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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

E. Buc [a], M. Der Vartanian [b,*], C. Darcha [c], P. Déchelotte [c], D. Pezet [a]

[a] *Service de Chirurgie Générale et Digestive, Hôtel-Dieu, 63058 Clermont-Ferrand, France*
[b] *Laboratoire de Microbiologie U454, INRA, Centre de Recherche de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France*
[c] *Service d'Anatomie et de Cytologie Pathologiques, Hôtel-Dieu, 63058 Clermont-Ferrand, France*

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.
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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

* Corresponding author. Tel.: +33 4 7362 4243; fax: +33 4 7362 4581.
E-mail address: dvartan@clermont.inra.fr (M.D. Vartanian).

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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

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