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The prevalence and clinical significance of chromogranin A and secretogranin II immunoreactivity in colorectal adenocarcinomas

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Abstract Colorectal adenocarcinomas may display features of endocrine differentiation, shown by argyrophil stains and by the expression of endocrine markers such as chromogranin A. We investigated chromogranin A and secretogranin II immunoreactivity in a series of 208 carcinomas of the large bowel to assess the prevalence and clinical significance of endocrine differentiation. Tumours expressing endocrine markers were classified as low expressors (< than 1 immunoreactive tumour cell/mm^2) and high expressors (> than 1 immunoreactive tumour cell/mm^2). There were 33 (16%) carcinomas showing both chromogranin A and secretogranin II immunoreactivity: 11 tumours (5%) were high expressors. Endocrine differentiation was not related to the disease stage, tumour location, grade, DNA ploidy and p53 protein accumulation. In the entire series chromogranin A immunoreactivity did not provide prognostic information using univariate and multivariate analysis. A worse overall survival (P=0.048) was demonstrated for the stage III patients with high expressor tumours, but there were only five patients in this group. The results of our investigation suggest that chromogranin A immunoreactivity is not a useful variable in the prognostic assessment of colorectal adenocarcinomas.

Key words Chromogranin A · Secretogranin II · Immunocytochemistry · Prognosis · Colorectal adenocarcinomas

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Introduction

The presence of cells containing secretory granules of endocrine type in carcinomas arising in the gastrointestinal tract has been documented convincingly using argyrophil stains [2, 18, 23, 32, 36] and, more recently, by the immunocytochemical demonstration of the presence of specific endocrine markers [1, 4, 9, 13, 15, 19, 27, 28]. The occurrence of endocrine cells in noncarcinoid carcinomas of the gastrointestinal tract, however, initially generated controversies about the origin of these cells. It was proposed that endocrine cells in the gastrointestinal tract were derived from the neural crest, therefore being of ectodermal origin [24, 29]. However, it is now firmly established that endocrine cells in gastric and colorectal carcinomas represent endocrine differentiation of endodermal cells [11, 21].

The controversy has now moved to the clinical arena, and, specifically, to the prognostic role of the presence of endocrine differentiation in gastrointestinal carcinomas. Smith and Hagitt [34] evaluated a series of colorectal adenocarcinomas applying the Churukian-Schenk stain for demonstrating argyrophil cells and concluded that the presence of endocrine differentiation was not associated with a worse prognosis. A few investigators have demonstrated the presence of serotonin and chromogranin A immunoreactive cells in colorectal carcinomas and assessed the clinical implications of endocrine differentiation in these tumours [1, 9, 15]. The clinical significance of these observations was variable; Arends et al. [1] reported that serotonin expression was present in tumours with a slightly worse prognosis, with borderline statistical significance, whereas Hamada et al. [15] and de Bruine et al. [9] reported that the presence of chromogranin A immunoreactivity was associated with a worse prognosis, statistically significant by univariate and multivariate analysis. The relationship of endocrine differentiation to other prognostic indicators, such as DNA ploidy and p53 protein accumulation, previously shown to be significantly associated with patient survival [7, 35], has not been reported.

The purpose of our investigation was two-fold: to assess whether endocrine differentiation in colorectal carcinomas is correlated to other clinicopathological variables and to evaluate its prognostic role. We immunostained colorectal tumours using two monoclonal antibodies (mAb) recognizing different epitopes of chromogranin A and a recently described mAb specific for secretogranin II, also known as chromogranin C (for nomenclature see [12]). We studied these endocrine markers in a well characterized series of patients in which we have previously reported the prognostic significance of the DNA ploidy status and of p53 protein accumulation [7].

Materials and methods

The study population included 208 patients with colorectal adenocarcinoma who were treated surgically at the Lahey Clinic Medical Center (Burlington, Mass.) between 1982 and 1984. To be included in the study, patients had to meet the following criteria: no history of previous malignancies (excluding basal cell and squamous cell carcinomas of the skin), colonic surgical resection margins negative for tumour, no preoperative treatment for the colorectal carcinoma and no perforation of the bowel by the tumour. Of the patients included in the study, one patient had synchronous colonic tumours (disease was classified according to the tumour with the most advanced stage), two patients had incidental carcinoid tumours found on pathological examination and one patient had carcinoma associated with chronic ulcerative colitis.

The slides and pathology reports from each patients were reviewed to confirm the pathological grading and staging. The stage was determined according to the American Joint Committee for Staging of Cancer [3]. Other data evaluated included patient age and sex, as well as the location of the tumours in the large bowel. Patients were followed up for at least 5 years and their survival and clinical status were obtained from the tumour registry or contact with the patient's personal physician or both.

For the immunolocalization of chromogranin A we used the mAb LK2H10 (Biogenex, San Ramon, Calif., USA) and A11 from our laboratory [30]. For the immunolocalization of secretogranin II we used the 3C12 mAb, elicited in our laboratory [31] against bovine secretogranin II and cross-reacting with human epitopes.

For immunocytochemistry one paraffin block was selected for each case, based on good morphological preservation and coexistence of neoplastic tissue with non-neoplastic colonic mucosa.

For the detection of chromogranin A and secretogranin II a standard avidin-biotin-peroxidase complex technique was used [16]. Briefly, the slides were dewaxed, rehydrated and incubated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity. After extensive washing, the slides were subsequently incubated with the following 20% non-immune human serum for 30 min at room temperature (RT); mAb (for LK2H10 at 1:300 dilution; for A11 and 3C12 at IgG concentrations ranging from 15 μg/ml to 17.5 μg/ml) overnight at 4° C; biotin-labelled antiserum against mouse IgG (Vector, Burlingame, Calif., USA) at 1:20 dilution for 40 min at RT; avidin-biotin-peroxidase complex (Vector) at 1:100 dilution for 40 min at RT. For mAb dilutions and for slide washings 0.05 M TRIS buffered pH 7.6 saline was used. Peroxidase activity was developed in the diaminobenzidine chromogenic substrate; slides were counterstained with haematoxylin.

The endocrine cells of the non-neoplastic mucosa served as positive control of the immunoreaction. Negative controls were obtained by substituting the immunoglobulin fraction of non-immune mouse serum for the primary antibody. The stained slides were independently evaluated by two of the authors (S.F., G.P.); in the few cases in which the evaluation provided different results, a consensus interpretation was reached after re-examination.

The number of chromogranin A immunoreactive tumour cells was evaluated with a gridded eye-piece, at ×630 magnification; immunoreactive tumour cells were counted in at least 30 tumour fields. The carcinomas were scored positive provided that immunoreactive tumour cells were detectable throughout the tumour. Neoplasm showing only a few scattered immunoreactive cells were scored as negative. Tumours were classified as follows: low expressors when there was <1 immunoreactive cell/mm^2 and high expressors when there was >1 immunoreactive cell/mm^2.

p53 protein accumulation has been evaluated by immunocytochemistry; the technique and the results have been previously reported [7]. Only cytoplasmic accumulation of p53 protein has been analysed in the current study, since it was previously reported as the only type of p53 accumulation providing independent prognostic information [7].

DNA ploidy was determined by image analysis using the CAS 200 (Cell Analysis Systems, Elmhurst, Ill., USA) image analyser and software. The technique and the histogram interpretation criteria were previously detailed [5].

For statistical analysis, the initial step was to compare all chromograin A immunoreactive cases with the negative ones; the two groups of chromogranin A immunoreactive tumours were also individually evaluated. For survival analysis the most significant results were obtained when the negative and low expressor tumours were grouped and compared with the high expressors. Thus, the latter grouping is used in part to present the results of the current investigation.

Statistical differences between variables were analysed with unpaired *t*-tests or analysis of variance, as appropriate. Contingency tables were analysed by Fishers' exact test or chi-square test. Survival and disease-free distributions were calculated by the Kaplan-Meier product-limit method. The statistical significance of differences between distributions was analysed by the generalized Savage (Mantel-Cox) and generalized Willcoxon (Breslow) methods. Multivariate analysis was performed with the Cox proportional hazard model. Statistical analysis was performed with BMDP statistical software (Los Angeles, Calif., USA).

Results

Chromogranin A immunoreactivity

In all cases chromogranin A immunoreactive cells were detected in non-neoplastic colonic mucosa. Granular cy-

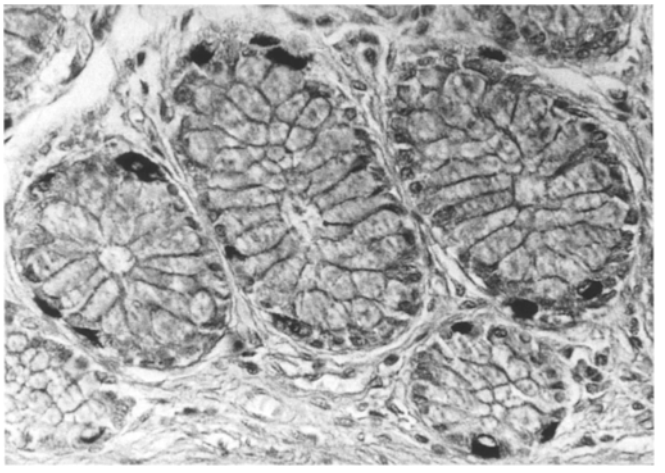

Fig. 1 Immunoreactivity for chromogranin A in non-neoplastic mucosa: staining is limited to a few cells at the bottom of crypts, ×450. Haematoxylin as nuclear counterstain

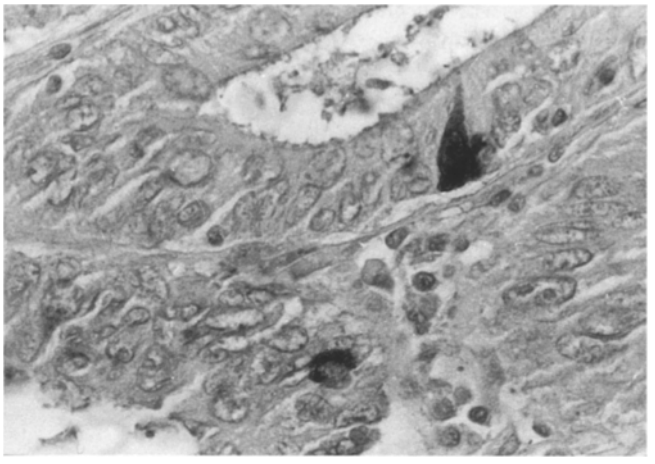

Fig. 2 Example of an adenocarcinoma displaying rare, scattered chromogranin A immunoreactive tumour cells, ×630. Haematoxylin as nuclear counterstain

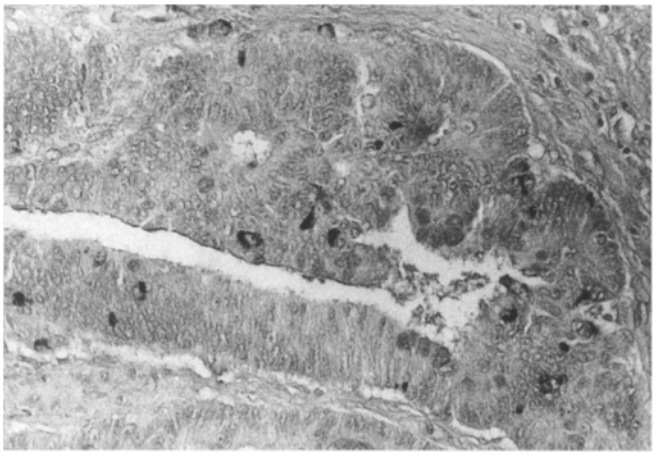

Fig. 3 Example of carcinoma with numerous chromogranin A immunoreactive tumour cells, ×300. Haematoxylin as nuclear counterstain

toplasmic staining was typical of gut endocrine cells (Fig. 1). Chromogranin A immunoreactive cells occurred singly and were more numerous in the left colon and rectum than in the right colon. Within neoplastic tissues, chromogranin A immunoreactive cells were detected in 33 (16%) colorectal adenocarcinomas. Neoplastic cells displaying endocrine differentiation showed diffuse cytoplasmic immunostaining. There were no discrepancies in the intensity of staining and in the number of immunoreactive cells using LK2H10 and A11 mAb. Moreover no significant differences were found studying different sections from the same block. An example of tumour with rare, scattered chromogranin A immunoreactive cells is shown in Figure 2, whereas the presence of diffuse chromogranin A immunoreactive cells is depicted in Figure 3.

Among chromogranin A immunoreactive tumours, 22 carcinomas (11%) were low expressors and 11 carcinomas (5%) were high expressors.

Table 1 Chromogranin A immunoreactivity and pathological correlations (*HE* high expressor tumours, *LE* low expressor tumours, *W* well differentiated, *M* moderately differentiated, *P* poorly differentiated, *R* rectum, *LC* left colon including sigmoid colon, *RC* right colon including transverse colon, *A* aneuploid, *T* tetraploid, *D* diploid)

		HE	LE	Total
Stage	I	2 (6%)	4 (11%)	35
	II	3 (4%)	11 (14%)	79
	III	5 (7%)	7 (10%)	71
	IV	1 (4%)	0	23
Grade	W and M	9 (6%)	18 (12%)	156
	P	2 (4%)	4 (8%)	52
Site	R	0	12 (20%)	61
	LC	6 (8%)	5 (6%)	77
	RC	5 (7%)	5 (7%)	70
DNA ploidy	A	5 (5%)	12 (11%)	106
	T	3 (7%)	2 (5%)	41
	D	3 (5%)	8 (13%)	61
p53	negative	4 (4%)	9 (9%)	97
	positive	7 (7%)	12 (12%)	99

Chromogranin A versus secretogranin II immunoreactivity

In non-neoplastic colonic mucosa the distribution of secretogranin II immunoreactive cells was similar to the pattern of chromogranin A staining. Among colorectal carcinomas, all cases that showed chromogranin A immunoreactivity also displayed secretogranin II staining. Conversely, tumours that did not show chromogranin A immunoreactivity were consistently negative when studied with the secretogranin II specific mAb. The number of secretogranin II immunoreactive neoplastic cells in each carcinoma was similar to that of chromogranin A immunoreactive cells, although staining serial sections from the same tumours demonstrated that the epitopes recognized by A11 and 3C12 did not coexist in the same cells. Since the tumours with endocrine differentiation identified by chromogranin A and secretogranin II were the same, clinicopathological correlations, as well as survival analyses, are described for chromogranin A immunoreactivity.

Chromogranin A immunoreactivity and clinicopathologic correlations

Adenocarcinomas displaying focal or diffuse endocrine differentiation were slightly less common among patients presenting with advanced stage (stage IV) and among poorly differentiated tumours. No statistically significant differences, however, could be demonstrated (Table 1).

The site of origin of the tumour (right colon versus left colon versus rectum) was not associated with endocrine differentiation. No chromogranin A high expressor carcinomas were identified arising in the rectum, but there were in turn more chromogranin A low expressor tumours than in the other sites (Table 1).

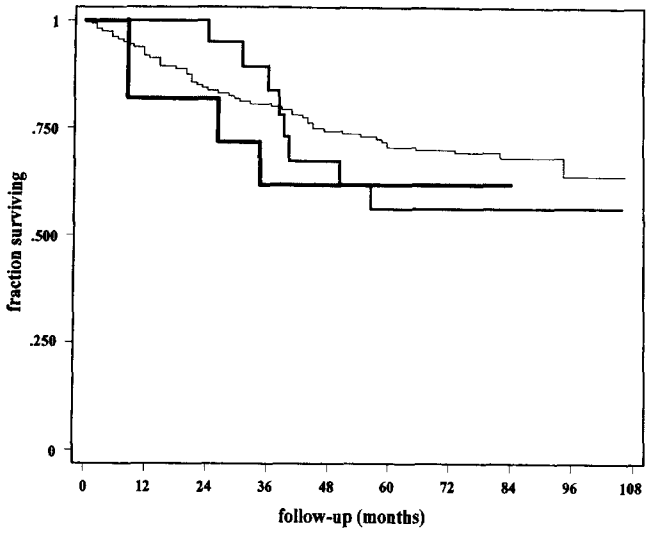

Fig. 4 Kaplan-Meier survival curves for the entire patient population by chromogranin A immunoreactive status. *Thin line* negative tumours, *intermediate line* chromogranin A high expressor tumours, *thick line* chromogranin A low expressor tumours

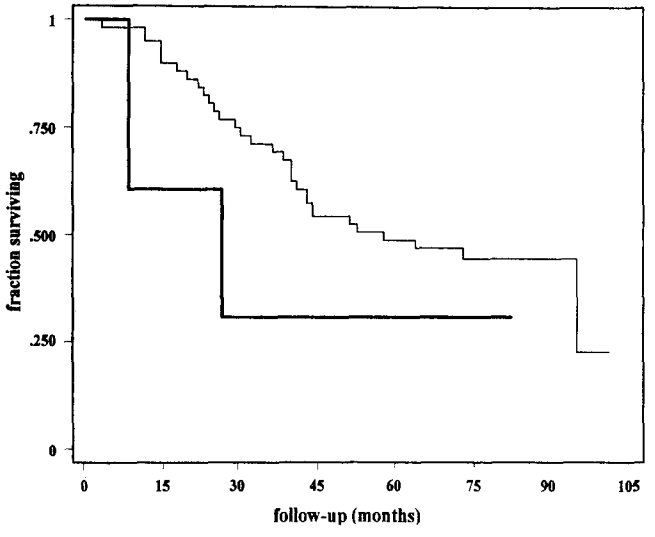

Fig. 5 Kaplan-Meier survival curves for the patients with stage III disease. *Thick line* chromogranin A high expressor tumours, *thin line* negative and chromogranin A low expressor tumours

No statistically significant associations were present between chromogranin A immunoreactive tumours and the DNA ploidy status or the presence or absence of cytoplasmic accumulation of p53 protein (Table 1).

Chromogranin A immunoreactivity and prognosis

In the entire series, patients with tumours displaying diffuse chromogranin A immunoreactivity demonstrated a slightly worse overall survival than the patients with carcinomas without endocrine differentiation; this trend did not reach statistical significance. The survival curve of the patients with tumours without chromogranin A immunoreactivity and that of the patients with low expressor tumours were also not statistically different (Fig. 4).

When the three chromogranin A groups of patients were analysed according to the disease stage, no differences in survival were observed for patients with stage I, II and IV (data not shown).

For disease stage III, however, patients with tumours showing diffuse chromogranin A immunoreactivity demonstrated a worse overall (P=0.048; Fig. 5) and disease-free survival (P=0.05) by the generalized Willcoxon method). There were five stage III patients with tumours showing diffuse chromogranin A immunoreactivity and three of them died of their disease.

Discussion

The results of the current investigation confirmed that tumour cells in colorectal carcinomas may display markers characteristic of endocrine differentiation. In our series the prevalence of neoplasms with endocrine features was 16%; only 5% of the carcinomas, however, displayed diffuse chromogranin A immunoreactivity (high expressors). It is noteworthy that tumours displaying chromogranin A immunoreactivity also showed secretogranin II expression. These two markers of endocrine differentiation were not coexpressed by the same tumour cells and there were no carcinomas showing only either chromogranin A or secretogranin II immunoreactivity. Thus, colorectal carcinomas, when displaying endocrine features, do so by expressing at least two endocrine markers in different tumour cells subpopulations. Several other endocrine markers have been investigated by de Bruine et al. [9]: chromogranin A expressing tumours frequently showed glucagon-like immunoreactivity and less commonly expressed a variety of other polypeptide hormones, such as serotonin, somatostatin, PYY and alpha- and beta-HCG. Therefore colorectal adenocarcinomas are capable of synthetizing several polypeptide hormones some of which, such as alpha- and beta-HCG, are even extraintestinal. The complete correspondence of secretogranin II and chromograin A immunoreactivity seen in the current investigation seems unique among polypeptide hormones and endocrine markers.

In the current study the prevalence of tumours with endocrine differentiation was slightly lower than the ones reported in other series, which range from 20% to 32.5% [9, 15, 34]. Since we used two different mAb to detect tumour cells for chromogranin A immunoreactivity, it is unlikely that tumours with endocrine differentiation went undetected. The strict criteria used to classify tumours with endocrine differentiation in our study may account for the somewhat lower prevalence of these neoplasms.

Our investigation failed to detect any associations between tumours with endocrine differentiation and other clinicopathological variables. Indeed, the prognostic significance of chromogranin A immunoreactivity was mar-

ginal at best. For the entire patient population, there were no statistically significant differences in overall and disease-free survival between the groups of chromogranin A low expressor tumours, chromogranin A high expressor tumours and negative tumours. No prognostic significance was noted in patients with stage I, II and IV disease. For disease stage III, patients with chromogranin A high expressor tumours demonstrated a worse survival than the patients with chromograin A low expressor tumours and negative tumours. The number of cases included in the chromogranin A high expressor group with disease stage III, however, was quite limited (7%), thus accounting for a minority of the patients presenting with stage III who died of disease. Multivariate analysis also failed to demonstrate prognostic significance of chromogranin A immunoreactivity.

Our results contradict the data of Hamada et al. [15] and de Bruine et al. [9]: they both reported a significant prognostic role of chromogranin A immunoreactivity when present in a diffuse pattern. Smith and Haggitt [34], however, evaluated endocrine differentiation by assessing the number of argyrophil cells in colorectal carcinomas and they also failed to find any prognostic significance. It must be noted that in each of these series, including the current one, the number of cases that were classified as tumours with diffuse endocrine differentiation is quite small and therefore the difference in survival of even a few patients may completely change the statistical evaluation. Although this could be the main explanation for the discrepancies previously noted, other factors should be taken into account. Particularly noteworthy is the geographic distribution of the reported series which included Japanese patients [15], Western European [9] and North Americans [34 and current series]. In fact, different aetiological factors may play a role in such diverse countries and in turn influence the subsequent pathway towards endocrine differentiation.

de Bruine et al. [8], using cell culture experiments with the NCI-H716 cell line, demonstrated that endocrine differentiation of cancer cells is linked to a combination of cell adhesion (probably mediated by cell adhesion molecules of the integrin family) and local factors that are bound to molecules of the extracellular matrix. Indeed, basic fibroblast growth factor (bFGF) binding to the extracellular matrix can enhance endocrine differentiation [8]. Abnormal expression of members of the integrin family has been described in colorectal carcinomas and reduced immunoreactivity for very late antigen a2 has been associated with advanced disease stage [22]. bFGF and angiogenin can be demonstrated in carcinomas arising in the gastrointestinal tract [25] and may be play a critical role in tumour angiogenesis [14, 20], which has been shown to have significant clinical implications in tumours of the breast and lung [6, 26, 37]. Furthermore, increased expression of proteolytic enzymes, such as cathepsin B, capable of digesting components of the extracellular matrix, has been related to advanced disease stage and poor patient survival [10]. Taken together, this data raise the possibility that the worse survival of patients with tumours showing endocrine differentiation is more likely to depend on aberration of integrin expression, degradation of the extracellular matrix and tumour angiogenesis: the expression of the endocrine markers may be only an epiphenomenon. Further studies are needed to address this hypothesis.

The clinical significance of endocrine differentiation in non-carcinoid carcinomas of the large bowel is likely to be quite limited, if any. Indeed, in all the investigations published, the number of patients with diffuse endocrine differentiation is small, and thus a large number of patients who eventually succumb to colorectal cancer is not correctly identified by the immunocytochemical evaluation of endocrine markers. Furthermore, other variables indicating a worse prognosis have been assessed: in particular, in the series of patients reported here, we have previously demonstrated that both DNA ploidy and the cytoplasmic accumulation of the tumor suppressor gene product p53 are independent prognostic factors in colorectal carcinomas [7]. Therefore these factors seem better suited to clinical application.

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