Chromogranin positive cells in colorectal carcinoma and transitional mucosa

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Abstract
Aims—Immunostaining of chromogranin identifies gastrointestinal mucosal endocrine cells. The detailed distribution and significance of chromogranin positive cells in colorectal carcinomas and in transitional mucosa remain unclear. The aim of this study was to clarify these aspects.
Methods—The distribution of chromogranin positive cells was studied by immunohistochemical methods in normal epithelium remote from carcinoma, in transitional mucosa, and in carcinomas of the colorectum. In selected cases northern or western blot analyses were performed.
Results—Chromogranin positive cells were seen in the lower third of the normal crypts and less frequently in transitional mucosa. Thirty five per cent (n = 38) of colorectal carcinomas showed immunohistochemically positive carcinoma cells in the tumour tissue. Northern and western blot analyses showed similar results. There was no difference in clinicopathological factors, including prognosis, between chromogranin positive cases of colorectal carcinoma (n = 38) and chromogranin negative cases (n = 70).
Conclusions—Neuroendocrine cell differentiation is controlled in transitional mucosa and the presence of chromogranin positive cells in carcinoma tissue does not influence the patient’s prognosis.

Keywords: Colorectal carcinoma, chromogranin immunostaining, northern blotting, western blotting, prognosis.

Neuroendocrine cells are present not only in normal tissues but also in malignant tissue in the human colon and rectum. A useful conventional staining method for identifying neuroendocrine cells is silver impregnation, using Grimelius’s technique. Recently, however, immunohistochemical staining for chromogranin A has been promoted. Chromogranins are glycoproteins with a molecular weight of 68 000, which were originally isolated from the chromafin granules of the adrenal medulla. Three types of chromogranins have been identified—A, B, and C. There seems to be a relationship between chromogranin A and Grimelius’s argyrophilia, so chromogranin A has mainly been used to identify neuroendocrine cells. Chromogranin A is a molecule of 431 amino acids and it coexists with catecholamines in the storage granules. It may be involved in the regulation of hormonal secretion or it may have a hormonal function of its own.

Whether or not colorectal adenocarcinomas containing neuroendocrine cells have the same prognosis as those without these cells has not yet been extensively studied, and there are few reports on the presence or distribution of neuroendocrine cells in transitional mucosa, which is characterised by increased amounts of sialomucin and elongation of the crypts.

We report here the results of chromogranin A expression as determined immunohistochemically by northern and western blot analyses in human colorectal carcinoma and transitional mucosa.

Methods
CASES
One hundred and eight patients with primary colorectal carcinoma were studied. These patients had been treated by surgical resection in Kyushu University Hospital, Fukuoka, Japan. The patients were followed for either five years or until death. Follow up was done by serum carcinoembryonic antigen (CEA) and common antigen (CA) 19–9 levels, computed tomography, echography, and barium enema. All information was obtained from the clinical records and from death certificates.

The colon or rectum was opened along its longitudinal dimension and fixed in 10% formalin. The central tissue slice taken from each tumour contained the largest longitudinal dimension. This slice was further cut into several blocks. Two to five blocks containing the tumour tissue and the non-carcinomatous mucosa adjacent to the carcinoma (transitional mucosa), and one block containing the normal tissue at least 5 cm distant from the tumour, were studied in each case. Haematoxylin and eosin stained sections of each tumour were examined by light microscopy to confirm the original histological diagnosis.

IMMUNOHISTOCHEMICAL STUDY
In all cases, the paraffin blocks were recut and stained immunohistochemically for chromogranin A. The sections were dewaxed in xylene, rehydrated through alcohol, and then immersed in 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. Sections were subsequently washed in phosphate buffered saline, and normal goat serum was applied for 10 minutes to reduce non-specific binding. The primary antibody is a monoclonal antibody to human chromogranin A from mouse-mouse hybrid
Chromogranin positive cells in colon cancer

Figure 1 Chromogranin A positive cells are seen mainly in the lower third of the crypts (immunostaining, × 93).

Figure 2 A few cells (arrow) are positive for chromogranin A in the transitional epithelium (immunostaining, × 75).

cells, and was purchased from Boehringer Mannheim. The sections were incubated with the primary antibody at 5 μg/ml at room temperature for 60 minutes in a humid chamber, then with biotinylated goat anti-mouse IgG (1:200 for one hour; Vector laboratories), and finally with avidin-biotin peroxidase complex (for 30 minutes; Vector Laboratories). Peroxidase labelling was developed with 3,3′-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with methyl green.

Each round of staining contained 23 slides from 20 cases and two control slides from human adrenal glands. To ensure consistency of chromogranin A staining between batches, one slide from the adrenal glands was used as a positive control. The other slide from the adrenal glands and duplicate slides from three cases were used for negative controls by replacing the primary antibody by normal mouse serum.

The number and position of cells positive for chromogranin A were determined in at least 15 well oriented crypts in transitional mucosa and normal mucosa away from the tumour. Positively stained cells were easily identified as cells with distinct brownish black cytoplasmic granules. The percentage of chromogranin A positive cells was calculated and mean values were calculated for transitional and normal (remote) mucosa.

In tumour tissues, positively stained tumour cells were also easily detected. The evaluation of chromogranin A immunoreactivity was done according to the method of Hamada et al. The frequency was calculated as follows: (total number of chromogranin positive carcinoma cells)/(tumour area (mm²)). The tumour area was measured using a digitiser connected to a computer. The tumours with positive chromogranin cells were divided into three grades: grade I, less than 0·5 positive cell/mm²; grade II, more than 0·5 and less than 1 positive cell/mm²; and grade III, more than 1 positive cell/mm².

NORTHERN BLOT ANALYSIS

Northern blot analysis was done in 32 cases according to methods described elsewhere. Briefly, total RNA was extracted using guanidine isothiocyanate. Equal amounts (15 μg) of total cellular RNA were applied onto each lane of 1·0% agarose-formaldehyde gels, electrophoresed overnight, and transferred to nylon membranes. A 2·1 kb cDNA PstI fragment containing the entire coding sequence for human chromogranin A was used in northern blot hybridisation. The probe was labelled with α32P-dCTP using a random primed DNA labelling kit (Boehringer Mannheim). Results were analysed using a Bio-Image analyser BAS2000 (Fuji).

WESTERN BLOT ANALYSIS

In 20 cases, protein was extracted by boiling tissue powder in a 1% sodium dodecyl sulphate (SDS) solution. The protein concentration was determined by the Bio-rad protein assay. Equal amounts of protein (50 μg) were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). Immunoblots were performed according to the method of Towbin et al. The chromogranin A was detected by the same antibody as that used in the immunohistochemical studies in a dilution of 1:2000. Blots were developed using an anti-mouse immunoglobulin G alkaline phosphatase conjugate (Promega).

STATISTICAL ANALYSIS

The percentages of chromogranin A positive cells in normal and transitional mucosa were compared by the Wilcoxon test. Clinicopathological data were stored in a mainframe computer. The Biomedical Computer Program
appeared to be components of the neoplastic compartment. The number and distribution of positive cells differed from gland to gland or from area to area, even within an individual tumour (figs 3 and 4). The numbers of tumours of grades I, II, and III were 15, 10, and 13, respectively.

There was no significant difference between positive \( (n = 38) \) and negative \( (n = 70) \) staining groups in terms of age, sex, location, tumour size, morphological type, histological type, depth of tumour invasion, lymphatic permeation, vascular permeation, lymph node metastasis, stage, and curability. There were also no differences among the cases of negative, grade I, grade II, and grade III tumours with respect to these factors. The five year survival of the positive group was 54·5% and that of the negative group was 58·7% (NS).

**NORTHERN BLOT**

Twenty seven of 32 normal colorectal mucosa samples showed a positive signal for chromogranin A. On the other hand in these 27 cases the tumour tissue showed a positive signal in only five, and the signal intensity was rather weak compared to that of the normal tissue samples (fig 5). The five positive tumours all had positive immunostaining of carcinoma cells histologically. However, four tumours with immunohistochemically positive carcinoma cells failed to show a positive signal in northern blot analysis. Five cases showed no signal in either normal or tumour tissues.

**WESTERN BLOT**

Western blot results in eight representative cases are shown in fig 6. Eighteen of 20 cases examined showed a reaction in the normal tissues and four cases showed a reaction in the tumour tissues. Three cases showed an almost equal reaction between normal and tumour tissues. Only one case showed a reaction in the tumour tissue but not in the normal tissue. The tumours that were positive by western blot showed positive carcinoma cells immunohistochemically. However, another four tumours that were positive by immunohistochemical study failed to show positive cells by western blot.

**Discussion**

Chromogranin A is widely distributed in peptide/amine endocrine cells of mammalian tissues such as the adrenal gland (adrenaline and noradrenaline cells), endocrine pancreas (A, PP, and \( \beta \) cells), gastrointestinal endocrine systems (EC, ECL gastrin, and others), thyroid (C cells), parathyroid and pituitary (TSH and gonadotropin cells), and some adrenergic nerves. Both light and electron microscope studies have shown a possible relationship between chromogranin A immunoreactivity and Grimelius's argyrophilia. In our experience, the positive cells containing neurosecretory granules were more easily detected by immunostaining for chromogranin A than by Grimelius staining.
Chromogranin from higher 4a...

Top: gland and the carcinoma cells and -A, Ii, ~~;6' ~;6'

Since the endocrine cells are thought to develop from the same endodermal progenitor cells as the other intestinal epithelial cells, the results of this study suggest that neuroendocrine cell differentiation may be controlled in the transitional mucosa.

It is controversial whether colorectal carcinomas that are rich in neuroendocrine cells have the same prognosis as those devoid of neuroendocrine cells. A limited number of studies addressing this question have been published. Smith and Haggitt reported that the presence of argyrophil cells, which were detected by the Churukian-Sehenk argyrophil stain, did not influence the prognosis of patients with colorectal carcinomas.1 On the other hand, Arends et al20 reported that colorectal carcinomas with serotonin positive enterochromaffin cells showed more aggressive behaviour than tumours without these cells. Hamada et al21 studied chromogranin positive colorectal carcinomas and concluded that patients with numerous endocrine cells had a significantly worse prognosis than patients without endocrine cells. We used almost the same method as Hamada; however, the results were different. Our results showed that the presence of chromogranin positive cells did not influence prognosis.2 The only difference in the methods was the number of slides examined, Hamada et al studied one paraffin block for each case, whereas we studied two to seven blocks which contained the longitudinal central slice of the tumour in each case. The information is therefore likely to be more precise in our study. In comparing grade III cases with negative cases, there was no difference in prognosis. We therefore consider that the presence or absence of neuroendocrine cells in colorectal carcinoma does not influence the prognosis.

Park et al22 found that immunohistochemistry was a more sensitive method than northern blot when a minority of the tumour cells are positive for a certain protein. This was recognised not only in our previous study of carbonic anhydrase I expression in colorectal carcinoma14 but also in this study of chromogranin A. Some immunohistochemically positive cases described here failed to be positive for chromogranin A when examined by northern or western blots. On the other hand, the Achilles' heel of immunohistochemistry is the interpretation of positive staining. The interpretation of the results of northern or western blots is more objective. We consider that immunohistochemistry can add significant in-

Figure 4 Top: The number and the distribution of positive cells for chromogranin A differed from gland to gland and from area to area (immunostaining, x 35). Bottom: A higher magnification of carcinoma cells positive for chromogranin A (immunostaining, x 140).

Figure 5 Northern blot analysis. Six of 32 cases examined are shown. Signals are seen in the normal tissue (N). In the tumour tissue (T) no signal is seen in many cases and it is weak in a few other cases. A, signal for chromogranin A; B, signal for p-actin as an internal control.
formation to molecular techniques such as northern or western blots to improve studies in surgical oncology.

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10 Murray SS, Deaven LL, Burton DW, O’Connor DI, Mellon PL, Defos LJ. The gene for human chromogranin-A (CgA) is located on chromosome 14. *Biochem Biophys Res Commun* 1987;142:141-6.


