found for the NE markers. ChrA expression decreased with increasing malignancy, being highest in NETs (80–90% LI) and lowest in high-grade NECs, with only some isolated tumor cells positive for ChrA [1, 2, 16, 17, 20, 21, 22]. The staining intensity of SNP was strong in NETs and in high-grade NECs, with 50–75% of the tumor cells showing positivity for SNP. Cells in NETs did not demonstrate coexpression of ChrA and MIB-1. Few double-labeled NE tumor cells were seen in low-grade NEC of the stomach and intestine (0.1–0.5%). In high-grade NEC, a mean of 9.7% of the tumor cells coexpressed ChrA and MIB-1 (range 0.9–39.6%). In view of the very scanty expression of the MIB-1 marker and the very high expression of SNP and ChrA in NETs and low-grade NECs, the low or missing incidence of double-labeled cells is not surprising.

Orthotopic benign NE cells of the gastro-intestinal mucosa and the prostate were always MIB-1 negative, whereas many non-NE epithelial cells were strongly MIB-1 positive, especially those of the gastro-intestinal mucosa adjacent to the tumor. Double-labeled cells were not seen. These results correspond to findings in the prostate, which showed that ChrA positive cells of the prostate represent postmitotic cells [5]. Therefore, it is unlikely that NETs derive from benign NE cells from the

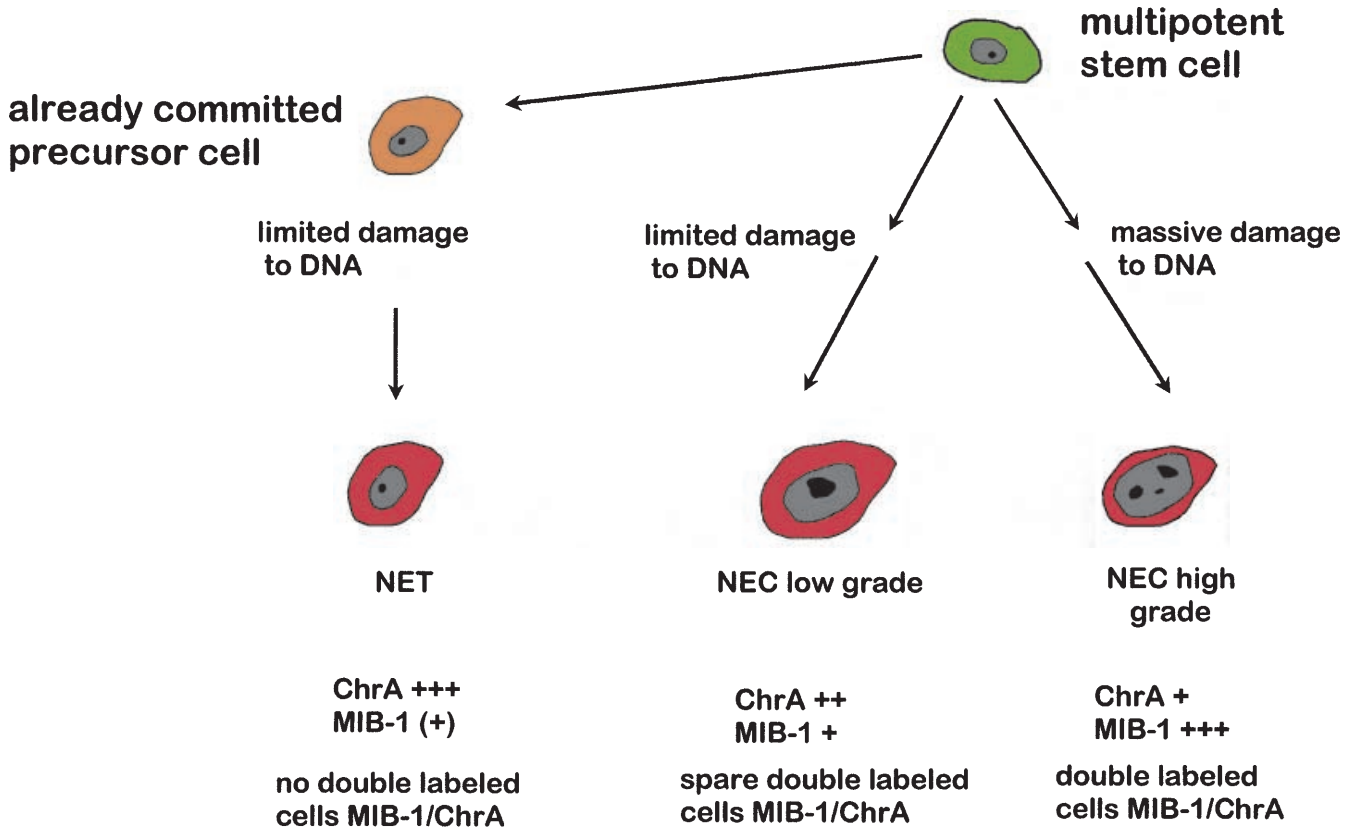

Fig. 4 Hypothetical concept of the origin and development of well-differentiated neuroendocrine tumors (NET) and neuroendocrine carcinomas (NEC)

nonmalignant epithelium adjacent to the tumor without any sign of mitotic activity [13, 14].

Recent studies of telomerase activity in pulmonary NETs revealed similarly low levels of telomerase activity in NETs as in non-neoplastic lung tissue. In contrast, all other pulmonary NETs showed high levels of telomerase activity [11]. This finding corresponds to the very low proliferative activity in our series of NETs. Most of the cells of these NETs are in the G_0-cell cycle phase. In contrast, low- and high-grade NECs overcome the mortality stage (M_2 phase) and are capable of indefinite cell proliferation [11]. Based on these data and our cell kinetic findings, we propose a hypothesis about the origin and development of NETs and NECs. It postulates that damage to stem cells may cause the development of high-grade NECs. If partially differentiated cells are damaged, low-grade NECs and NETs develop (Fig. 4). In support of this hypothesis is the lack of a low-grade NEC to high-grade NEC sequence in our material. In addition, transformation from low-grade NET to high-grade NEC has not yet been described in the literature. However, whether this assumption is correct can only be clarified by means of molecular pathology.

In conclusion, high-grade NECs, independent of their site of origin, have to be regarded as an entity distinct from low-grade NECs and NETs. There are no indications for the tumor progression from NET to NEC low grade and finally to NEC high grade (NET–NEC sequence). The well-differentiated and poorly differentiated NE tumors may therefore derive from different precursor cell populations. However, the final steps of the carcinogenesis of NETs can only be clarified by molecular pathological investigations.

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