Clinica Chimica Acta 441 (2015) 133–141

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

# Elevated levels of 14-3-3 proteins, serotonin, gamma enolase and pyruvate kinase identified in clinical samples from patients diagnosed with colorectal cancer

Paul Dowling [a,b,*], David J. Hughes [c], Anne Marie Larkin [b], Justine Meiller [b], Michael Henry [b], Paula Meleady [b], Vincent Lynch [b], Barbara Pardini [d], Alessio Naccarati [d], Miroslav Levy [e], Pavel Vodicka [f,g], Paul Neary [h], Martin Clynes [b]

[a] Department of Biology, National University of Ireland, Maynooth, Maynooth, Co. Kildare, Ireland
[b] National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland
[c] Department of Physiology and Medical Physics and Centre for Systems Medicine, Royal College of Surgeons in Ireland, Dublin 2, Ireland
[d] Human Genetics Foundation, Turin, Italy
[e] 1st Medical Faculty of Charles University and Thomayer University Hospital, Prague, Czech Republic
[f] Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[g] Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Prage, Czech Republic
[h] Department of Colorectal Surgery, AMNCH Hospital, Dublin 24, Ireland

ARTICLE INFO

*Article history:*
Received 23 September 2014
Received in revised form 12 November 2014
Accepted 5 December 2014
Available online 23 December 2014

*Keywords:*
Biomarkers
Colorectal cancer
Proteomics
Mass spectrometry
14-3-3 Proteins
Pyruvate kinase

ABSTRACT

*Background:* Colorectal cancer (CRC), a heterogeneous disease that is common in both men and women, continues to be one of the predominant cancers worldwide. Lifestyle, diet, environmental factors and gene defects all contribute towards CRC development risk. Therefore, the identification of novel biomarkers to aid in the management of CRC is crucial. The aim of the present study was to identify candidate biomarkers for CRC, and to develop a better understanding of their role in tumourogenesis.
*Methods:* In this study, both plasma and tissue samples from patients diagnosed with CRC, together with non-malignant and normal controls were examined using mass spectrometry based proteomics and metabolomics approaches.
*Results:* It was established that the level of several biomolecules, including serotonin, gamma enolase, pyruvate kinase and members of the 14-3-3 family of proteins, showed statistically significant changes when comparing malignant versus non-malignant patient samples, with a distinct pattern emerging mirroring cancer cell energy production.
*Conclusion:* The diagnosis and management of CRC could be enhanced by the discovery and validation of new candidate biomarkers, as found in this study, aimed at facilitating early detection and/or patient stratification together with providing information on the complex behaviour of cancer cells.


## 1. Introduction

Colorectal cancer (CRC) is the second most common cause of cancer-related death worldwide and places an enormous cost to society in direct healthcare expenditures. Globally, CRC is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cases and 608,700 deaths estimated to have occurred in 2008 [1]. The highest incidence rates of CRC are in Oceania, Europe and North America, whereas the lowest rates are found in Africa, South-Central Asia and Latin America [1,2]. More than 90% of colorectal carcinomas are adenocarcinomas originating from epithelial cells of the colorectal mucosa. Other types of cancer that can occur here include neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas [3]. In most people, CRC develops slowly over a period of typically a few decades, presenting an opportunity for early detection and intervention to improve patient outcomes [4]. In this regard, biomarkers can potentially be used clinically to aid early detection, diagnosis and disease monitoring or to guide therapy selection for CRC patients.

The discipline of proteomics is an important means to help solve the complex physiological and biochemical mechanisms/pathways with the ultimate goal of identifying new opportunities for developing new

*Abbreviations:* ELISA, Enzyme-linked immunosorbant assay; HPLC, High-performance liquid chromatography; MS/MS, Tandem Mass Spectrometry; *m/z*, mass-to-charge ratio.

* Corresponding author at: Department of Biology, National University of Ireland, Maynooth, Maynooth, Co. Kildare, Ireland. Tel.: +353 1 7086368; fax: +353 1 7083845.
*E-mail address:* paul.dowling@nuim.ie (P. Dowling).

http://dx.doi.org/10.1016/j.cca.2014.12.005
0009-8981/

diagnostics and therapeutic strategies [5]. Serum and plasma are the most readily accessible clinical sample for the investigation of biomarkers. The fact that the serum and plasma proteomes have a large dynamic range in protein concentration, perhaps as high as 15 orders of magnitude, hampers the development of clinical biomarkers [6,7]. A range of fractionation techniques are available to facilitate researchers to this end, including traditional chromatography [8], immunodepletion [9], nanoparticle enrichment [10] and ProteoMiner™ technology [11], which has been used in this investigation.

State-of-the-art technologies for metabolomics analysis, has sparked renewed interest in this important research area, and this may complement proteomic analysis [12]. Identifying, quantifying and understanding variations in the concentration of various metabolites, in combination with proteomics screening, is a powerful strategy for understanding the complex mechanisms in such biological systems.

This study combines proteomic and metabolomic approaches to identify circulating biomolecules that discriminate malignant from non-malignant (polyps, adenomas) patients. Differential protein and metabolite expression analysis, by measuring upregulated or downregulated biomolecules in CRC, may contribute to a better understanding of disease mechanisms and could provide clinicians with a suite of biomarkers to complement existing strategies for the management of CRC patients. Interestingly, the data presented in this study on a series of proteins and metabolites, identified a noticeable link to changes associated with the characteristic metabolic profile of most tumour cells [13].

## 2. Materials and methods

### 2.1. Patient selection and sample collection

Cases with positive colonoscopy results for malignancy, confirmed by histology as colon or rectal carcinomas, were recruited between December 2007 and December 2010 at the Departments of Gastroenterology and Surgery, Adelaide and Meath Hospital, Dublin, Ireland and at the Thomayer Teaching Hospital in Prague, Czech Republic. Control subjects or subjects diagnosed with polyps or adenomatous polyps were acquired during the same period from individuals undergoing colonoscopy for various gastrointestinal complaints (macroscopic bleeding, positive faecal occult blood test or abdominal pain of unknown origin). The participating subjects gave written informed consent in accordance with the Declaration of Helsinki at the participating site that was approved by local ethics committees. See Table 1 for clinical information on samples used. The samples were collected according to standard phlebotomy procedures from consented patients. A total of 10 ml of blood was collected into an EDTA plasma tube and centrifuged at 1000 ×g for 30 min at 4 °C. Plasma was aliquoted in the cryovial tubes, labeled and stored at −80 °C until time of analysis. The time from sample procurement to storage at −80 °C was less than 3 h. Each plasma sample underwent not more than three freeze/thaw cycles prior to analysis.

**Table 1**
Patient data table.

| | n (M/F) | Average age |
|---|---|---|
| *Discovery set* | | |
| Control | 10 (5/5) | 67 ± 11 |
| CRC Stage III | 8 (4/4) | 57 ± 9 |
| CRC Stage IV | 8 (4/4) | 60 ± 5 |
| *Validation set* | | |
| Control | 20 (12/8) | 63 ± 8 |
| Polyps | 10 (6/4) | 58 ± 8 |
| Adenoma | 10 (6/4) | 61 ± 11 |
| CRC Stage I | 10 (5/5) | 62 ± 9 |
| CRC Stage II | 10 (5/5) | 60 ± 14 |
| CRC Stage III | 10 (5/5) | 67 ± 11 |
| CRC Stage IV | 10 (5/5) | 63 ± 12 |

Clinical information on the discovery and validation plasma sample cohorts. M denotes male; F denotes female.

### 2.2. Metabolomics analysis

Plasma specimens (250 μl) were shipped on dry ice to Metabolon Inc., Durham, North Carolina, USA, where the metabolomic profiling was performed. Metabolon incorporates three independent complimentary analysis platforms to maximise the number of small molecules and metabolites that the combined systems can identify and measure. Two independent ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS2) injections (one optimised for basic compounds and the other for acidic compounds) and one Gas chromatography–mass spectrometry (GC/MS) injection per sample are performed. Firstly, small molecules were extracted from plasma specimens using methanol to allow precipitation of proteins. The extract supernatant was then split into four equal aliquots; two for UHPLC/MS, one for GC/MS and one reserve aliquot. Aliquots were then dried overnight to remove solvent.

For the UHPLC methods, one aliquot was reconstituted in 50 μl 0.1% formic acid and the other in 50 μl 6.5 mM ammonium bicarbonate pH 8.0. For GC/MS analysis, aliquots were prepared using equal parts N,O-bistrimethylsilyltrifluoroacetamide and a solvent mixture of acetonitrile/dichloromethane/cyclohexane (5:4:1) with 5% triethylamine at 60 °C for 1 h. All reconstitution solvents contained instrument internal standards used to monitor instrument performance [14,15].

UHPLC/MS was carried out using a Waters Acquity UHPLC coupled to an LTQ mass spectrometer equipped with an electrospray ionization source. Two independent UHPLC/MS injections were performed on each sample. The acidic injections were monitored for positive ions and the basic injections were monitored for negative ions. The derivatised samples for GC/MS were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS. The resulting MS/MS2 data were then searched against Metabolon's reference standard library. This library was generated from 1500 standards and contains the retention time/index, mass to charge (m/z), and MS/MS spectral data for all molecules in the library, including their associated adducts, in-source fragments, and multimers. The library allows identification of experimentally detected metabolites based on a multi-parameter match basis. All identifications and quantifications were subjected to quality control (QC) to verify the quality of the identification and peak integration.

### 2.3. ProteoMiner™ fractionation

Plasma protein equalization was performed using ProteoMiner™ enrichment kit according to the manufacturer procedure (Bio-Rad Laboratories, CA, USA). In summary, the storage solution was first washed out from the spin column containing 100 μl of peptide beads with deionised water. Thereafter, the column was washed with the 10 mM $NaH_2PO_4$, 150 mM NaCl, pH 7.4 solution provided with the kit. When the spin column was ready for sample binding, 1 ml of centrifuged plasma sample was added to the column and equilibrated at room temperature for 2 h. The unbound proteins were removed with the wash buffer and the captured proteins were eluted by 3 × 100 μl of 8 M urea containing 2% CHAPS dissolved in 5% acetic acid.

Following vortexing, sonication and centrifugation, the protein concentration of control and stage III/IV CRC patient samples was determined. Volumes of protein suspensions were equalised using label-free solubilisation buffer and then reduced for 30 min with 10 mM dithiothreitol (DTT) and alkylated for 20 min in the dark with 25 mM iodoacetamide in 50 mM ammonium bicarbonate. The proteolytic digestion of proteins was carried out in two steps. Firstly, digestion was performed with sequencing grade Lys-C at a ratio of 1:100 (protease/protein) for 4 h at 37 °C, followed by dilution with four times the initial