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European Journal of Cancer 41 (2005) 1618–1627

European Journal of Cancer

www.ejconline.com

Guanylyl cyclase C as a reliable immunohistochemical marker and its ligand *Escherichia coli* heat-stable enterotoxin as a potential protein-delivering vehicle for colorectal cancer cells

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Received 17 November 2004; received in revised form 10 February 2005; accepted 17 February 2005
Available online 24 May 2005

Abstract

mRNA-based technologies and preclinical research in a variety of animal models have shown that guanylyl cyclase C (GCC) is a highly sensitive and specific molecular marker for the diagnosis of colorectal cancer (CRC). GCC is also a receptor for *Escherichia coli* (*E. coli*) heat-stable enterotoxin (STa) and can be used for STa-directed delivery of small-sized imaging agents to human CRC tumours. In this study, we have evaluated GCC as a new immunohistochemical (IHC) marker for CRC tissues and STa as a suitable vector for delivering high-sized protein molecules to CRC cells. Firstly, we have developed a highly sensitive EnVision^{+}-based IHC staining method for detecting GCC in serial paraffin-embedded sections of primary and metastatic CRC (38 cases) or non-CRC (14 cases) adenocarcinomas. Carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) were chosen as controls. Our results indicate that GCC staining was positive in 100% of CRC tumours and was comparable to CEA (95%) or CK20 (92%). In contrast to CEA and CK20, GCC was negative in all of the extra-intestinal non-CRC tumours examined. GCC appears to display higher specificity than either CEA or CK20 while retaining high sensitivity, suggesting that it is a better CRC marker than CEA or CK20. Secondly, STa was genetically coupled to green fluorescent protein (GFP) and the resulting GFP-tagged STa was characterized for expression in *E. coli* and enterotoxicity in mouse. The binding characteristics of GFP-STa in CRC Caco-2 cells were followed by immunofluorescence microscopy. In this work we show that GFP-tagged STa is biologically active and has retained its ability to internalise into Caco-2 cells making it a potential vehicle for the delivery of anticancer therapeutic protein agents.

Keywords: Colorectal cancer; Guanylyl cyclase C; Heat-stable enterotoxin; Immunohistochemistry; Immunofluorescence; Fusion protein; Protein delivery

1. Introduction

Colorectal cancer (CRC) still lacks a tumour-specific marker capable of improving patient care through better screening, diagnosis, prognosis and prediction of disease recurrence. No marker exists to achieve greater success in treatment by exploiting it as a therapeutic target. The great majority of once promising tumour markers have failed because of their lack of specificity and sensitivity when used in the general population [1]. Currently, carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) are the two most frequently used CRC tumour markers in routine clinical practice. However, despite high sensitivity, the lack of specificity of both CEA and CK20 in immunohistochemical (IHC) and reverse-transcriptase polymerase chain reaction (RT-PCR) assays remains a major concern. CEA and CK20 have

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doi:10.1016/j.ejca.2005.02.031

been reported to be positive in various types of normal tissues, several other types of cancer cells and blood cells from healthy volunteers [1–5]. The lack of colon tissue-specificity limits the use of CEA and CK20 as relevant CRC markers. In contrast, due to its restricted expression pattern, guanylyl cyclase C (GCC), the transmembrane receptor for ligands guanylin, uroguanylin, lymphoguanylin and *Escherichia coli* (*E. coli*) heat-stable enterotoxin (STa) [6], has been proposed to be a new target for the diagnosis, staging, recurrence prediction and post-operative surveillance of patients with CRC [2,7–14]. GCC is specifically only expressed in intestinal mucosa cells from the duodenum to the rectum and protein expression is retained after neoplastic transformation into adenocarcinoma cells [7]. Indeed, in contrast to extra-intestinal tissues, tumours or cell lines, all histologically confirmed adenomatous polyps, primary/metastatic colorectal tumours and CRC cell lines examined by RT-PCR express GCC [7,8]. Importantly, GCC-specific RT-PCR detection of micrometastases in histologically negative lymph nodes was associated with a greater risk of CRC-related mortality [9–11]. It was also found that GCC RT-PCR assays could detect circulating colonic epithelial cells in both peripheral and portal venous blood of CRC patients [2,12–14] and emphasise the clinical utility of GCC in CRC staging and post-operative surveillance.

Surprisingly, no study on the potential value of GCC as a new IHC marker for human colonic adenocarcinomas has been published until now. Some studies have shown GCC detection by IHC in animal tissues and cell lines [15–17]. However, most reports on the expression of GCC in human CRC cells use mRNA-based technologies, although new and very sensitive IHC assays have recently become available (e.g. the EnVision$^+$ technique and the antigen retrieval methods). IHC is simple, quick, inexpensive, currently used in routine clinical practice and has the advantage of eliminating sampling error from test tissues by confirming tumour cells with microscopic observation. On the other hand, use of RT-PCR as a routine tool in clinical medicine remains questionable, especially with regard to its simplicity, quickness, cost, standardisation across laboratories, automation and hypersensitivity [4,12,18]. In this study, we have developed an EnVision$^+$ based technique [19] IHC procedure for targeting GCC, CEA and CK20 in paraffin-embedded sections of normal and malignant colorectal or extra-colorectal tissues.

In addition, several studies have now indicated that radionuclide-conjugated STa is capable of selectively targeting GCC in CRC metastases in mice [20,21] and suggest the potential utility of STa to deliver imaging or chemotherapy agents directly to metastatic CRC cells in humans. We have previously established that STa that is genetically fused to another heterologous protein still retains its native biological properties (secretion, enterotoxicity, folding, GCC recognition) [22,23]. In this work, we have fused human STa to the green fluorescent protein (GFP) and examined its ability to deliver GFP into GCC-expressing CRC Caco-2 cells by immunofluorescence. From these data, and given that native STa undergoes ligand-dependent GCC receptor-mediated endocytosis in human CRC cell lines [24], we hypothesise that the small (19 amino acids), poorly antigenic, non-immunogenic STa toxin is a highly attractive tool as a cell targeting and delivering vector, not only for small therapeutic molecules like radionuclides, but also for large proteinaceous anticancer agents.

2. Materials and methods

2.1. Patients and tissue processing

Tissue samples were collected from patients who had undergone colorectal carcinoma resection in Hotel-Dieu Hospital, Clermont-Ferrand, France from 2002 to 2004. The specific details of these tissues and patients are indicated in the Section 3 of this paper. None of the patients received chemotherapy or radiotherapy prior to surgery. Histopathology confirmed the presence or absence of adenocarcinoma and was used to determine the degree of differentiation, TNM classification or size of the tumour. A sample (1 cm^3) of non-necrotic tumour tissue and unaffected tissue at a standard distance from the tumour were also taken from each resected specimen. Both tissues were either snap frozen in liquid nitrogen and stored at −80 °C or fixed for 20 h and processed in an automatic processor for paraffin sectioning. Tissues were dehydrated in various grades of ethanol (70% v/v for 1 h, 95% v/v for 1 h, absolute for 2×2 h), cleared three times (0.5, 1, and 1.5 h) with toluene, infiltrated three times (1, 1.5, and 2 h) with paraffin heated at 56 °C and embedded with paraffin wax in cassettes. Serial cryostats and paraffinised sections were cut at 5 μm and placed on Fisher Starfrost® slides (two sections per slide). Frozen tissue slides were stored at −20 °C, while paraffinised tissue slides were incubated at 56 °C for 45 min, then at 37 °C for 5 days and stored at room temperature (RT).

2.2. Immunohistochemistry

IHC staining was performed on frozen and paraffin-embedded tissue sections using the Dako (Copenhagen, Danemark) EnVision$^+$ System peroxidase kit protocol. This is a two-step IHC method in which application of the primary antibody is followed by a polymeric conjugate consisting of a large number of secondary antibodies (goat anti-mouse) bound directly to a dextran backbone containing horseradish peroxidase (HRP). One such conjugate contains up to 100 HRP molecules

Table 1
Primary antibodies and applications used in this study

Antibody (designation)	Isotype	Applications	Source (Reference)
Anti-GCC (GCC:4D7)	$IgG_{2a\kappa}$	IHC	[17]
Anti-STa (20C1)	IgG	IF, WB	[30]
Anti-CEA	$IgG_{1\kappa}$	IHC	Dako (#M7072)
Anti-CK20	$IgG_{2a\kappa}$	IHC	Dako (#M7019)
Anti-glucose oxidase	$IgG_{1\kappa}$	IHC	Dako (#X0931)
	$IgG_{2a\kappa}$	IHC	Dako (#X0943)
Anti-GFP	$IgG_{2a\kappa}$	IF, WB	Clontech (#8371)
Anti-His tag	IgG	WB	Clontech (#8909)

GCC, guanylyl cyclase C; STa, heat-stable enterotoxin; CEA, carcinoembryonic antigen; CK20, cytokeratin 20; GFP, green fluorescent protein; His tag, histidine tag; IHC, immunohistochemistry; IF, immunofluorescence; WB, Western blotting. GCC:4D7 is raised against the intracellular protein kinase-like domain of GCC and 20C1 is directed against the C-terminal part of STa. Anti-CEA and anti-CK20 were used as colorectal cancer standards, and anti-glucose oxidase isotypes were used as negative controls ($IgG_{2a\kappa}$ *vs.* anti-GCC or anti-CK20, and $IgG_{1\kappa}$ *vs.* anti-CEA). Glucose oxidase, an enzyme produced by *Aspergillus niger*, is neither present nor inductible in mammalian tissues. All antibodies are mouse monoclonal, except for anti-His tag that is a rabbit polyclonal.

Table 2
Effect of fixatives and fixation conditions on the IHC staining of GCC in frozen CRC tissues

Fixative	Temperature	Time (min)	Staining
Acetone	−20 °C; 4 °C; RT	0.50; 0.75; 2; 5; 10	0
Acetone–methanol (1:1)	−20 °C; RT	0.75; 10	0
Acetone–ethanol (1:1)	−20 °C	0.75	0
Acetone–methanol–16% PAF (7.5:7.5:1)	4 °C; RT	0.50; 0.75	0
Acetone–methanol–16% PAF (1.5:1.5:1)	4 °C; RT	0.50; 0.75	0
100% Ethanol	RT	10	0
100% Ethanol–H_2O (1:1)	RT	10	0
PBS–0.5% PAF	RT	5; 10	0
PBS–1% PAF	RT	5; 10	0
PBS–4% PAF	RT	10	–
PBS–1% PAF–0.1% Triton X-100	RT	10	0
PBS–2% PAF–0.2% Triton X-100	RT	10	0
H_2O-0.5% PAF	RT	5	–
Bouin	RT	5	+
Hollande's bouin	RT	5; 10	+++
Carnoy	RT	5	–
Zamboni	RT	10	0
Davidson's AFA	RT	10	++
10% Formalin	RT	5	–
10% Formalin saline	RT	5	–
10% Neutral formalin	RT	5	–
10% Lillie's neutral formalin	RT	5	–
10% Neutral buffered formalin	RT	5; 10	–
Zinc–10% formalin	RT	10	0
Zinc formalin–free	RT	10	0
Zinc–1% PAF	RT	10	0
0.1% Acetic acid–2.5% zinc chloride–15% formalin	RT	5; 10	0
5% Acetic acid–85% ethanol–10% formalin	RT	5; 10	0
PBS–2.5% formalin	RT	5; 10	–
PBS–5% formalin	RT	5; 10	–
PBS–10% formalin	RT	5; 10	–
Buffered 10% formalin–45% acetone	4 °C	0.50	0
Buffered 1% PAF–45% acetone	4 °C; RT	0.50; 0.75	0
Buffered 4% PAF–45% acetone	4 °C; RT	0.50; 0.75	0

IHC using EnVision$^+$ system and GCC:4D7 antibody was performed in duplicate. For some fixatives, incubation was carried through different temperatures and times. Staining was graded from 0 to 3+ according to its specificity, intensity, and evaluation. –, no staining; 0, not specific, moderately or strongly intense, evaluation not possible; +, specific, weakly intense and evaluation possible with some difficulty; ++, specific, weakly intense and evaluation possible without problem; +++, specific, strongly intense, and evaluation very easy. CRC, colorectal cancer; RT, room temperature; PAF, paraformaldehyde; PBS, phosphate-buffered saline pH 7.2–7.6; AFA, alcohol–formalin–acetic.

and up to 15 antibodies [19]. All primary antibodies used in this study are presented in Table 1. Staining was examined with a colour camera (Sony) equipped with a light microscope (Olympus BH-2) and images were analysed and captured with Biocom software (Biocom Technologies, WA, USA) and then assembled using Microsoft Power Point (Microsoft Corp.).

2.2.1. Detection of GCC in frozen tissues

The sections were air-dried for 30 min, fixed as indicated in Table 2, rinsed up-and-down two times in Tris-buffered saline pH 7.6 (TBS), rehydrated twice in TBS-0.1% Triton X-100 v/v for 5 min, and subjected to the staining procedure. The sections were incubated overnight at 4 °C with antibody GCC:4D7 or with positive or negative controls (Table 1) at 20 μg/ml each in TBS-0.1% Triton X-100 v/v, and washed twice in the same buffer for 5 min at RT. Sections were then incubated with the goat anti-mouse EnVision-HRP-enzyme conjugate for 30 min at RT, rinsed up-and-down in TBS, washed in TBS for 5 min and incubated with the EnVision-3,3′-diaminobenzidine plus (DAB^+) substrate-chromogen solution in the dark for 3 min at RT. After quick dipping in TBS then in distilled water, slides were counterstained with Meyer's hematoxylin (Dako) for 45 s, washed in tapwater and coverslipped with Faramount (Dako).

2.2.2. Detection of GCC in paraffin-embedded tissues

The slides were dried overnight at 37 °C, placed in a 56 °C hotplate for 10 min, and deparaffinised in two changes of xylene-substitute (D-limonene) for 10 min each. The sections were hydrated twice for 1 min in absolute ethanol, once for 2 min in 90% and 70% ethanol v/v, once for 5 min in distilled water and twice for 5 min in TBS. Heat treatment with Dako's target retrieval solution (a modified citrate buffer, pH 6.1) was performed in a water bath at 98.5 °C for 10 min followed by a 20-min cool down period at RT. The sections were washed twice for 5 min in distilled water and once for 5 min in TBS. They were then incubated at 37 °C for 1 h with antibody GCC:4D7 or with positive or negative controls (Table 1) at 5 μg/ml each in TBS. After two washes in TBS for 5 min at RT, IHC procedure was continued as described above for frozen tissues.

2.3. STa-mediated cell uptake of GFP

2.3.1. Construction and characterization of GFP-tagged STa

Histidine (His)-tagged GFP (HisGFP) was obtained by cloning the His tag-encoding *Hin*dIII/*Asp*718 fragment of the pHAT12 plasmid (Clontech, CA, USA) into the GFP-encoding *Hin*dIII/*Asp*718-linearised pGFPuv vector (Clontech) to place the GFP just downstream of the His tag sequence. His-tagged GFP-STa (His-GFP-STa) fusion was created in two steps. First, the STa-encoding *Hpa*I/*Eag*I fragment of the pProSTC28 plasmid [22,23] was inserted between the *Ecl*136II and *Eag*I sites of pGFPuv thus positioning STa downstream of GFP. The resulting construct was digested by *Hin*dIII and *Asp*718 and ligated to the His tag-encoding-*Hin*dIII/*Asp*718 fragment of pHAT12 to place the GFP between the His tag and STa peptides. After constructs were checked by DNA sequencing and transformed into *E. coli* $DH5_\alpha$ (Invitrogen, France), the production of His-GFP and HisGFP-STa was induced for 3–4 h with 1 mM isopropylthio-β-D-galactoside (IPTG) when the cell culture was at optical density $A_{600\ nm} = 0.6$. His-tagged proteins from cell lysates were purified under non-denaturing conditions using an immobilized cobalt ion affinity chromatography resin column according to the protocol by Clontech (# PT3250-1). The purified proteins were quantified with the Advanced Protein Assay reagent from Cytoskeleton (Denver, CO, USA) and checked by Western blotting [23] using anti-His tag, anti-GFP and anti-STa primary antibodies (Table 1) and goat anti-mouse or anti-rabbit HRP-conjugated IgG as secondary antibody.

The enterotoxic activity of fusion proteins was evaluated in the suckling mouse assay as previously described [22,23]. Briefly, 0.1 ml aliquot of PBS-diluted protein samples was injected intragastrically into 3 days old Swiss OF1 mice which were killed 3 h after injection and examined for increased gut-to-carcass weight ratio (G/C). A G/C ratio $\geqslant 0.090$, corresponding to an unambiguous accumulation of fluid in the gut lumen, indicated positive enterotoxicity and effective STa–GCC interaction. HisGFP (this work) and commercially supplied STa (Sigma, MO, USA) were used as negative and positive controls respectively.

2.3.2. Immunofluorescence of GFP-tagged STa binding and internalisation in CRC cells

The GCC-expressing Caco-2 cell line [8], derived from human colorectal adenocarcinoma with epithelial morphology and purchased from the American Type Culture Collection (ATCC, VA, USA), was cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) 10% fetal bovin serum v/v (Gibco-BRL, MD, USA), 1% non-essential amino acids (NEAA) w/v, penicillin (50 U/ml) streptomycin (50 μg/ml) in an atmosphere of 5% CO_2 and 95% air. Caco-2 cells were grown in 4-well tissue culture Lab-Tek® II-chamber slides™ (Fisher, France) to 75–100% confluence. Cell monolayers were washed consecutively with 37 °C-prewarmed serum-free DMEM 1% NEAA w/v and DMEM 1% NEAA w/v 0.1% bovine serum albumin (BSA) w/v, and incubated in the latter medium at 37 °C for 1 h. STa (Sigma), His-GFP or HisGFP-STa (2 μM) were added to the cells which were then incubated for 45 min at 4 or 37 °C.

Cells were washed with phosphate-buffered saline, pH 7.2 (PBS) 0.1% BSA w/v and PBS, fixed in freshly prepared PBS 4% formaldehyde v/v for 15 min, permeabilized with PBS 0.2% Triton X-100 v/v for 5 min, washed twice in PBS and incubated in PBS 10% goat serum v/v, 1% BSA w/v at RT for 45 min. Alternatively, the cells were acid-washed with 0.2 M glycine–HCl, pH 2.2, prior to fixation. The cells were PBS-washed, incubated with anti-STa (10 μg/ml) or anti-GFP (1 μg/ml) in PBS 1% BSA w/v at RT for 1 h and washed twice with PBS 1% BSA w/v. A goat anti-mouse IgG-Alexa Fluor® 488 conjugate (Molecular Probes, OR, USA) was then applied at 10 μg/ml in PBS 1% BSA w/v for 45 min at RT in the dark. Anti-GFP was used in conjunction with this secondary antibody to augment GFP fluorescence. After two washes in PBS and nuclear counterstaining with 100 ng/ml of 4,6-diamidino-2-phenyl-indole (DAPI), the slides were mounted in Gel-Mount antifade medium (Biomeda, CA, USA). Cells were visualised and photographed with a black-and-white camera (Cohu)-equipped, incident fluorescence microscope (Olympus BH-2). Samples were illuminated with a 100-W mercury lamp and images were captured with the appropriate filters in place, analysed with Biocom software and assembled using Microsoft Power Point.

3. Results

3.1. IHC detection of GCC in CRC tissues

Many preliminary experiments were done to optimise fixative media and fixation conditions using frozen CRC tissues. The protocol was optimised to give better immunostaining results with respect to tissue morphology, anti-GCC antibody specificity, intensity and evaluation. These conditions were then tested on paraffinised sections. The results are summarized in Table 2. Most fixatives produced moderate or strong non-specific staining that was not distinguishable from the specific labelling. In some cases, fixatives such as acetone-based media often created deleterious morphological changes. For some fixatives, staining was absent or at best very weak while for others it was specific but weakly intense or not easily evaluable. Finally, Hollande's bouin (2.5% copper acetate, 4% picric acid, 1.5% acetic acid, 4% formaldehyde) proved to be the best fixative for clearly demonstrating GCC by IHC. It generated strong specific staining mainly at the apical border of the epithelial cells in both adenocarcinomas (Fig. 1E) and adjacent normal tissues (Fig. 1A) as compared to the negative control (Fig. 1B and F). In addition, Hollande's bouin was also suitable for CK20 (Fig. 1C and G) and CEA (Fig. 1D and H) antigens and produced no false-positive due to endogenous peroxidase when used in conjunction with the EnVision^{+} system. For these reasons, this fixative was selected for all further IHC assays on paraffinised sections.

In 36 Hollande's bouin-fixed, paraffin-embedded sections of malignant and adjacent normal tissues of the rectum and colon were analysed by IHC for expression of GCC, CK20 and CEA. Clinical and biological status of all CRC patients are indicated in Table 3. The detection level of GCC was higher in adenocarcinomas than in normal tissues, while that of CK20 or CEA was similar in both tissues. Indeed, GCC, CK20 and CEA were detected in 100%, 94% and 94% of adenocarcinomas in comparison to 80%, 100% and 94% of normal tissues, respectively. In all cases, there was no obvious correlation between the percentages of staining and the degree

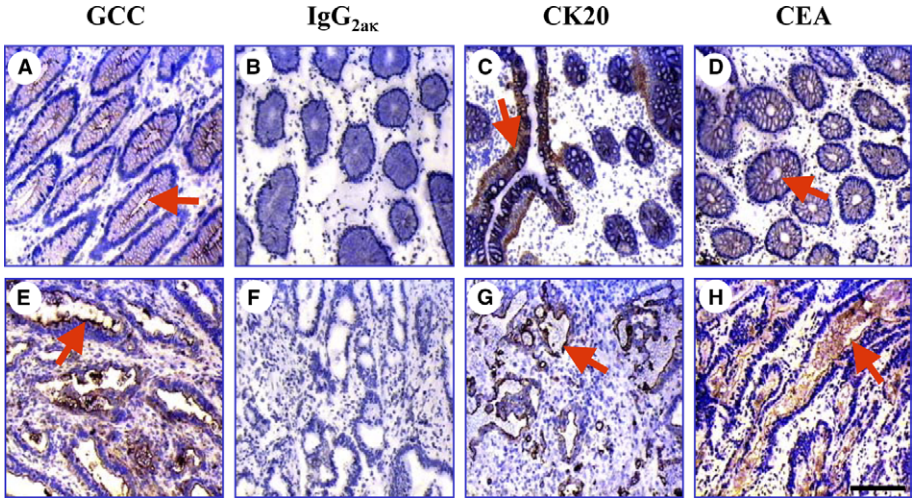

Fig. 1. IHC expression in frozen, Hollande's bouin-fixed tissues. Sections of (A–D) normal colons and (E–H) colon adenocarcinomas were labelled with (A and E) anti-GCC, (C and G) anti-CK20 and (D and H) anti-CEA. (B and F) $IgG_{2a\kappa}$ isotype was used as negative control. The red arrowheads indicate the apical border (in brown colour) of the epithelial cells. Squale bar: 10 μm.

Table 3
Clinicopathological characteristics for all CRC patients and IHC detection in normal tissues and tumours

Clinicopathology	
Gender	
Male	19 (53%)
Female	17 (47%)
Age (years)	
Mean (min–max)	65 (31–87)
TNM stage	
I	8 (22%)
II	14 (39%)
III	8 (22%)
IV	6 (17%)
Tumour size (cm)	
Mean (min–max)	5 (2–12)
Tumour type	
Colon	31 (86%)
Rectum	5 (14%)
Differentiation	
Well	10 (28%)
Moderate	21 (58%)
Poor	5 (14%)
IHC detection	
GCC	
Normal	29/36 (80%)
Adenocarcinoma	36/36 (100%)
CK20	
Normal tissue	36/36 (100%)
Adenocarcinoma	34/36 (94%)
CEA	
Normal	34/36 (94%)
Adenocarcinoma	34/36 (94%)

IHC on paraffin-embedded sections was performed in duplicate and repeated two or three times.

of histological differentiation, TNM classification, sex, size or site of the tumour. GCC, CK20 and CEA were localized into the crypt regions in mucosal epithelia of normal colons (Fig. 2A–C) and colon adenocarcinomas (Fig. 2D–F), but never in the lamina propria, submucosal and serosal layers. Staining intensities of GCC, CK20 and CEA were usually higher in paraffin-embedded tissues (Fig. 2) than in frozen tissues (Fig. 1). We further evaluated the tissue-specificity of GCC by determining if it could be also detected in extra-colorectal tumours. All results are indicated in Table 4 and some of them are shown in Fig. 2. GCC was targeted exclusively in primary adenocarcinoma of the duodenum and in colon adenocarcinoma metastatic to lymph node and liver (Table 4 and Fig. 2G and J). By contrast, both CK20 and CEA were detected in primary adenocarcinoma of the oesophagus, oesophagus–stomach junction, and stomach (Table 4), and in colon adenocarcinoma metastatic to lymph node (Table 4 and Fig. 2H and I), but not in duodenum (Table 4). Only CK20 was labelled in pancreas metastatic to lymphe node (Table 4) and not in colon adenocarcinoma metastatic to liver (Table 4 and Fig. 2K) which, in contrast, expressed CEA (Table 4 and Fig. 2L).

3.2. STa mediates entry of GFP in CRC cells in vitro

To assess if STa toxin was able to deliver GFP into CRC cells, a fusion protein between STa and GFP was constructed, characterized and followed by immunofluorescence in Caco-2 cell line (Fig. 3).

Fig. 3A shows structures and protein sequences of STa (Fig. 3A (a) and (b)), HisGFP (Fig. 3A(c) and (d)) and HisGFP-STa fusion (Fig. 3A(e) and (f)), which consist of 19 (2.0 kDa), 282 (31.8 kDa) and 307 (34.3 kDa) amino acid residues, respectively. Once expressed in *E. coli*, both fluorescent proteins were purified to greater than 95% as seen by Coomassie staining, checked by Western blotting for expression, and examined in suckling mice for activity. As indicated in Fig. 3B, HisGFP-STa (Fig. 3B(c)) and STa alone (Fig. 3B(a)), but not HisGFP (Fig. 3B(b)), exhibited enterotoxicity in mouse. HisGFP-STa was immunoreactive towards antibodies raised against the three components forming the entire hybrid protein (Fig. 3B(c)) while HisGFP reacted with anti-His tag and anti-GFP antibodies only (Fig. 3B(b)). Fig. 3C shows immunofluorescence images of the localisation of STa, HisGFP and HisGFP-STa in Caco-2 cells. Proteins were applied to Caco-2 cells at 4 °C (which prevents endocytosis) or 37 °C (which induced endocytosis) and cells were washed with PBS, fixed and processed as indicated in Section 2. Alternatively, the cells were washed with low pH buffer prior to fixation, which allowed to reveal endocytosis by removing cell-surface-bound proteins while preserving internalized proteins. Cells treated with HisGFP at 37 °C displayed no fluorescence, even without an acid wash. (Fig. 3C(g)). On the other hand, cells incubated at 4 °C with HisGFP-STa showed a high level of binding all around the cells (Fig. 3C(a), as indicated by arrows), which was strongly decreased after acid washing (Fig. 3C(b)). By contrast, with or without an acid wash, cells incubated at 37 °C with HisGFP-STa (Fig. 3C(c) and (d)) or STa alone (Fig. 3C(e) and (f)) produced a similar pattern of fluorescence (Fig. 3C(c) *vs.* (e) and (d) *vs.* (f)) consisting of bright, speckled fluorescent foci concentrated mainly in a well delimited area of the cell (Fig. 3C(c–f), as indicated by arrows). However, fluorescence was more diffuse in acid-unwashed cells (Fig. 3C(c) and (e)) than in acid-washed cells within which spotted fluorescent foci were more distinguishable in the absence of cell-surface-bound proteins (Fig. 3C(d) and (f)).

4. Discussion

In all previous studies of GCC expression in human CRC, GCC was detected by either functional assays [25,26] or RT-PCR analysis [2,7–14]. The specificity and sensitivity of GCC detection by IHC in colorectal or extra-colorectal tissues have not been published until

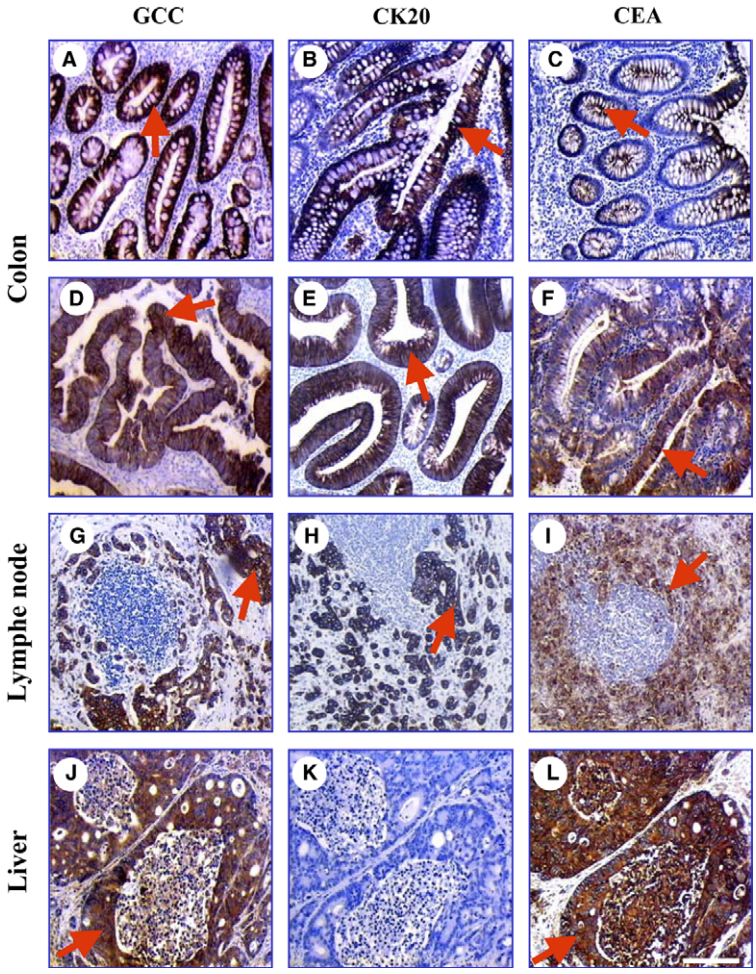

Fig. 2. IHC expression in paraffin-embedded tissues. Sections of (A–C) normal colons, (D–F) colon adenocarcinomas and (G–I) colon adenocarcinomas metastatic to lymphe node and (J–L) liver were labelled with (A, D, G and J) anti-GCC, (B, E, H and K) anti-CK20 and (C, F, I and L) anti-CEA. The red arrowheads indicate mucosal epithelia (in brown colour). Squale bar: 10 μm.

Table 4
IHC detection in extra-colorectal malignant tissues[a]

	GCC		CK20		CEA	
Oesophagus						
Adenocarcinoma (1)[b]	–		+		+	
Oesophagus–stomach junction						
Adenocarcinoma (2)	–	(0/2)	+	(2/2)	+	(2/2)
Stomach						
Adenocarcinoma (6)	–	(0/6)	+	(6/6)	+	(6/6)
Duodenum						
Adenocarcinoma (1)	+		–		–	
Lung						
Adenocarcinoma (2)	–	(0/2)	–	(0/2)	–	(0/2)
Lymph node						
Metastatic pancreas (1)	–		+		–	
Metastatic CRC[c] (1)	+		+		+	
Liver						
Hepatocarcinoma (1)	–		–		–	
Metastatic CRC[c] (1)	+		–		+	

[a] IHC on paraffin-embedded tissues was performed in duplicate and repeated two to four times.
[b] In parenthesis is the number of tissue specimens.
[c] As illustrated in Fig. 2.

now. The first aim of the present study was to evaluate the value of GCC as an IHC marker for targeting human colorectal tissues as compared to the well established CEA and CK20. For this purpose, we adapted a newly developed IHC system EnVision$^+$ [19] for detecting GCC in paraffin-embedded tissues using a GCC-specific antibody. This system was chosen based on its easy application, short assay time, staff workload and excellent sensitivity that allowed high dilutions of the primary antibodies to minimise background and non-specific staining while maintaining the specificity of the reaction [19]. Preliminary studies were done to determine the effects of fixation on the immunostaining of GCC in frozen CRC tissues by investigating a variety of fixatives, fixation times and temperatures. Results showed differential patterns of expression depending on the fixative. By contrast, immunostaining of CEA and CK20 was less dependent on fixative protocol. These findings indicated that tissue fixation had a significant impact on GCC antibody immunoreactivity in

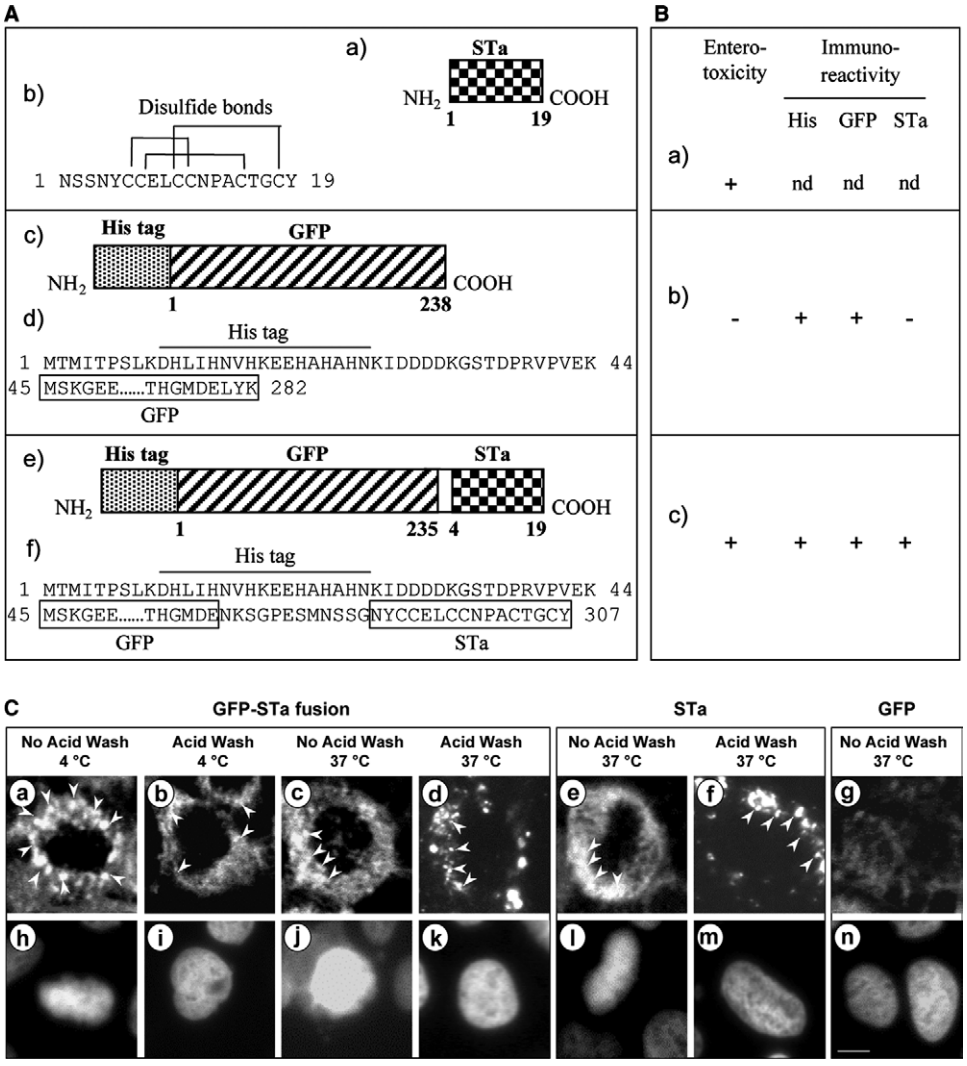

Fig. 3. STa-mediated cell delivery of GFP. (A) Structures (a, c and e) and protein sequences (b, d and f) of STa (a and b), HisGFP (c and d) and HisGFP-STa fusion (e and f). STa contains three disulfide bonds, toxic domain (C^{6}–C^{18}) and GCC receptor-binding region (^{11}CNPAC^{15}). The numbers in bold face below the GFP and STa boxes illustrating the HisGFP-STa protein (e) are the positions of the amino acid residues relative to native STa (a) and wild-type GFP (c). Fusion between GFP and STa resulted in the loss of the three last amino acid residues (LYK) of the wild-type GFP and in the addition of a glycine residue between the three first residues (NSS) and the fourth residue of native STa. (B) Enterotoxicity (in suckling mice) and immunoreactivity (Western blotting using antibodies to His tag, GFP and STa) of STa (a), HisGFP (b) and HisGFP-STa (c). (+) and (−) positive and negative responses; nd, not detectable. (C) Caco-2 cells were incubated with HisGFP-STa (a–d), STa (e and f), or HisGFP (g) at 4 °C (a and b) or 37 °C (c–g) without (a, c, e and g) or with (b, d and f) a low pH acid wash. Binding and internalisation of proteins were visualised by immunofluorescence microscopy using anti-GFP (a–d and g) and anti-STa (e and f) antibodies. All cells were nuclear counterstained with DAPI (h–n). Arrows indicate bright, punctuated fluorescent foci. Squale bar: 40 μm (a–e and g) or 50 μm (f).

both normal and malignant colonic tissues, underlining the importance of fixation in GCC epitope accessibility or degradation. Finally, out of 33 fixatives, only Hollande's bouin maintained GCC recognition while preserving CEA and CK20 epitopes. IHC was then adapted and finalised for the detection of GCC, CEA and CK20 in Hollande's bouin-fixed, paraffin-embedded sections of primary adenocarcinomas and adjacent normal tissues from 36 CRC patients. In both tissues, GCC, CEA and CK20 were predominantly localised to the cytoplasm and mainly at the apical border of the epithelial cells. All these markers were detected in adenocarcinomas in a similar large proportion, demonstrating that they had comparable high sensitivity in labelling mucosal epithelia. In addition, there was no association between detection of any of the three markers and clinical parameters such as TNM classification, degree of histological differentiation, sex, size or site of the tumour. Our results indicated that there was not significant advantage of detecting GCC in primary CRC tumours as compared to CEA and CK20. However, unlike CEA and CK20, the detection rate of GCC was higher in tumours than in adjacent normal tissues. The first explanation for this difference is that the

intracellular GCC epitope in adenocarcinoma was more accessible to anti-GCC antibody due to a change in tissue integrity as compared to normal tissue. Another possibility is that GCC expression was significantly higher in CRC tissues as supported by a recent quantitative RT-PCR-based study [27] indicating that, in 19 paired samples from normal colon and colorectal tumour, GCC was expressed at twice the level in tumour tissue (40,000 molecules) as in normal colon (20,000 molecules).

In order to investigate the tissue-specificity of GCC, we determined if it could be IHC-detected in various extra-colorectal malignant tissues. In contrast to CEA and CK20, GCC was specifically targeted exclusively in primary and metastatic tumours originating from intestine, suggesting that GCC is more tissue-specific than CEA and CK20. However, this must be confirmed in a larger series of extra-intestinal sites in patients with or without CRC diseases from which a great number of samples from normal or tumour tissues will have to be examined. Indeed, Bustin and colleagues [18] detected GCC, CEA and CK20 mRNAs in 13%, 47% and 89% of 149 lymphes nodes from 17 patients with benign disease and in 97%, 100% and 93% of 30 histologically positive lymphes nodes from CRC patients, respectively. In accordance with our findings, these data indicate that GCC displays significantly higher specificity than either CEA or CK20 while retaining high sensitivity. However, they also mean that GCC cannot be considered as an absolute specific CRC marker. Nevertheless, all together, our results are in line with RNA expression [2,7–14] and functional [25,26] studies emphazing GCC as an attractive marker for the diagnosis of CRC, thus demonstrating the reliability and relevance of our IHC findings.

Another aim of this prospective study was to investigate the potential of STa to deliver foreign proteins into CRC cells. For this purpose, using GFP as an imaging reporter-protein, we constructed a GFP-tagged STa fusion protein and characterised it for enterotoxicity in mouse prior to following its binding and internalisation in human CRC Caco-2 cells by immunofluorescence microscopy. We demonstrated that fluorescent GFP-tagged STa retained its wild-type enterotoxic activity in mouse, suggesting that its structural conformation was not drastically affected by the GFP moiety and that it was still capable of recognising and activating GCC. These results are in accordance with our earlier studies with high-sized STa chimeras [22,23] and strengthen the idea that STa can be used as an active carrier for various foreign proteins. Immunofluorescence assays indicated that Caco-2 cells, treated at 37 °C with STa alone or GFP-tagged STa and acid washed, presented the same spotted pattern of fluorescence typical of endocytosed ligands [28]. Acid wash of cells following antibody internalisation did not alter the labelling of STa or GFP-tagged STa, confirming that they do not communicate with the cell surface. This contrasted with the diffuse fluorescence additionally displayed at 37 °C by acid-unwashed cells, which could be a result of both cell surface and internalised proteins. Conversely, no fluorescence was observed at 37 °C with GFP devoid of STa, even without a cell-acid wash as it could not be taken up by these cells. In addition, cells incubated at 4 °C with GFP-tagged STa prior to an acid wash showed a very low level of fluorescence, supporting the hypothesis that at 37 °C the fusion protein was endocytosed, since endocytosis is usually prevented at 4 °C, as exemplified by the temperature-dependent endocytosis of the native STa toxin [24]. Together, all these results suggest that STa mediates internalisation of GFP after engagement to its surface receptor GCC on Caco-2 cells. Given that STa alone and GFP-tagged STa produced the same spotted fluorescent pattern and that native STa undergoes ligand-dependent GCC receptor-mediated endocytosis in human CRC cells [24], we speculate that GFP-tagged STa is moving through the same endocytic pathway and delivered in the same intracellular compartment as STa.

In conclusion, this study leads us to promote our GCC-specific IHC method for identifying regional lymphe node CRC metastasis that is a key factor for precise disease staging, ''sentinel'' lymphe node examination, post-operative surveillance and further treatment strategy. As well, it encourages us to use STa for delivering anticancer therapeutic protein agents into metastatic CRC tumours based on the following attractive advantages: (i) STa is small in size, heat stable, proteases resistant, and poorly antigenic and immunogenic, (ii) STa carrying a foreign protein as a fusion still retains full activity while preserving its passenger, and (iii) STa is capable of delivering small and large active molecules into CRC cells. Coupling of STa to an anti-neoplastic protein, such as *E. coli* cytosine deaminase (CD) or herpes simplex virus thymidine kinase (HSV-tk) [29] and mediating GCC-dependent cell internalisation of the resulting conjugate could potentially inhibit CRC metastases. In addition, introduction of conjugated STa into the systemic circulation should not affect the intestinal GCC receptors present on normal cells, because they are localized to apical membranes, facing the lumen, and isolated from STa by the mucosal barrier.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported in part by grants from Sanofi-Synthelabo, France and the Ligue Nationale

Contre le Cancer, France. We thank Prs. R. A. Gianella [30] and S. S. Visweswariah [17] for kindly supplying the mouse anti-STa and anti-GCC monoclonal antibodies (20C1 and GCC:4D7, respectively). The authors are grateful to E. Brunet (Service d'Anatomie et de Cytologie Pathologiques, Hôtel-Dieu, 63058 Clermont-Ferrand, France) for excellent technical assistance in regard to the human tissue processing for IHC.

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