

# Gastrin and gastrin receptor activation: an early event in the adenoma-carcinoma sequence

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## Abstract

**Background and aims**—Gastrin and the cholecystokinin type B/gastrin receptor (CCKBR) have been shown to be expressed in colorectal adenocarcinoma. Both exogenous and autocrine gastrin have been demonstrated to stimulate growth of colorectal cancer but it is not known if gastrin affects the growth of colonic polyps. The purpose of this study was to determine if gastrin and CCKBR are expressed in human colonic polyps and to determine at which stage of progression this occurs.

**Methods**—A range of human colonic polyps was assessed for gastrin and CCKBR gene and protein expression.

**Results**—Normal colonic mucosa did not express gastrin or CCKBR. Gastrin and CCKBR reverse transcription-polymerase chain reaction products were detected and verified by specific hybridisation with an oligo probe on Southern blots. Gastrin and CCKBR were expressed in 78% and 81% of polyps, respectively. Both genes were coexpressed in 97% of cases. Immunohistochemistry identified progastrin in 91%, glycine extended gastrin 17 in 80%, and amidated gastrin 17 in only 47% of polyps. CCKBR was present in 96% of polyps. Expression of gastrin and CCKBR was seen in all histological types and sizes of polyps.

**Conclusions**—This study is the first to show widespread expression of both gastrin and its receptor in colorectal polyps. Their activation occurs early in the adenoma-carcinoma sequence. Gastrin may promote progression through the adenoma-carcinoma sequence.

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Colorectal carcinoma is one of the most common cancers and is the second leading cause of cancer mortality in the USA.<sup>1</sup> The work of Muto and colleagues<sup>2</sup> revealed that the majority of colorectal carcinomas develop from an adenomatous polyp. Progression from adenoma to carcinoma requires a combination of genetic mutations which are either hereditary and/or occur as a result of environmental risk factors. Gastrin has been extensively studied as a growth factor for colorectal adenocarcinoma but few studies have investigated its role in the adenoma-carcinoma sequence.

Numerous studies have demonstrated the proliferative effect of exogenous hypergastrin-

naemia on in vitro colonic cancer cell lines<sup>3-6</sup> and in tumours in vivo.<sup>7-9</sup> The action of gastrin in colorectal cancer is potentially mediated by several receptor subtypes. The presence of a high affinity binding site has been seen in 57% of samples,<sup>10</sup> although gastrin/cholecystokinin type B receptor (CCKBR) mRNA has only been demonstrated in less than 20% of samples.<sup>11, 12</sup> However, other receptor isoforms may be responsible for the proliferative action of gastrins, including the cholecystokinin type C receptor,<sup>11</sup> the glycine extended gastrin 17 (Gly-G17) receptor,<sup>13</sup> or potentially truncated isoforms of CCKBR.<sup>14, 15</sup>

Following malignant transformation of the colonic epithelial cell, there is activation of the gastrin gene<sup>16</sup> and the cell associated gastrin can act in an autocrine/paracrine manner.<sup>17</sup> The presence of tumour associated gastrin has been demonstrated in several human tumour series.<sup>18-23</sup> These series showed that the non-amidated precursors progastrin and Gly-G17 are present in the majority of specimens, with a wide variation in the proportion demonstrating complete processing of gastrin. The precursor peptides have assumed greater importance with recent reports demonstrating that Gly-G17 and progastrin have proliferative effects.<sup>13, 24</sup> Ciccotosto *et al* demonstrated that non-amidated and total gastrin levels are elevated in patients with colorectal cancer<sup>20</sup> and this excess systemic non-amidated gastrin may arise from the tumour.

The point in the adenoma-carcinoma sequence when the gastrin autocrine pathway is activated has not been determined. In normal colonic mucosa, gastrin production is confined to neuroendocrine cells.<sup>18</sup> Therefore, the gastrin autocrine pathway must be activated during the adenoma-carcinoma sequence. If autocrine gastrin activation is an early event, its presence may promote polyp progression, and furthermore, expression of CCKBR may allow the adenoma to be affected by circulating gastrin concentrations.

Only one limited study has examined expression of gastrin mRNA in six adenomatous polyps by northern blotting.<sup>18</sup> The aim of the present study, using a range of colorectal polyps, was to determine if colonic adenomas express either gastrin, CCKBR, or both, in a larger series of polyps. We have determined the

**Abbreviations used in this paper:** Gly-G17, glycine extended gastrin 17; CCKBR, gastrin/cholecystokinin type B receptor; RT-PCR, reverse transcription-polymerase chain reaction; DEPC, diethyl pyrocarbonate; dNTP, deoxynucleoside triphosphate; APC, adenomatous polyposis coli; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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point in the adenoma-carcinoma sequence when gastrin and CCKBR gene activation occur.

## Materials and methods

### PATIENTS

Polyp samples were collected from 60 patients taking part in the Nottingham colorectal cancer screening study between 1984 and 1991. Samples were formalin fixed and paraffin embedded. The histological type and size of polyp, confirmed by a consultant histopathologist, are shown in table 1. Median age of the patients was 67 years (interquartile range (IQR) 63–70), and there were 37 males and 23 females. Gastrin and CCKBR expression was assessed by both reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry.

### REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Sections of 10 µm thickness were cut from the tissue block and two 10 µm sections were placed in an Eppendorf tube. Xylene 1 ml was added, the sample was vortexed for five minutes, and placed in a warming cabinet at 50°C for at least 30 minutes. The sample was then homogenised, followed by five minutes of centrifugation at 13 000 *g*. Xylene was eluted and the process was repeated four times. Ethanol (100%) 1 ml was added to the sample, vortexed, and centrifuged at 13 000 *g*. Ethanol was eluted and the process was repeated. The Eppendorf tube was then placed in a heated block at 50°C to allow evaporation. Once dry, RNA extraction was undertaken.<sup>25</sup>

RNAzol-B (Biogenesis, Poole, UK) 1 ml was added to the sample and homogenised. Chloroform 100 µl (Sigma, Poole, UK) was added and the Eppendorf tube shaken vigorously for 15 seconds. The Eppendorf tube was placed on ice for five minutes, followed by 30 minutes centrifugation at 13 000 *g* at 4°C. The upper colourless aqueous layer, composed of approximately 400 µl, was carefully removed to a clean Eppendorf. An equal volume of isopropanol was added to the aqueous phase to precipitate RNA from the eluate, and mixed and kept on ice for at least 30 minutes. The sample was centrifuged at 13 000 *g* for 30 minutes. The supernatant was decanted, the pellet washed with 1 ml of 75% ethanol, and centrifuged at 8000 *g* at 4°C for seven minutes. Following the final wash, ethanol was allowed to evaporate from the tube and the pellet was resuspended in 50 µl of 0.1% diethyl pyrocarbonate (DEPC) treated water.

Total RNA preparations were divided into two 25 µl aliquots, one of which became the RT negative control by omission of the RT enzyme. Random hexamer primer (90 U/ml) 3 µl was then added and heated to 70°C for 10 minutes.

The tubes were cooled on ice, after which the RT reaction buffer was added, which was composed of 5 µl 10× PCR buffer (Flowgen, Litchfield, UK); 1.5 µl of 5 mM deoxynucleoside triphosphate (dNTP) (Pharmacia, Little Chalfont, UK); 5 µl of 0.1 M deoxythymidine

triphosphate (GibcoBRL, Irvine, UK); 1 µl of 200 U Superscript RT (GibcoBRL); and DEPC treated H<sub>2</sub>O to make the reaction mixture volume 50 µl. In the negative control, the RT enzyme was replaced with water, vortexed, incubated for 10 minutes at room temperature and then for one hour at 37°C and 95°C.

Details of all primers and probes have been described previously.<sup>26</sup> PCR, gel electrophoresis, and Southern blotting were performed using protocols described previously.<sup>26</sup> In this study different cDNA volumes were used for individual reactions. Reactions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and gastrin mRNA detection (50 µl) were prepared using the following: 1.0 U of Dynazyme (Flowgen) in 1× Dynazyme PCR buffer (Flowgen) with 40 µmol of dNTPs (Pharmacia) and 10 µmol of upper and lower primers in a 50 µl final volume. The volumes of cDNA preparations were 1 µl for GAPDH and 5 µl for gastrin. The reaction for total CCKBR mRNA (the primer pairs were designed to detect long/short and truncated isoforms) detection was as follows: 2.5 U Amplitaq Gold (PE Applied Biosystems, Warrington, UK) in 1×PCR buffer (PE Applied Biosystems) and 1.5 mM MgCl<sub>2</sub> (PE Applied Biosystems) with 40 µmol of dNTPs (Pharmacia) and 10 µmol of upper and lower primers, to which 10 µl of CCKB cDNA were added.

### IMMUNOHISTOCHEMISTRY: GASTRIN PEPTIDES AND GASTRIN/CCKB RECEPTOR

Paraffin embedded sections (5 µm) were adhered to glass coated slides, dewaxed, deparaffinised, and rehydrated in alcohol. The sections were then incubated in 10% hydrogen peroxide in methanol for 15 minutes at room temperature to quench endogenous peroxidase followed by two further alcohol washes and a one minute water soak.<sup>27</sup>

The sections were then blocked in swine serum (DakoPatts, Cambridge, UK) for 20 minutes at room temperature, excess serum was removed, and sections were incubated with either a 1/100 dilution of rabbit antiserum directed against carboxy amidated gastrin, 1/80 diluted rabbit antiserum against glycine extended gastrin, rabbit 1/40 diluted antiserum against progastrin (provided by Andrea Varro), or 1/40 rabbit anti-CCKBR (GRP1) antiserum (directed against the amino terminal domain of the human CCKBR) with and without pre-absorption (Aphtron Corporation, California, USA) for 60 minutes. The antisera against the different gastrin species have previously been characterised in studies by immunoprecipitation and high pressure liquid chromatography.<sup>28</sup> The anti-CCKBR antiserum has been characterised by western blotting.<sup>29</sup>

The methodology for primary and secondary antibody staining has been described previously.<sup>27</sup> For assessment of the presence, degree, and intensity of staining for gastrin peptides in colonic samples, image analysis was performed by the use of the Leica Qwin Image processing and analysis system run on a Leica Q5001W PC. A program was used enabling cross sectional area of DAB staining to be specifically

Table 1 Immunohistochemistry results (mean (SD)) for each group of polyps; 6–10 fields were examined on each sample

Polyp (adenoma) type	No of samples	Immunohistochemistry (% positive staining)			
		Pro-gastrin	Gly-G17	G17-NH2	CCKBR
Normal mucosa	5	5 (1.8)	2 (1.1)	3 (0.9)	6 (2.6)
Tubular <1 cm	5	53 (12.3)	25 (8.7)	15 (6.1)	51 (13.6)
Tubular 1–1.9 cm	5	35 (7.8)	20 (7.2)	18 (7.3)	34 (10.8)
Tubular >2 cm	5	33 (13)	21 (8.3)	12 (5.9)	43 (12.3)
Tubular villous 1–1.9 cm	5	56 (14.2)	14 (5.4)	17 (6.5)	40 (8.3)
Tubular villous >2 cm	5	30 (8.5)	17 (11.3)	13 (8.5)	38 (15.1)
Villous 1–1.9 cm	5	38 (9.2)	25 (6.1)	7 (3.8)	44 (8.2)
Villous >2 cm	5	48 (11.7)	31 (9.8)	9 (6.7)	32 (11.3)
Severe dysplasia <1 cm	5	41 (10.1)	25 (7.8)	20 (8.9)	37 (10)
Severe dysplasia 1–1.9 cm	5	32 (6.7)	21 (7.2)	12 (7.3)	44 (11)
Severe dysplasia >2 cm	5	57 (12.3)	31 (7.5)	17 (6.5)	42 (10.3)
Polyp cancer	5	41 (11.2)	27 (6.7)	9 (4.3)	57 (14)

measured and expressed as a function of total cross sectional area of tumour tissue. The positively stained areas appear black/brown and were selected by density and wavelength of staining and expressed as a percentage of total adenoma tissue on the section. Six to 10 fields were assessed per section. Interobserver variation was found to be 5.5% and intraobserver variation 7.2%. This was performed by assessing the variation in the scoring of a complete section either twice by the same individual or by two observers. Identical fields were therefore not used for this analysis.

## Results

### REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

GAPDH was obtained from 42/60 cDNA samples analysed. The absence of GAPDH implied failure either to extract messenger RNA from the sample or of RT. Normal human gastric antrum cDNA samples gave an expected gastrin PCR product of 125 bp which was detected on Southern blots by a specific oligo probe. There was no expression of gastrin in the normal mucosa from non-malignant cases (5/5).

Gastrin was expressed in 29/37 polyps where GAPDH was obtained. Gastrin PCR products from the products showed a two band pattern that has been reported previously in colonic adenocarcinoma.<sup>30</sup> The expected product, derived from a mature mRNA, of 215 bp was coexpressed with a higher molecular weight band of 345 bp. Negative control RT-PCR lanes showed no hybridisation on Southern blots, confirming that the 345 bp band was derived from a gastrin RNA species. The gastrin PCR primers bind to regions on exons 2 and 3 of the cDNA and therefore the extra band is derived from retention of the intron as previously reported.<sup>30</sup>

The primers used for CCKBR RT-PCR detected both CCKB and the truncated CCKB isoforms. The expected CCKBR RT-PCR products were detected and verified by specific hybridisation with an oligo probe on Southern blots in 30/37 samples in which GAPDH was identified. The receptor was coexpressed with gastrin in 29/30 cases.

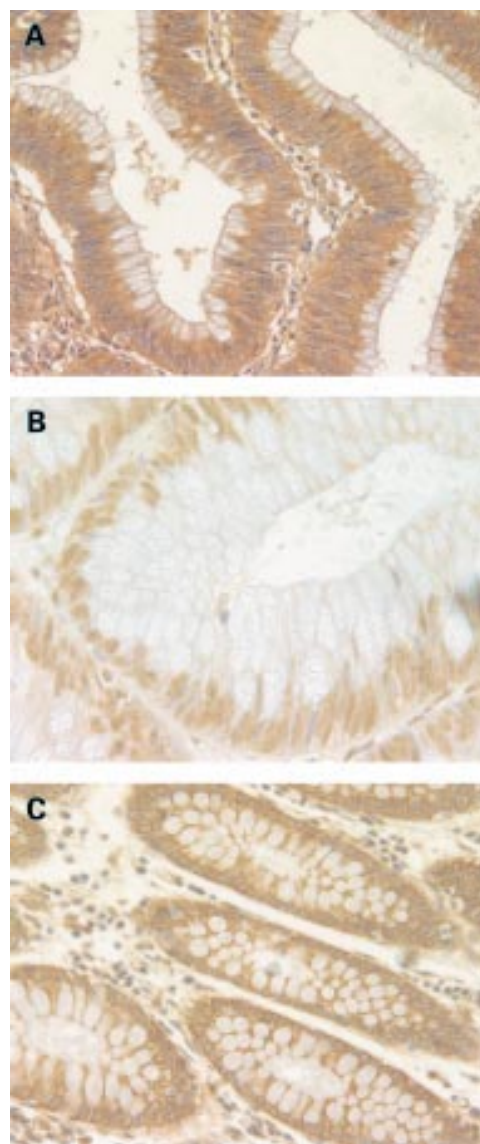


Figure 1 (A) Progastrin staining was seen in the cytoplasm in 91% of polyps. (B) Cytoplasmic staining of glycine extended gastrin 17 was seen in 80% of polyps. (C) Gastrin/cholecystokinin type B receptor was present in 96% of polyps.

### IMMUNOHISTOCHEMISTRY

The distribution of staining for gastrin and CCKBR in the control gastric antrum and fundus has previously been reported.<sup>27</sup>

Immunohistochemistry results for normal mucosa and polyps are presented in table 1. Positive staining is expressed as a percentage of total adenoma tissue on the section. A value of 10% staining was used as the minimum to indicate the presence of the peptide. In the normal control colonic mucosa there was no staining of gastrin peptides or CCKBR. Progastrin staining was seen in the cytoplasm in 91% of polyps (fig 1A), with a mean of 41% (SD 11.2%) coverage. Cytoplasmic staining of Gly-G17 was seen in 80% of polyps (fig 1B) (mean 23% (SD 7.9%) coverage), and amidated gastrin 17 only in 47% (13.2% (SD 6.8) coverage). CCKBR was present in 96% (fig 1C) with 43% (SD 13.3) coverage. Staining for

CCKBR was located within the cytoplasm but the intensity was greatest at the nucleus.

Expression of gastrin peptides and CCKBR occurred in small tubular adenomas <1 cm. Expression of the peptides was seen in all histological types of polyp; there was no difference in the proportion and level of expression between the histological types.

### Discussion

Ours is the first study to show widespread expression of both gastrin and its receptor in colorectal polyps. Immunohistochemistry demonstrated that the gastrin protein was expressed in epithelial cells in the majority of human colonic polyps, irrespective of histological type or size. CCKBR was also expressed in the majority of polyps. Staining intensity for CCKBR was greatest at the nucleus due to rapid internalisation and translocation following ligand binding to the receptor, as previously reported.<sup>31-32</sup>

In all samples expressing gastrin there was accumulation of gastrin pre-mRNA. Gene specific accumulation, and also export to the cytosol, has been reported for the CD44 gene in gastrointestinal tract tumours.<sup>33</sup> It is not known if gastrin and CD44 pre-mRNAs are retained via the same mechanism. If this is the case then gastrin pre-mRNA may also be expected to be exported from the nucleus and translated into a novel protein product where any potential biological activity may be assayed. Its high prevalence in tumour cells would be consistent with it imparting some advantage on tumour growth.

As expression of gastrin occurs in all polyps, including simple tubular adenomas less than 1 cm in size, this indicates that activation of the gastrin autocrine pathway may be an early event in the adenoma-carcinoma sequence. As the potential components necessary for an autocrine pathway to be functional are present, there may be enhanced proliferation/progression of the tumours. In addition, there may be expression of alternate receptors that may mediate the action of precursor gastrins, but as these remain to be characterised it was not possible to assess these in the present study.

Mutational change in the adenomatous polyposis coli (APC) gene is an early event in the adenoma-carcinoma sequence.<sup>34</sup> A mutant APC gene has been shown to upregulate additional genes.<sup>35</sup> Potentially the APC gene may partly control gastrin gene expression. Indirect evidence of this potential interaction has been seen in the APC1638N model of polyposis coli where there was expression of gastrin in the histological normal colonic mucosa of mutant mice.<sup>36</sup>

Furthermore, enhanced expression of CCKBR may make the polyps susceptible to changes in the concentration of circulating gastrin (particularly gastrin 17) that occurs in conditions of hypergastrinaemia. Hypergastrinaemia may increase the polyp proliferation rate and as a result promote progression through the adenoma-carcinoma sequence.

It has been shown that hypergastrinaemia increases the proliferation rate of the normal colonic mucosa.<sup>37</sup> Long term exposure to

hypergastrinaemia, due to increased cell turnover of the mucosa, may increase the chance of a spontaneous mutation and eventual tumour formation. This was confirmed in a recent nested case control study by Thorburn and colleagues.<sup>38</sup> They demonstrated that gastrin levels above normal were associated with an increased risk of colorectal malignancy (odds ratio 3.9; 95% confidence interval 1.5–9.8).

In conclusion, we postulate that gastrin may act at several points in the adenoma-carcinoma sequence. Hypergastrinaemia may increase the turnover of the normal colonic epithelium and lead to an increased incidence of polyps. Following transformation of the epithelium, tumour cells express their own gastrin which can further promote growth. In addition, polyps may have enhanced susceptibility to exogenous circulating gastrin due to expression of CCKBR.

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