Article is available online at http://www.webio.hu/por/2007/13/4/0321

ARTICLE

# Chromogranin A-Positive Tumor Cells in Human Esophageal Squamous Cell Carcinomas

Aping YUAN,[1,2] Jinzhong LIU,[3] Yiqing LIU,[1] Guanglin CUI[1,2]

[1]Department of Medicine, the Second Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China, [2]Laboratory of Gastroenterology, Institute of Clinical Medicine, Faculty of Medicine, University of Tromsø, Tromsø, Norway, [3]Department of Pathology, the Fourth Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

**Gastrointestinal cancers have frequently shown neuroendocrine (NE) differentiation, but whether NE differentiation occurs in esophageal squamous cell carcinoma (ESCC) remains unclear. In this study, tissue sections obtained from 43 patients with ESCC from a high-incidence area of Northern China were used for the assessing of NE differentiation by immunohistochemistry using antibody against chromogranin A (CGA). In addition, the malignant characteristics and proliferation capacity of CGA-positive cells were also examined by immunohistochemistry. The clinicopathological significance of these CGA-positive tumor cells in ESCC was assessed. Of 43 ESCC samples, CGA-immunoreactive tumor cells were detected in 10 cases (23.26%). However, the CGA-positive tumor cells were scattered at a very low number among non-immunoreactive tumor cells and were rarely constituted a major part of cancer cell nests. Only 4.65% (2/43) cases showed a high density (>10 cells but <1% of total tumor cell mass) of CGA-positive tumor cells. P53 immunoreactivity was frequently shown, while Ki67 was hard to detect in these CGA-positive cells. In addition, no relationship between CGA positivity rate and clinicopathological parameters was found. Thus, we concluded that low-density CGA-positive tumor cells can be detected in ESCC, supporting the notion that heterogeneous NE differentiation also exists in tumors that lack neuroendocrine cells in their normal epithelial counterparts. (**Pathology Oncology Research Vol 13, No 4, 321–325)

*Key words:* esophagus; squamous cell carcinoma; neuroendocrine; differentiation

## *Introduction*

Tumor cells with neuroendocrine (NE) differentiation properties can be observed in many human cancers derived from different organs, by immunohistochemistry and ultrastructural techniques using different NE differentiation markers. The occurrence of NE differentiation was mostly observed in tumors arising in organs that normally contain NE cells, such as the prostate, breast and thyroid etc. In the digestive tract, gastrointestinal (GI) tract mucosa has been found to harbor numerous dispersed NE cells, and is recognized as the largest endocrine organ. NE differentiation is particularly common in adenocarcinomas deriving from the gastrointestinal tract i.e. the stomach, colon and pancreas. Interestingly, NE differentiation has been reported in hepatocellular cell carcinoma (HCC) that had been considered previously as non-NE containing tumor. Until now, whether NE differentiation existed in tumors deriving from the esophagus has largely been ignored in the majority of studies, the reason probably being that NE cells are fairly unusual in normal esophageal mucosa, and thus NE differentiation in esophageal cancer was considered to be rare. However, NE differentiation has recently been found in some tumors that lack neuroendocrine cells in their normal epithelial counterparts, i.e. in mucinous cys-

*Received:* Jan 23, 2007; *accepted:* Oct 20, 2007

*Correspondence*: Dr. GUANGLIN CUI, Laboratory of Gastroenterology, Institute of Clinical Medicine, Faculty of Medicine, University of Tromsø, N-9037, Tromsø, Norway. Fax: +47 77644650, e-mail: guanglin.cui@fagmed.uit.no

***Acknowledgments:*** This study was partially supported by Henan Medical Health Committee Foundation. We express our sincere gratitude to our colleagues at Esophageal Surgery Section and Department of Pathology of the People's Hospital of Linzhou for supplying biopsies.

© 2007 Arányi Lajos Foundation

tadenocarcinoma of the ovary. One of the hypotheses is that NE cells share the same stem cell origin as other types of tumor cells. Thus, a single tumor stem cell under the favorable stimuli of the tumor microenvironment can have the potential of developing to all tumor cell types including NE tumor cells.

Histologically, esophageal cancers have been classified into two main histological types: esophageal squamous cell carcinomas (ESCC) representing the great majority in some Asian countries such as China, and adenocarcinoma which is more frequently seen in Western countries and showed a dramatically increasing rate recently. NE differentiation in Barrett's esophagus epithelium and esophageal adenocarcinoma has been reported. It was found, however, that NE properties documented by immunohistochemical evidence may also be exhibited in an uncommon and specific type of squamous cell carcinoma (basaloid squamous carcinoma) of the esophagus, which was traditionally considered of non-NE nature. However, it is unclear whether the NE differentiation exists in ordinary ESCC.

Based on the above background, therefore, the objective of this study was to evaluate the prevalence and the clinical implications of NE differentiation by using immunohistochemical assays for chromogranin A, a reliable NE marker, in ESCC patients from Linzhou (formerly Linxian), a county in Henan Province, located in North-Central China, which has one of the highest rates of esophageal cancer in the world. The dominant histological type of esophageal cancers in Linzhou is ESCC, providing an opportunity to evaluate ESCC. We also correlated NE differentiation with a variety of biologic risk factors for ESCC, including indicators of tumor gene mutation (P53) and tumor proliferation capacity (Ki67). Our results indicated that NE differentiation was detected in tumor cells of ESCC.

## *Patients and methods*

A total of 43 cases of advanced ESCC were randomly selected from the Department of Pathology of the People's Hospital of Linzhou for this study. Mean age at treatment was 57.3 years (ranging from 32 to 76 years). The male/female ratio was 26/17. No patient received preoperative radiotherapy and/or chemotherapy. Curative surgery was performed in all 43 patients. Resected specimens from ESCC patients were longitudinally sliced, fixed in 10% formalin and embedded in paraffin. Representative sections were cut at 4 mm and stained with hematoxylin-eosin (H&E) for routine histological diagnosis. Basic information on histological and clinical findings is summarized in *Table 1*. This work was approved and supported by the Medical Research Committee of Henan Provincial Medical Health Department.

### *Immunohistochemistry (IHC)*

Sections for IHC were deparaffinized in xylene, rehydrated in graded ethanol, and incubated in 0.3% $H_2O_2$ solution in methanol for 15 minutes to block endogenous peroxidase. Antigen retrieval was achieved by boiling sections for 15 minutes in 0.01 M citrate buffer, pH 6.0. Nonspecific binding was blocked by incubating sections in phosphate-buffered saline (PBS) containing 4% normal bovine serum and 0.25% Triton-X 100. The slides were rinsed three times with PBS with 0.25% Triton-X 100 (PBS-T) for 3 min and incubated overnight 4ºC with a rabbit anti-porcine chromogranin A (CGA) antibody (working dilution 1:500, Immunostar, Hudson, WI, USA). The slides were then washed with PBS-T 3 times and detection was performed with a commercial LSAB-2 system-HRP kit (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions and our published method. 3-Amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen and slides were counterstained with Mayer's hematoxylin. Negative control slides for IHC were performed routinely by (1) substituting primary antibody with isotype-matched control antibodies; (2) substituting secondary antibody with PBS-T. Sections from antral mucosa were used as positive controls.

### *Serial sections to show P53 and Ki67 immunoreactivity in CGA-positive tumor cells*

To characterize CGA-positive tumor cells, serial sections were stained with anti-CGA and anti-P53 (1:25; DAKO) antibodies or anti-CGA and anti-Ki67 (1:70; BD Pharmingen., San Jose, CA, USA) antibodies according to the method described above. Antigen retrieval for P53 IHC was achieved by boiling sections in 0.01 M citrate buffer, pH 6.0 at high power for 20 minutes and for Ki67 IHC at middle power for 15 minutes. Primary antibodies were incubated at 4°C overnight and developed with LSAB-2 system-HRP kit (DAKO) as described above.

***Table 1.*** **Basic histological and clinical information of ESCC patients**

| | *N* | *Position* | | | *Differentiation* | | *Infiltration in layers* | |
|---|---|---|---|---|---|---|---|---|
| | | *Upper* | *Middle* | *Lower* | *Well* | *Moderately* | *All* | *Muscular* |
| ESCC | 43 | 6* | 29** | 8 | 2 | 41 | 36 | 7 |

*Two cases mixed with middle part; ** two cases mixed with lower part.

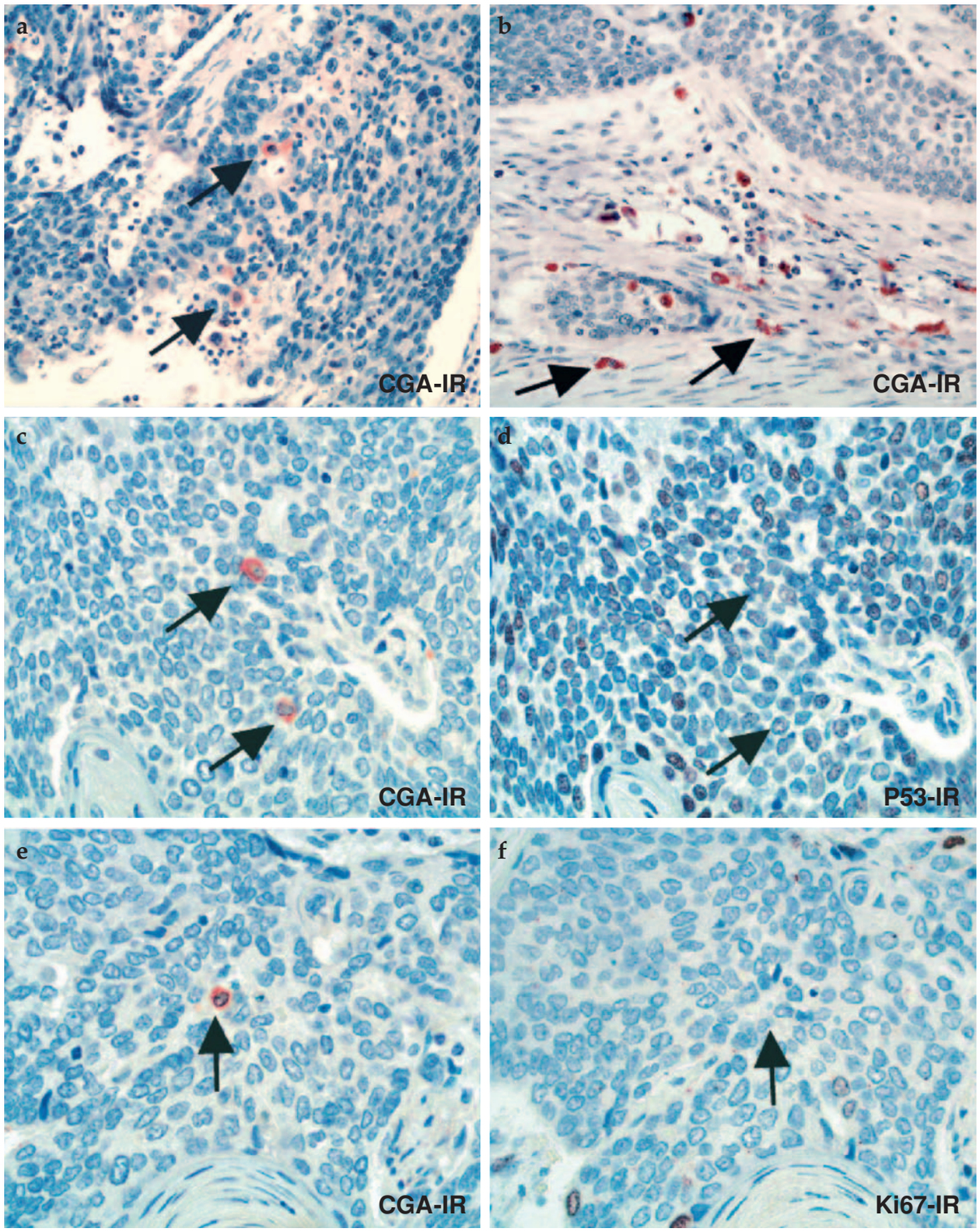


***Figure 1.*** *Immunohistochemical examination of CGA expression in ESCC. Tumor cells with CGA immunoreactivity were mostly observed at a low density* ***(a),*** *while CGA-positive cells were also seen in the stroma between tumor cell nests* ***(b).*** *Serial sections stained for CGA and P53 or CGA and Ki67, demonstrating that some of the CGA-positive tumor cells* ***(c, e)*** *also expressed P53* ***(d),*** *but not Ki67* ***(f).*** *(IHC, counterstained with hematoxylin ×400)*

*Table 2.* **Clinicopathological features of ESCC positive for CGA immunoreactivity**

| *ID* | *Age (year)* | *Gender* | *Differentiation* | *Infiltration* | *Tumor location* | *CGA* |
|---|---|---|---|---|---|---|
| 1 | 58 | m | M | Muscular | L | + |
| 2 | 67 | f | M | All | Upp | + |
| 3 | 65 | m | M | All | L | + |
| 4 | 66 | m | M | All | Upp | +++ |
| 5 | 70 | m | M | All | Upp+Mid | + |
| 6 | 56 | m | M | All | Mid+L | + |
| 7 | 70 | m | M | All | Mid | + |
| 8 | 59 | f | M | All | Mid | + |
| 9 | 43 | m | W | All | L | + |
| 10 | 56 | m | M | All | Upp | +++ |

M: male; F: female; M: moderately differentiated; W: well-differentiated; All: all layers; Upp: upper part of esophagus; Mid: middle part of esophagus; L: lower part of esophagus

*Morphometric evaluation*

Slides were examined under light microscopy, and only the slides with CGA immunoreactivity in tumor cells were identified as positive. Since the CGA-positive tumor cell number in ESCC cancerous tissues were generally not as high as observed in gastrointestinal cancers, the numbers of positive tumor cells in five independent high-power fields (HPF;  400) with abundant distribution were counted and scored as follows: (-), no immunoreactive cells; (+), 1–5; (++), 5–10; (+++), >10 cells but <1% of positive cells in the total cell mass; (++++), >1% positive cells.

## *Results*

In ESCC cancerous tissues, CGA-positive tumor cells were detected in 10 of 43 cases (23.26%). However, the number of CGA-positive tumor cells was generally low (<10/HPF with abundant distribution), only 2 of 43 cases (4.65%) showing a high density of CGA-positive tumor cells (>10/HPF). CGA-positive tumor cells were randomly scattered among non-reactive tumor cells (*Fig. 1A*). Additionally, some of the CGA-positive cells in the stroma between tumor nests were likely nerve fibers according to their morphological appearance and location (*Fig. 1B*). The occurrence of CGA-positive tumor cells in ESCC did not significantly correlate with the patients' gender and tumor location (*Table 2*). Since most cases included in this study were moderately differentiated ESCCs, it was hard to find a correlation between differentiation and the presence of CGA-positive tumor cells.

Staining for CGA and P53 or CGA and Ki67 in adjacent sections clearly demonstrated that P53 immunoreactivity (*Fig. 1D*) was also demonstrated in these CGA-positive tumor cells (*Fig. 1C*), while Ki67 was hard to detect in them (*Fig. 1E and F*).

## *Discussion*

NE differentiation in human tumors can be identified under light microscope by several techniques. Some of these, i.e. silver impregnation techniques (Masson-Fontana method and the Grimelius techniques) are specific for identifying NE cells; however, they require a skilled technician and experienced eyes to recognize positive cells. To date, immunohistochemistry using specific antibodies recognizing NE cells has become the preferred technique. The anti-CGA antibody, staining granule proteins in NE cells, has been recognized as a reliable tool and became the most commonly used marker for the histological detection of NE differentiation. By using anti-CGA antibody, our current results demonstrate that NE differentiation exists in ESCC at a low density.

As noted earlier, NE differentiation can be found not only in tumors deriving from normal tissues containing NE cells, but also in tumors arising in normal tissues that do not contain NE cells. In the former case, as gastrointestinal cancers, the cellular origin of NE differentiation was most likely a stem cell shared with non-NE tumor cells. However, in the latter case, as in ESCC, an alternative explanation may be applied. The epithelial stem cells do not give rise to NE differentiation in the normal epithelial counterparts, but may hold a capacity of NE differentiation that depends on the external stimuli. Some factors i.e. cytokines, extracellular matrix and peptides in tumor microenvironment may play an important role in regulating NE differentiation. It has been found that components of extracellular matrix, cytokines and growth factors were frequently altered during the esophageal epithelial carcinogenesis; these factors might promote stem cells favor the direction of NE differentiation in particular conditions or microenvironments.

Although the innervations of cancer are not appreciated, nerve fibers in esophageal cancer have been found sometimes. In our present study using immunohistochemical staining with anti-P53 and anti-CGA antibodies in serial sections, most CGA-positive cells clearly had potential malignant oncogenetic changes (P53 positivity). This confirmed that most CGA-positive cells were real malignant ESCC cells. Whether the endocrine cells residing in the gastrointestinal tract have proliferation capacity remained as an unsettled issue. Our finding that Ki67 immunoreactivity was rarely shown in CGA-positive tumor cells might indicate that CGA-positive tumor cells in ESCC have low proliferative capacity.

The clinical significance of NE differentiation in human cancers has been extensively discussed. One of the main reasons is that tumor cells with NE characterization may produce peptides to simulate their growth via autocrine/paracrine ways. Recently we were able to demonstrate an increased progastrin peptide expression in ESCC (manuscript in submission). Its overexpression might have a biological and therapeutic importance for ESCC, since it has been shown that progastrin has a stimulatory effect on gastrointestinal and pancreatic cancers. In spite of interest in its biological role, the prognostic significance of NE differentiation in tumors remains largely unsettled. Some studies found that NE differentiation was related to poor survival, but others were unable to confirm such a connection. In our current study, we could not find any difference in NE differentiation related to gender, age, tumor location or type of invasion. Since the ESCCs included in this study were mostly moderately differentiated, it was difficult to assess the correlation between NE cells and the degree of histological differentiation.

In conclusion, our observation on CGA-positive tumor cells in patients with ESCC indicated that NE differentiation could be detected in human cancers that lack NE cells in their normal epithelial counterparts. However, these cells had a low density as compared with gastrointestinal tumors.

### *References*


1. *Barrett P, Hobbs RC, Coates PJ, et al:* Endocrine cells of the human gastrointestinal tract have no proliferative capacity. Histochem J 27:482-486, 1995
2. *Blumenfeld W, Chandhoke DK, Sagerman P, et al:* Neuroendocrine differentiation in gastric adenocarcinomas. An immunohistochemical study. Arch Pathol Lab Med 120:478-481, 1996
3. *Bosman FT:* Neuroendocrine cells in non-endocrine tumors: what does it mean? Verh Dtsch Ges Pathol 81:62-72, 1997
4. *Bosman FT, Louwerens JW:* Neuroendocrine (APUD) cells in ovarian teratomas and testicular teratocarcinomas. Cell Biol Int Rep 5:464, 1981
5. *Chang KT, Huang CY, Tsai CM, et al:* Role of IL-6 in neuroendocrine differentiation and chemosensitivity of non-small cell lung cancer. Am J Physiol Lung Cell Mol Physiol 289:L438-445, 2005
6. *Cho KJ, Jang JJ, Lee SS, et al:* Basaloid squamous carcinoma of the oesophagus: a distinct neoplasm with multipotential differentiation. Histopathology 36:331-340, 2000
7. *Cui G, Koh TJ, Chen D, et al:* Overexpression of glycine-extended gastrin inhibits parietal cell loss and atrophy in the mouse stomach. Cancer Res 64:8160-8166, 2004
8. *de Bruine AP, Dinjens WN, van der Linden EP, et al:* Extracellular matrix components induce endocrine differentiation in vitro in NCI-H716 cells. Am J Pathol 142:773-782, 1993
9. *Hamilton K, Chiappori A, Olson S, et al:* Prevalence and prognostic significance of neuroendocrine cells in esophageal adenocarcinoma. Mod Pathol 13:475-481, 2000
10. *Hayashi H, Ooi A, Nakanishi I:* Biological and clinicopathological significance of endocrine differentiation of gastric adenocarcinoma evaluated by double immunohistochemical labeling for chromogranin A and bromodeoxyuridine. Jpn J Clin Oncol 20:339-348, 1990
11. *Jaskiewicz K, Louw J, Anichkov N:* Barrett's oesophagus: mucin composition, neuroendocrine cells, p53 protein, cellular proliferation and differentiation. Anticancer Res 14:1907-1912, 1994
12. *Li MX, Cheng SJ:* Carcinogenesis of esophageal cancer in Linxian, China. Chin Med J (Engl) 97:311-313, 1984
13. *Li Z, Shimada Y, Uchida S, et al:* TGF-alpha as well as VEGF, PD-ECGF and bFGF contribute to angiogenesis of esophageal squamous cell carcinoma. Int J Oncol 17:453-460, 2000
14. *Linder S, Myrvold K, Falkmer UG, et al:* Neuroendocrine cells in pancreatic duct adenocarcinoma: an immunohistochemical study. J Exp Clin Cancer Res 25:213-221, 2006
15. *Louwerens JK, Schaberg A, Bosman FT:* Neuroendocrine cells in cystic mucinous tumours of the ovary. Histopathology 7:389-398, 1983
16. *Lu SH, Zhou Y, Que HP, et al:* Peptidergic innervation of human esophageal and cardiac carcinoma. World J Gastroenterol 9:399-403, 2003
17. *Ohashi K, Nemoto T, Nakamura K, et al:* Increased expression of matrix metalloproteinase 7 and 9 and membrane type 1-matrix metalloproteinase in esophageal squamous cell carcinomas. Cancer 88:2201-2209, 2000
18. *Ooi A, Hayashi H, Katsuda S, et al:* Gastric carcinoma cells with endocrine differentiation show no evidence of proliferation. Hum Pathol 23:736-741, 1992
19. *Ooi A, Mai M, Ogino T, et al:* Endocrine differentiation of gastric adenocarcinoma. The prevalence as evaluated by immunoreactive chromogranin A and its biologic significance. Cancer 62:1096-1104, 1988
20. *Parkin DM, Bray FI, Devesa SS:* Cancer burden in the year 2000. The global picture. Eur J Cancer 37 Suppl 8):S4-S66, 2001
21. *Rengifo-Cam W, Singh P:* Role of progastrins and gastrins and their receptors in GI and pancreatic cancers: targets for treatment. Curr Pharm Des 10:2345-2358, 2004
22. *Seifert P, Spitznas M:* Tumours may be innervated. Virchows Arch 438:228, 2001
23. *Syversen U, Halvorsen T, Marvik R, et al:* Neuroendocrine differentiation in colorectal carcinomas. Eur J Gastroenterol Hepatol 7:667-674, 1995
24. *Szumilo J, Swatek J, Chroscicki A, et al:* Colonic adenocarcinoma with numerous paneth and endocrine cells. Pol J Pathol 56:89-92, 2005
25. *Tajima Y, Nakajima T, Sugano I, et al:* Hepatocellular carcinoma containing endocrine cells. An autopsy report of triple cancer involving the liver, kidney and thyroid. Acta Pathol Jpn 42:904-910, 1992
26. *Tezel E, Nagasaka T, Tezel G, et al:* Characterization of scattered neuroendocrine cells in ductal carcinoma of the pancreas. Pancreas 25:136-141, 2002
27. *Untergasser G, Plas E, Pfister G, et al:* Interferon-gamma induces neuroendocrine-like differentiation of human prostate basal-epithelial cells. Prostate 64:419-429, 2005
28. *Waldum HL, Brenna E:* Non-proliferative capacity of endocrine cells of the human gastro-intestinal tract. Histochem J 28:397-398, 1996
29. *Wang JH, Dhillon AP, Sankey EA, et al:* 'Neuroendocrine' differentiation in primary neoplasms of the liver. J Pathol 163:61-67, 1991
30. *Wang KL, Yang Q, Cleary KR, et al:* The significance of neuroendocrine differentiation in adenocarcinoma of the esophagus and esophagogastric junction after preoperative chemoradiation. Cancer 107:1467-1474, 2006
31. *Wang LS, Chow KC, Wu CW:* Expression and up-regulation of interleukin-6 in oesophageal carcinoma cells by n-sodium butyrate. Br J Cancer 80:1617-1622, 1999
32. *Zhao M, Laissue JA, Zimmermann A:* "Neuroendocrine" differentiation in hepatocellular carcinomas (HCCs): immunohistochemical reactivity is related to distinct tumor cell types, but not to tumor grade. Histol Histopathol 8:617-626, 1993