Expression of granulocyte colony stimulating factor receptor in human colorectal cancer

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Aims: To discover if human colorectal cancer expresses granulocyte colony stimulating factor receptor (G-CSFR) and if granulocyte colony stimulating factor (G-CSF) plays an important part in the development and progression of human colorectal cancer.

Methods: Forty two specimens of colorectal cancer and normal colorectal mucosa were investigated, taken from the colon or rectum in group of colorectal cancer patients. Immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) technique were used to show G-CSFR expression. The relation between expression of G-CSFR and clinical or pathological factors was analysed.

Results: Immucohistochemical analyses showed that G-CSFR was expressed in the human colorectal cancer (25 of 42, 59.52%) and seemed to be up-regulated compared with the normal mucosa (14 of 42, 33.33%, $p<0.001$). In pronounced contrast with mostly strongly positive tumours, corresponding normal colorectal mucosa was negative or weakly positive. A significant correlation was found between G-CSFR expression and tumour stage ($p=0.001$), tumour differentiation ($p<0.001$), but there was no significant relation between the expression of G-CSFR and the age, sex, and tumour size ($p=0.346$, $p=0.686$, $p=0.459$). In RT-PCR, 21 of all 42 tumours had G-CSFR mRNA expression, while only 11 of 42 normal colorectal mucosa had such expression.

Conclusions: These data show that G-CSFR is commonly expressed in human colorectal cancers, thus supporting a possible role for G-CSF in colorectal cancer physiology.

R ecombinant human granulocyte colony stimulating factor (rhG-CSF) is a peptide hormone known to be responsible for the in vitro and in vivo proliferation of bone narrow progenitor cells into mature differentiated cells. It is clinically used to overcome neutropenic periods during chemotherapy.[1] [2] In vitro studies using cell lines as a model system have recently suggested that G-CSF can promote cancer growth. G-CSFR was first studied on haematopoietic cells, such as myeloid progenitor cell,[3] [4] mature neutrophils,[5] platelets, monocytes, and some T and B lymphoid cell lines.[6] [7] Several non-haematopoietic cells also express G-CSFR including endothelial cell,[8] [9] placenta,[10–12] trophoblastic cell.[13] The role of G-CSF in malignant tumour cells as well as their significance in cancer development is not clear. The incubation of different cancer cell lines and primary tumours with recombinant human (rh) G-CSF induced diverse reactions. While the leukaemia cell line U-937 suffered growth inhibition,[14] the murine pro-B cell line BAF-3 reacts with a 60% raised DNA synthesis.[15] [16] Cell proliferation was also seen for the murine cell line NFS-60,[17] the osteosarcoma cell line MG63,[18] and bladder cancer cells.[19] Colorectal cancer (CRC) is a common malignancy. Whether it expressed G-CSFR has not been reported. The aim of the work is to investigate whether CRC cells as well as corresponding normal mucosa express G-CSF protein and mRNA using immunohistochemistry and RT-PCR.

METHODS

Patients and tissue samples

The case list of our study includes CRC specimens from 42 different patients with histologically confirmed CRC admitted to the department of general surgery of Jinling Hospital in Nanjing, China from September 2003 to December 2003. The age and sex of patients, tumour size, tumour differentiation, and tumour stage were recorded in all cases (table 1). Tumour tissue and normal mucosa tissue (taken 10 cm apart from the tumour) were obtained in all cases. Histological examination was performed routinely in all of the cases. Immunohistochemical analyses were also performed in all 42 cases. Tumour histotype and grade of differentiation were defined according to the WHO criteria.

Immunohistochemistry

For formalin fixed and paraffin wax embedded tissue specimens, consecutive 4 μm thick sections were cut and used for immunohistochemistry (IHC).

The sections were immunohistochemically stained by the labelled streptavidin-biotin peroxidase method (LSAB2 Kit; Dako Japan, Kyoto, Japan) with the following primary antibodies: mouse monoclonal antibodies G-CSFR (Serotec, United Kingdom, dilution 1:80). The slides were immersed for 10 minutes in 0.3% hydrogen peroxide/methanol to deplete endogenous peroxidase. Then, non-specific binding sites were blocked with 0.3% normal goat serum for 10 minutes. The primary antibody was then applied, and the sections were incubated overnight at 4℃. After washing with phosphate buffered saline (PBS) (0.01 mol/l pH 7.4), biotinylated goat antimouse IgG was applied to the tissue sections and incubated at room temperature for 10 minutes. After washing with PBS, a streptavidin peroxidase reagent was applied and incubated at room temperature for 10 minutes. Finally, the reaction product was visualised using developing colour by incubating the slides in a solution of 0.3% hydrogen peroxide and AEC chromogen. The sections were counterstained slightly with haematoxylin. Negative controls included parallel sections treated without the primary antibody, in addition to negate an adjacent section of the same block in which the primary antibody was

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Table 1 Summary of clinical data of 42 carcinomas and RT-PCR, immunohistochemical analyses

| | | | | | | | G-CSFR detection | | | |
| | | | | | | | RT-PCR | | IHC(0–3) | |
Case number	Age (y)	Sex	Position	Tumour size(cm)	Differentiation	Stage (I–IV)	T	N	T	N
1	63	M	R	1×2	poorly	II	+	+	3	2
2	70	M	R	4×5	poorly	III	+	−	3	0
3	75	F	R	2×3	moderately	IV	+	−	3	1
4	70	M	S	3×3	moderately	II	−	−	1	0
5	58	F	R	3×4	moderately	I	−	−	0	0
6	65	M	D	3×2	moderately	II	−	−	0	0
7	61	F	R	4×3	well	III	+	+	2	2
8	37	F	R	2×3	well	I	−	−	0	0
9	90	M	R	2×2	poorly	IV	+	+	3	2
10	39	M	R	4×5	well	II	−	−	0	0
11	55	M	D	3×4	moderately	II	+	+	2	2
12	73	M	R	3×4	moderately	I	−	−	0	0
13	46	F	R	3×3	poorly	I	+	−	3	1
14	60	F	A	4×5	poorly	III	+	+	3	3
15	56	M	S	3×5	moderately	III	−	−	0	0
16	47	F	R	2×3	poorly	I	+	−	2	0
17	77	M	R	3×4	moderately	II	+	+	2	1
18	55	M	T	2×3	moderately	I	−	−	1	0
19	46	M	R	4×4	well	II	−	−	0	0
20	65	F	R	5×5	well	II	−	−	0	0
21	73	F	A	3×5	moderately	II	−	−	0	0
22	65	F	R	2×3	moderately	III	+	−	2	0
23	48	M	R	1×3	well	I	−	−	0	0
24	80	M	R	5×6	moderately	II	−	−	1	0
25	46	F	S	1×1	moderately	I	−	−	1	0
26	33	M	R	2×3	poorly	II	+	+	3	2
27	44	M	R	2×2	moderately	II	+	−	1	0
28	49	F	A	4×4	well	I	−	−	0	0
29	67	F	R	5×6	moderately	II	+	−	2	0
30	76	M	R	3×5	moderately	III	+	+	3	1
31	71	M	R	1×2	well	I	−	−	0	0
32	63	M	A	5×6	poorly	IV	+	+	3	2
33	74	M	R	4×5	moderately	II	+	−	2	1
34	80	F	D	1×2	moderately	I	+	−	1	0
35	72	M	R	2×3	well	II	−	−	0	0
36	43	M	A	2×2	well	II	−	−	0	0
37	50	F	R	4×5	moderately	III	+	−	2	0
38	60	M	D	2×3	moderately	II	−	−	0	0
39	64	M	R	3×3	well	I	−	−	0	0
40	48	F	R	3×4	moderately	IV	+	+	3	2
41	50	F	R	2×2	moderately	II	−	−	0	0
42	15	M	R	6×7	poorly	III	+	+	3	3

M, male; F, female; R, rectum; S, sigmoid colon; D, descending colon; A, ascending colon; T, transverse colon; well, well differentiation; moderately, moderately differentiation; poorly, poorly differentiation; RT-PCR, reverse transcription polymerase chain reaction; IHC, immunohistochemistry; T, tumour; N, normal colorectal mucosa.

replaced by PBS. The immunoreactions were graded as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3) according to the immunostaining intensity by at least two pathologists who were blinded to this study.
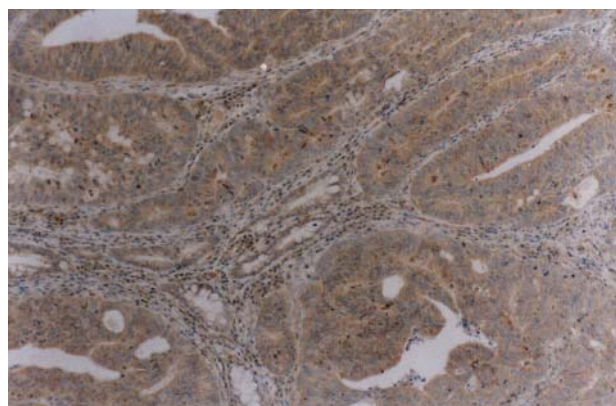
RNA extraction

Forty two freshly frozen colorectal cancer tissue samples and normal colorectal mucosa, 80 mg of each, were collected and minced and total RNA was extracted with the guanidinium thiocyanate method. RNA integrity was checked by agarose gel electrophoresis and ethidium bromide staining as well as monitoring absorbance at A260/280 before each RT-PCR. RNA concentration was determined by spectrophotometric analysis at 260 nm and before each RT-PCR.

RT-PCR

The key points of the RT-PCR method are summarised as follows. One step RNA PCR Kit was used for PCR amplification in 50 μl volume containing 5 mmol/l $MgCl_2$, 0.8 U/μl RNase inhibitor, 0.1 U/μl AMV reverse transcriptase XL, 0.4 μmol/l sense and antisense primer, 0.2 mmol/l of dNTP, 0.1 U of AMV-opitimised Taq polymerase, and 5 μl 10 × reaction buffer (TaKaRa Biotechnology, Dalian, China). RNA (1 mg) was converted into cDNA by incubating 30 minutes at 50°. The mixture was incubated for two minutes at 94° to inactivate reverse transcriptase XL. PCR amplification was
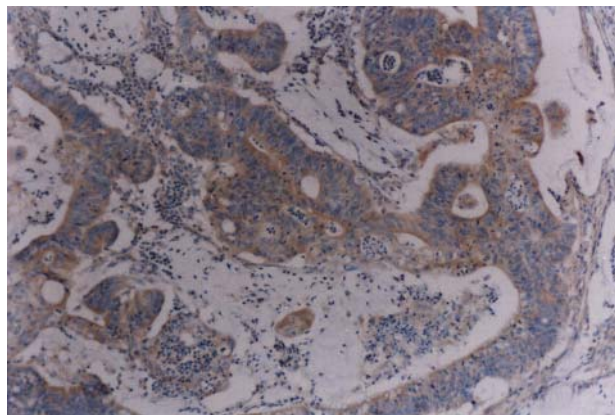

Figure 1 G-CSFR positive immunostaining of colorectal cancers (SP×100).

Figure 2 G-CSFR positive immunostaining of colorectal cancers (SP×100).

performed for 30 cycles consisting of 30 seconds at 94°, one minute at 62°, and one minute at 72° plus elongation seven minutes at 72°. After amplification 20 μl of the PCR reaction mix were electrophoresed in a 2% (wt/vol) agarose gel. The PCR-amplicon had a size of 276 bp (figs 2 and 3). For the PCR reaction, the following primers were used: 5'-CCTGGAGCTGAGAACTACCG -3' (sense) and 5'-TCCCGGCTGAGTTATAGG -3' (antisense). PCR primers were designed according to the published sequence of G-CSF receptor cDNA. The quality of cDNA for amplification reaction was proved by β actin amplification by the same PCR protocol. As a result a 599 bp-amplicon was obtained. The β actin primers were designed as follows: sense primer: 5'-CATTTCCGGTGCACGATGGAG-3' and the antisense primer: 5'-GCCATCCTAGCGTCTGGACCTG-3'.

Statistical analysis

The Mann-Whitney U and Kruskal-Wallis tests were used to examine the correlation between various clinical or pathological parameters and the expression of G-CSFR of tumours. Differences in G-CSFR expression between tumours and normal colorectal mucosa as determined by IHC analysis were evaluated using a Wilcoxon signed ranks test. A p values less than 0.05 were considered significant. All the calculations were performed by SPSS 10.0 for Windows.

RESULTS

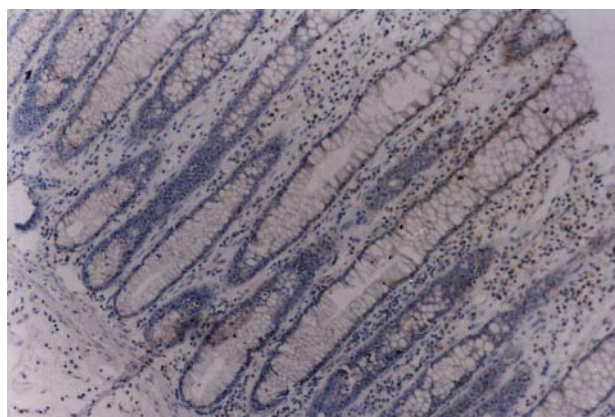Table 1 summarises the patient and tumour characteristics. The 42 patients ranged in age from 15 years to 90 years

(median 59). There were 25 male patients and 17 female patients. Tumours ranged from 1 mm^2 to 42 mm^2 in area. Most of the differentiation of tumours was moderately differentiation (n = 22), and the remainder were well differentiation (n = 11), poorly differentiation (n = 9). The patients included stage I (12), stage II (18), stage III (8), and stage IV (4).

Immunohistochemistry

Table 1 summarises the G-CSFR immunostaining results. A variable degree of cytoplasmic staining of tumour cells was seen in 25 of the 42 analysed tumours (59.52%) (figs 1 and 2). Weak or moderate cytoplasmic staining also occurred in some stromal cells (figs 1 and 2). Seventeen of all 42 tumours were negative. Only 14 of 40 normal mucosa expressed the G-CSFR protein (33.33%).The corresponding normal colorectal mucosa was negative or weakly positive (figs 3 and 4), sharply contrasting with mostly strongly positive tumours (figs 1 and 2). A significant inverse correlation was found between G-CSFR expression and tumour stage (p = 0.001), tumour differentiation (p<0.001). There was no significant difference between G-CSFR expression and age (p = 0.346), sex (p = 0.686), and tumour size (p = 0.459).

RT-PCR

Amplified fragments of the expected sizes (276 bp) were detected in 21 of all 42 tumour samples examined (fig 5 and table 1). In 11 cases, G-CSFR mRNA expression was also

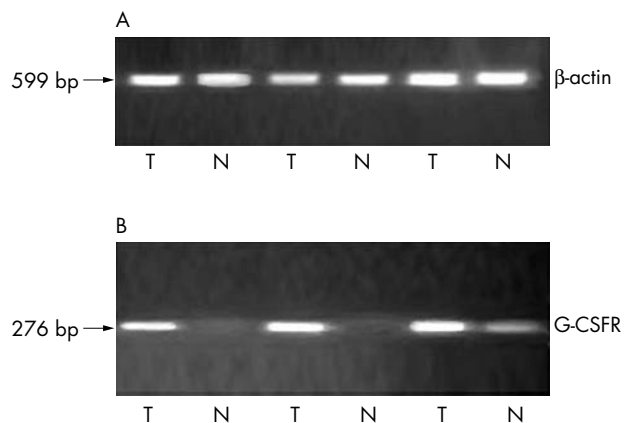**Figure 4** Negative G-CSFR immunostaining of the normal colorectal mucosa (SP×100).

Figure 3 Negative G-CSFR immunostaining of the normal colorectal mucosa (SP×100).

Figure 5 (A) RT-PCR of β actin; (B) representative RT-PCR of G-CSFR expression in three colorectal cancer tissues and corresponding colorectal mucosa.
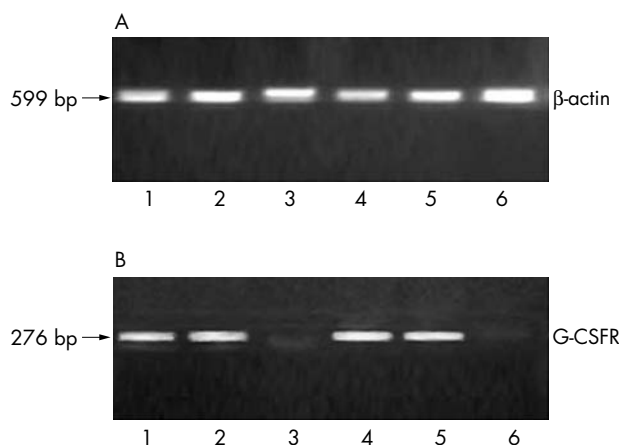
Figure 6 (A) RT-PCR of β actin; (B) RT-PCR of G-CSFR expression in six colorectal cancer cases.

assessed in normal colorectal mucosa, and the remaining 31 cases had no G-CSFR mRNA expression (fig 6 and table 1).

DISCUSSION

The expression of cytokine receptors on malignant tumour cells[20][21] as well as the cytokine balance in the tumour microenvironment[22][23] may be relevant for cell proliferation, differentiation, or apoptosis. Receptors for G-CSF have been found on cell lines derived from human small cell carcinoma of the lung,[24] in astrocytoma cell lines,[25] ovarian cancer cell lines,[26] and on primary bladder cancer[27] and nasopharyngeal carcinoma.[28] In this work, we investigated the expression of G-CSFR on CRC. Immunohistochemistry studies showed the presence of G-CSFR on 31 of 42 colorectal cancers, while only 20 of 42 corresponding normal colorectal mucosa expressed G-CSFR. These results confirm the expression of G-CSFR in CRC and show for the first time that its expression seemed to be up-regulated compared with the normal mucosa. The adjacent normal mucosa showed no or weak immunoreactivity for G-CSFR, but most carcinomas showed strong or moderate diffuse cytoplasmic staining. Thus, G-CSFR is expressed in CRC cells in vivo. This finding reinforces our understanding of the role of G-CSF in CRC.

The behaviour of colorectal tumours expressing G-CSFR may therefore be influenced by G-CSF local concentration. G-CSF can be produced either by white blood cells, which frequently infiltrate colorectal tumours,[29] or even by cancer cells themselves,[30][31] as has been shown for cultured bladder carcinoma[31] and astrocytoma cell lines.[32] We had not looked for G-CSF production in this report. However, it is important to point out that certain conditions might be required to trigger G-CSF production in these cells. Autocrine/paracrine mechanisms have been suggested.

The downstream G-CSF signalling in non-haematopoietic cells is not well understood.[33] In contrast, it has been intensively studied in haematopoietic cells.[34] Several phosphorylation cascades,[35] including the ras-MAP kinase route and JAK tyrosine kinases, are activated by ligand binding to G-CSFR. Distinct regions of the G-CSF receptor[36] cytoplasmic domains are required for interaction with specific molecules of each pathway. The differential expression of the five G-CSFR isoforms,[36–38] which differ in their cytoplasmic domains, is speculated ultimately to regulate proliferation and maturation of myeloid cells. In this study, the function of G-CSFR has not been detected. The proliferative effect of G-CSF to CRC in vivo and in vitro and the mechanism were also not studied. In fact, Takeshi Natori et al[39] found that G-CSF promoted tumour growth and neovascularisation in vivo,

whereas G-CSF had no effect on cell proliferation in vitro. Sara Calatayud et al[40] reported diminished spontaneous apoptosis or sulindac sulphide induced apoptosis of human colon epithelial HT-29 cells in the presence of CSF. Their results suggest that G-CSF probably promotes tumour growth by stimulating neovascularisation or reducing apoptosis.

To further understand the mechanisms involved, additional studies are necessary, including analyses of benign colorectal lesions and precancerous conditions.

Recently, a significant association between G-CSFR expression in cancer cells and a poor prognosis in patients with oral and nasopharyngeal squamous cell carcinoma has been reported. To improve clinical approaches with patients suffering CRC it seems important to find out if similar associations are also present. To find correlations between G-CSFR expression in colorectal cancer and clinical parameters as well as prognostic factors, further studies with higher number of cases are needed.

In summary, the results presented here show that G-CSFR is frequently expressed in CRC cells. Further research is required before deciding if our findings may have clinical implications for the use of G-CSF on CRC.

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