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

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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.

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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

sample volume in 50 mM ammonium bicarbonate. Secondly, further digestion was based on incubation with sequencing grade trypsin at a ratio of 1:25 (protease/protein) overnight at 37 °C. The protease-treated plasma protein suspensions were diluted 3:1 (v/v) with 2% trifluoroacetic acid in 20% acetonitrile. To ensure an even suspension of peptide populations from control and stage III/IV CRC patient samples, the samples were briefly vortexed and sonicated.

2.4. Label-free LC-MS/MS analysis

The nano LC-MS/MS analysis of control versus stage III/IV CRC patient samples was performed using an Ultimate 3000 nanoLC system (Dionex (UK) Ltd., Camberley, Surrey, UK) coupled to a an LTQ Orbitrap XL mass spectrometrer (Thermo Fisher Scientific, Dublin, Ireland) in the Proteomics Facility of the National Institute for Cellular Biotechnology, Dublin City University. The optimised methodology has been as previously described in detail [16]. Peptide mixtures (5 μl volume) were loaded onto a C18 trap column (C18 PepMap, 300 μm id × 5 mm, 5 μm particle size, 100 Å pore size; Dionex). Desalting was achieved at a flow rate of 25 μl/min in 0.1% TFA for 10 min. The trap column was switched on-line with an analytical PepMap C18 column (75 μm id × 500 mm, 3 μm particle and 100 Å pore size; Dionex). Peptides generated from plasma proteins were eluted with the following binary gradients: solvent A (2% ACN and 0.1% formic acid in LC-MS grade water) and 0–25% solvent B (80% ACN and 0.08% formic acid in LC-MS grade water) for 240 min and 25–50% solvent B for a further 60 min. The column flow rate was set to 350 nl/min. Data were acquired with Xcalibur software, version 2.0.7 (Thermo Fisher Scientific). The MS apparatus was operated in data-dependent mode and externally calibrated. Survey MS scans were acquired in the Orbitrap in the 300–2000 *m/z* range with the resolution set to a value of 30,000 at *m/z* 400 and lock mass set to 445.120025 u. CID fragmentation was carried out in the linear ion trap with the three most intense ions per scan. Within 60 s, a dynamic exclusion window was applied. Normalised collision energy of 35%, an isolation window of 3 *m/z* and one microscan were used to collect suitable tandem mass spectra.

2.5. Quantitative profiling by label-free LC-MS/MS analysis

Processing of the raw data generated from LC-MS/MS analysis was carried out with Progenesis label-free LC-MS software (version 3.1; Non-Linear Dynamics, Newcastle upon Tyne, UK). Data alignment was based on the LC retention time of each sample, allowing for any drift in retention time given an adjusted retention time for all runs in the analysis [16]. A reference run was established with the sample run that yielded most features (i.e. peptide ions). The retention times of all of the other runs were aligned to this reference run and peak intensities were then normalised. Prior to exporting the MS/MS output files to MASCOT (www.matrixscience.com) for protein identification, a number of criteria were employed to filter the data including: (i) peptide features with ANOVA < 0.05 between experimental groups, (ii) mass peaks (features) with charge states from +2, +3, and (iii) greater than one isotope per peptide. A MASCOT generic file was generated from all exported MS/MS spectra from Progenesis software. The MASCOT generic file was used for peptide identification with MASCOT (version 2.2) and searched against the UniProtKB-SwissProt database (downloaded in January 2013) with 16,638 proteins (taxonomy: *Homo sapiens*). The following search parameters were used for protein identification: (i) MS/MS mass tolerance set at 0.5 Da, (ii) peptide mass tolerance set to 20 ppm, (iii) carbamidomethylation set as a fixed modification, (iv) up to two missed cleavages were allowed and (v) methionine oxidation set as a variable modification. For further consideration and re-importation back into Progenesis LC-MS software for further analysis, only peptides with ion scores of 40 and above were chosen. Importantly, the following criteria were applied to assign a plasma associated protein as properly identified: (i) an ANOVA score between experimental groups of ≤0.05, (ii) proteins with ≥1 peptides matched and (iii) a MASCOT score >40.

2.6. Enzyme-linked immunosorbent assay

Plasma samples were screened using different enzyme-linked immunosorbent assays for 14-3-3, NSE (gamma enolase), M2-PK and Serotonin. Serotonin levels were analysed using a colorimetric competitive enzyme immunoassay with absorbances read at 405 nm (Enzo Life Sciences, Farmingdale, NY, USA: ADI-900-175). NSE and the 14-3-3 family of proteins were assayed using the quantitative sandwich enzyme immunoassay technique (CSB-E07961h and CSB-E12010h Cusabio Biotech, Wuhan, China). M2-PK levels were analysed using the ScheBo® • Tumor M2-PK™ EDTA Plasma Test (ScheBo Biotech AG, Giessen, Germany).

2.7. Immunohistochemistry

A commercially available colon cancer Tissue Microarray (TMA), (AccuMax A713(VII), ISU/ABXIS Co., Seoul, Korea) comprising 24 cores of colon cancer tissues with corresponding normal tissues was stained for 14-3-3 ε expression using an anti-14-3-3 ε polyclonal antibody suitable for Immunohistochemistry (IHC) (NBP1-89827; Novus Biologicals Littleton, CO, USA). Staining was performed using an automated staining apparatus for IHC (Autostainer, Dako, Glostrup, Denmark) according to the manufacturer's guidelines. Briefly following de-paraffinisation and rehydration, the slides were subjected to an antigen retrieval step consisting of 20-min incubation in pH 6.0 buffer (Target Retrieval, Dako, Glostrup, Denmark). The slides were counterstained with haematoxylin, dehydrated in graded alcohols and glass-mounted (DPX, Sigma). Negative control (primary antibody omitted, replaced by TBS) and positive control (tonsil) slides were processed at the same time. TMA cores were scored according to the intensity of the 14-3-3 ε immunoreactivity observed (categorised as weak, moderate, or strong).

2.8. Statistical analysis

The Mann–Whitney *U* Test (Wilcoxon Rank-Sum Test) was used to determine the p-values for specific proteins between control, polyps, adenoma and stages I-IV CRC patient plasma samples. ELISA data were analysed by constructing a standard curve as a result of plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and drawing a best fit curve through the points on the graph. Receiver-operating characteristic (ROC) curve analysis was performed as it is a useful tool in assessment of biomarker accuracy. The ROC plots were obtained by plotting all sensitivity values (true positive fraction) on the y-axis against their equivalent (1-specificity) values (false positive fraction) for all available thresholds on the x-axis (MedCalc, version 13-0-0-0 64-bit, Medcalc Software, Mariakerke, Belgium). The area under the curve (AUC) was calculated to provide a summary of overall classifier effectiveness.

3. Results

3.1. Patient data

The discovery set of samples included controls = 10, (5/5), 67 ± 11 (number, (male/female), average age ± standard deviation), CRC Stage III = 8 (4/4), 57 ± 9 and CRC Stage IV = 8 (4/4), 60 ± 5. The validation set of samples included controls = 20 (12/8), 63 ± 8, Polyps = 10 (6/4), 58 ± 8, Adenomas = 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 and CRC Stage IV = 10 (5/5), 63 ± 12. Discovery label-free mass spectrometry was performed on controls (n = 10) and CRC (n = 16; 8 stage III/8 stage IV) patient plasma samples fractionated using

Proteominer beads. Discovery metabolomics was performed on control (n = 8) and CRC (n = 16; 8 stage III/8 stage IV) patient plasma samples.

3.2. Proteomic profiling

Prior to label-free MS analysis, plasma samples were enriched using ProteoMiner™ technology. ProteoMiner™ protein enrichment represents an effective and novel sample preparation method that can concentrate low-abundance proteins and reduce the dynamic range of protein concentrations in a sample using combinatorial hexapeptide ligand libraries (CPLL) [17,18]. CPLL are synthesised on beads by the split-couple-recombine method using the 20 naturally occurring amino acids, theoretically resulting in 64 million different ligands, with each ligand fixed to a single bead. Specific binding of proteins to the CPLL is thought to depend on the physicochemical properties of the protein (e.g. conformation, hydrophobicity, and pI). High abundant proteins, such as albumin and haptoglobin, will quickly saturate their ligand quickly, whereas low abundant proteins will not saturate their binding sites and consequently can be quantitatively analysed when eluted from the matrix.

ProteoMiner™ fractionated samples were analysed by label-free MS analysis, an established powerful method for peptide/protein Quantitation. Progenesis LC–MS software was used to compare healthy controls to stage III/IV CRC patient samples (Table 1). We included proteins that were only identified by a single peptide (the so-called "one-hit" wonders) as the complexity of the plasma sample even after fractionation was very high and resulted in much single hit identification (Table 2). Of particular interest from the protein list was the number of 14-3-3 family members identified, 14-3-3 protein theta (θ/τ), 14-3-3 protein gamma (γ), 14-3-3 protein epsilon (ε), 14-3-3 protein zeta/delta (ζ/δ) and 14-3-3 protein beta/alpha (β/α), which were all found to be increased in the stage III/IV CRC patient plasma samples (2.52-fold, 2.14-fold, 2.17-fold, 3.31-fold and 3.78-fold, respectively) compared to the control group. The 14-3-3 family of proteins are not present in high concentrations, especially in the circulation, but their overexpression and probably the ProteoMiner™ enrichment facilitated their identification and quantitation in this study. Both pyruvate kinase isozymes M1/M2 (6.62-fold, p = 0.0003) and gamma enolase (4.50-fold, p = 0.02) were found to be increased in CRC patient plasma samples respectively.

3.3. Metabolomic profiling

Of the molecules that were analysed by UHPLC–MS/MS in the negative and positive ion mode, mode and GC–MS, a number of these analytes were found at statistically significant altered levels when comparing healthy controls to stage III/IV CRC patient plasma samples (Table 3). Serotonin (5-HT) was discovered to be 3.11-fold increased in CRC patient samples (p = 0.001). Two long chain fatty acid, Stearate (18:0) (1.20-fold, p = 0.037) and oleate (18:1n9) (1.69-fold, p = 0.002) were found to be higher in healthy control plasma compared to CRC patients. The gamma-glutamylated amino acids, gamma-glutamylvaline (2.27-fold, p < 0.001) and gamma-glutamylleucine (1.89-fold, p < 0.001) were also found to be higher in healthy control specimens.

3.4. ELISA analysis of M2-PK, gamma enolase, 14-3-3 (pan) and serotonin

ELISA analysis of M2-PK, gamma enolase, 14-3-3 (pan) and serotonin in plasma samples was performed in control (n = 20), polyps (n = 10), adenoma (n = 10), stage I/II CRC (n = 20) and stage III/IV (n = 20) patients. Box-and-whisker plots were constructed from this ELISA data showing the median and interquartile range (Fig. 1). M2-PK, gamma enolase, 14-3-3 (pan) and serotonin were found to be statistically significant in multiple comparisons, including: M2-PK

Table 2
Protein table.

Accession	Platform	Protein name	Peptide count	Confidence score	ANOVA (p)	Fold change	Highest mean condition	Lowest mean condition
P08567	LC/MS	Pleckstrin	1	80	0.0003	4.11	CRC	Control
P14618	LC/MS	Pyruvate kinase isozymes M1/M2	1	47	0.0003	6.62	CRC	Control
P27348	LC/MS	14-3-3 protein theta	2	120	0.0008	2.52	CRC	Control
P60709	LC/MS	Actin, cytoplasmic 1	8	495	0.002	3.36	CRC	Control
P61981	LC/MS	14-3-3 protein gamma	1	63	0.002	2.14	CRC	Control
P02774	LC/MS	Vitamin D-binding protein	6	314	0.002	2.92	CRC	Control
P68032	LC/MS	Actin, alpha cardiac muscle 1	3	165	0.003	3.35	CRC	Control
P62158	LC/MS	Calmodulin	2	153	0.003	2.90	CRC	Control
P10720	LC/MS	Platelet factor 4 variant	2	87	0.003	3.01	CRC	Control
Q562R1	LC/MS	Beta-actin-like protein 2	1	75	0.005	3.09	CRC	Control
P06733	LC/MS	Alpha-enolase	6	383	0.006	5.13	CRC	Control
P62258	LC/MS	14-3-3 protein epsilon	1	71	0.007	2.17	CRC	Control
P06702	LC/MS	Protein S100-A9	2	149	0.007	9.03	CRC	Control
P63104	LC/MS	14-3-3 protein zeta/delta	3	230	0.010	3.31	CRC	Control
P67936	LC/MS	Tropomyosin alpha-4 chain	1	90	0.01	5.85	CRC	Control
Q9Y490	LC/MS	Talin-1	5	290	0.01	2.96	CRC	Control
P04406	LC/MS	Glyceraldehyde-3-phosphate dehydrogenase	3	236	0.01	6.88	CRC	Control
P01009	LC/MS	Alpha-1-antitrypsin	1	55	0.01	2.01	CRC	Control
P31946	LC/MS	14-3-3 protein beta/alpha	1	76	0.01	3.78	CRC	Control
P07437	LC/MS	Tubulin beta chain	1	49	0.02	2.23	CRC	Control
P04350	LC/MS	Tubulin beta-4A chain	1	49	0.02	2.23	CRC	Control
Q86UX7	LC/MS	Fermitin family homolog 3	1	64	0.02	2.73	CRC	Control
P0DJI8	LC/MS	Serum amyloid A-1 protein	2	101	0.02	2.07	CRC	Control
P09104	LC/MS	Gamma-enolase	1	60	0.02	4.50	CRC	Control
P23528	LC/MS	Cofilin-1	1	41	0.02	4.18	CRC	Control
P07996	LC/MS	Thrombospondin-1	7	451	0.03	2.14	CRC	Control
P28676	LC/MS	Grancalcin	2	132	0.03	2.61	CRC	Control
O43707	LC/MS	Alpha-actinin-4	3	138	0.04	3.02	CRC	Control
Q08043	LC/MS	Alpha-actinin-3	1	45	0.05	3.40	CRC	Control

List of proteins found to show statistically significant differences between control (n = 10) and CRC (n = 16; 8 stage III/8 stage IV) patient plasma samples fractionated using Proteominer beads. Information provided in the table includes accession number, discovery platform used, protein description, the number of unique peptides for quantitation, a mascot score for protein identification (confidence number), ANOVA p-values (≥0.05), fold change in protein abundance (≥2-fold) and highest/lowest mean change.

Table 3
Metabolite table.

HMDB	Platform	Biochemical name	ANOVA (p)	Fold change	Highest mean condition	Lowest mean condition
HMDB06344	LC/MS pos	phenylacetylglutamine	0.005	2.88	CRC	Control
HMDB00259	LC/MS pos	serotonin (5HT)	0.001	3.11	CRC	Control
HMDB00682	LC/MS neg	3-indoxyl sulfate	0.028	2.03	CRC	Control
HMDB02302	LC/MS neg	indolepropionate	0.002	4.26	CRC	Control
HMDB11172	LC/MS pos	gamma-glutamylvaline	<0.001	2.27	Control	CRC
HMDB11171	LC/MS pos	gamma-glutamylleucine	<0.001	1.89	Control	CRC
HMDB00827	LC/MS neg	stearate (18:0)	0.037	1.20	Control	CRC
HMDB00207	GC/MS	oleate (18:1n9)	0.002	1.69	Control	CRC
HMDB00848	LC/MS pos	stearoylcarnitine	0.004	1.45	Control	CRC
HMDB05065	LC/MS pos	oleoylcarnitine	<0.001	1.75	Control	CRC
HMDB00660	GC/MS	fructose	0.010	2.12	CRC	Control
HMDB00209	LC/MS neg	phenylacetate	0.018	1.68	CRC	Control

List of metabolites found to show statistically significant differences between control (n = 8) and CRC (n = 16; 8 stage III/8 stage IV) patient plasma samples. Included in the table is the Human Metabolome Database (HMDB) entry, platform used to analyse the biochemicals, biochemical name, ANOVA p-values (≥0.05), fold-change and highest/lowest mean change.

(polyps vs. adenoma, p = 0.02; polyps vs. stage I/II p = 0.01; polyps vs. stage III/IV, p = 0.01), gamma enolase (control vs. stage I/II, p < 0.0001; control vs. stage III/IV, p = 0.002; polyps vs. stage III/IV, p = 0.0003; adenoma vs. stage III/IV, p = 0.0001; stage I/II vs. stage III/IV, p < 0.0001), 14-3-3 (pan) (control vs. adenoma, p = 0.04; stage I/II vs. stage III/IV p < 0.0001) and serotonin (control vs. stage III/IV, p < 0.0001; polyps vs. stage III/IV, p = 0.0003; adenoma vs. stage III/IV, p = 0.0002; stage I/II vs. stage III/IV, p = 0.003).

The most significant AUC-values for Control vs. Stage I/II was 0.82 (14-3-3 (pan)), Control vs. Stage III/IV 0.89 (Gamma Enolase), Polyps vs. Stage I/II 0.78 (M2-PK), Polyps vs. Stage III/IV 0.91 (Serotonin), Adenoma vs. Stage I/II 0.70 (14-3-3 (pan)) and Adenoma vs. Stage III/IV 0.93 (Serotonin). These results indicate that all four candidate biomarkers investigated post discovery phase have significant discriminatory power when comparing control, polyps or adenoma to stage I/II and stage III/IV CRC patient samples (Table 4).

3.5. Immunohistochemistry

14-3-3 ε was selected for further analysis as this protein was found to be statistically significant from the proteomics profiling of plasma samples from control and CRC patients (p = 0.0008). A tissue microarray comprising of 24 cores of colon cancer tissues with corresponding normal tissues was stained for 14-3-3 ε expression using an anti-14-3-3 ε polyclonal antibody. A representative immunohistochemical analysis of 14-3-3 ε is shown in Fig. 2. Each core was appraised using basic annotation parameters for evaluation of staining intensity (negative, weak, moderate or strong). Results indicated a higher level of expression for 14-3-3 ε in malignant compared to non-malignant tissue based on the following counts: for malignant: negative (0/12, 0%), weak (0/12, 0%), moderate (5/12, 42%) or strong (7/12, 58%) and adjacent normal: negative (0/12, 0%), weak (5/12, 42%), moderate (6/12, 50%) or strong (1/12, 8%).

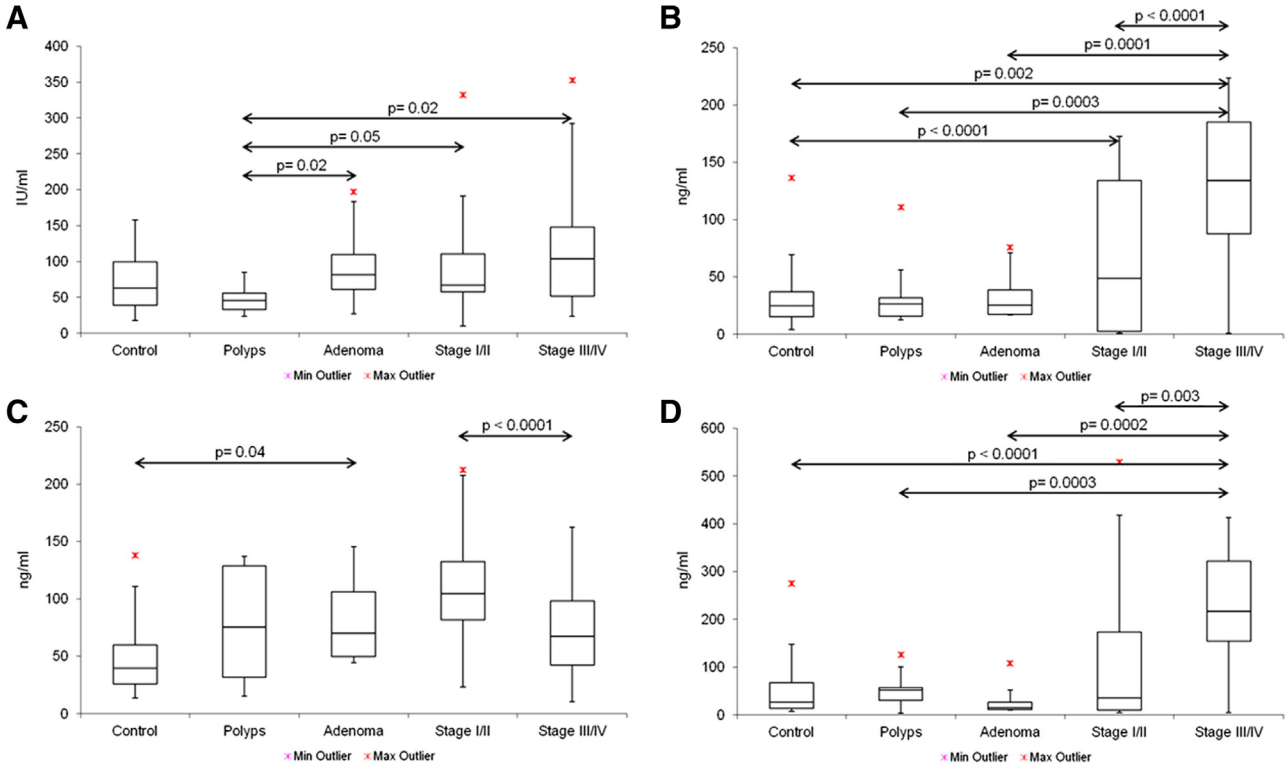

Fig. 1. Box and whisker plots for (A) M2-PK, (B) gamma enolase, (C) 14-3-3 (pan) and (D) serotonin. ELISA analysis of M2-PK, gamma enolase, serotonin and 14-3-3 (pan) in plasma samples from control (n = 20), polyps (n = 10), adenoma (n = 10), stage I/II CRC (n = 20) and stage III/IV (n = 20) patients. The figures show statistically significant p-value for various comparisons between the different sample groups. This ELISA measurement for 14-3-3 detects all known isoforms of mammalian 14-3-3 proteins (β/α, γ, ε, η, ζ/δ, θ/τ and σ).

Table 4
AUC (area under curve) values.

	M2-PK	Gamma enolase	14-3-3 (pan)	Serotonin
Control vs. Stage I/II	0.60	0.55	0.82	0.57
Control vs. Stage III/IV	0.68	0.89	0.67	0.88
Polyps vs. Stage I/II	0.78	0.53	0.65	0.55
Polyps vs. Stage III/IV	0.78	0.89	0.53	0.91
Adenoma vs. Stage I/II	0.56	0.55	0.70	0.65
Adenoma vs. Stage III/IV	0.58	0.92	0.57	0.93

AUC (area under curve) values associated with comparisons of control, polyps and adenoma to both stage I/II and stage III/IV CRC for M2-PK, gamma enolase, 14-3-3 (pan) and serotonin.

4. Discussion

Circulating levels of disease-related biomolecules have the potential to be used for a number of important applications in the management of cancer patients including more precise diagnosis, stratification, prognosis, surveillance and monitoring response to treatment. Blood based biomarkers may also give insight into tumour biology, with many of the biomolecules secreted or shed from tumour cells or surrounding cells in the microenvironment providing important information into the pathogenic mechanisms. Carcinoembryonic antigen (CEA) used for surveillance of patients following primary surgical resection and carbohydrate antigen 19-9 (CA19-9) as a prognostic indicator are the most widely used serum biomarkers in CRC [19–21].

More accurate biomarkers are needed that reflect CRC tumour biology for early detection and monitoring treatment response as new therapeutic strategies emerge. Conventional colonoscopy (CC) is considered the "gold standard" by which we measure any other CRC screening test, but with low patient acceptance rates and waiting lists for colonoscopy, this technique cannot be considered a realistic method for serial monitoring. Ideally, a set of measurable blood-based companion biomarkers, reflecting tumour development and burden, together with the advantage of being cost-effective, routine to perform and with short analysis times would be beneficial to conduct in parallel.

The analysis performed in this report identifies a link between higher expression levels reported in the literature in CRC tissue specimens and in this study, with increased circulatory levels of 14-3-3 proteins. 14-3-3 proteins are established intracellular proteins, mainly found in the cytosol, nucleus or associated with the plasma membrane. Tumour cells secret or shed a larger selection of important molecules, such as hormones, growth factors, and cytokines through well characterised mechanisms. However, many intracellular proteins are likely to be released into the extracellular environment due to vesiculation, cell lysis, apoptosis, and necrosis [22,23]. The ELISA used in this investigation was a pan 14-3-3 assay, evaluating all family members in a single measurement. Given the conflicting reports on the over- and under-expression levels for specific family members (e.g. 14-3-3σ), ideally this analysis would be performed using specific ELISAs to each of the family members individually [24,25]. It is very likely that some of the family members will play important roles in tumour biology at various stages during progression, from early development to establishing metastatic colonies. Tissue levels for 14-3-3 ε were also examined as this protein was amongst the most statistically significant. Overall, the data indicate increased staining levels in CRC tissue compared to surrounding control tissue, as other groups have reported [26].

14-3-3 proteins are highly conserved and ubiquitously expressed with seven isoforms, beta/alpha (β/α), gamma (γ), epsilon (ε), eta (η), zeta/delta (ζ/δ), theta/tau (θ/τ) and sigma (σ) identified in mammals. Through their amino-terminal α helical region, 14-3-3 proteins form homo- or heterodimers that interact with a wide variety of signalling proteins including transcription factors (forkhead box O), metabolic enzymes, kinases (c-Raf Ser/Thr kinase), phosphatases (cdc25 tyrosine phosphatases) and cytoskeletal proteins [27–29].

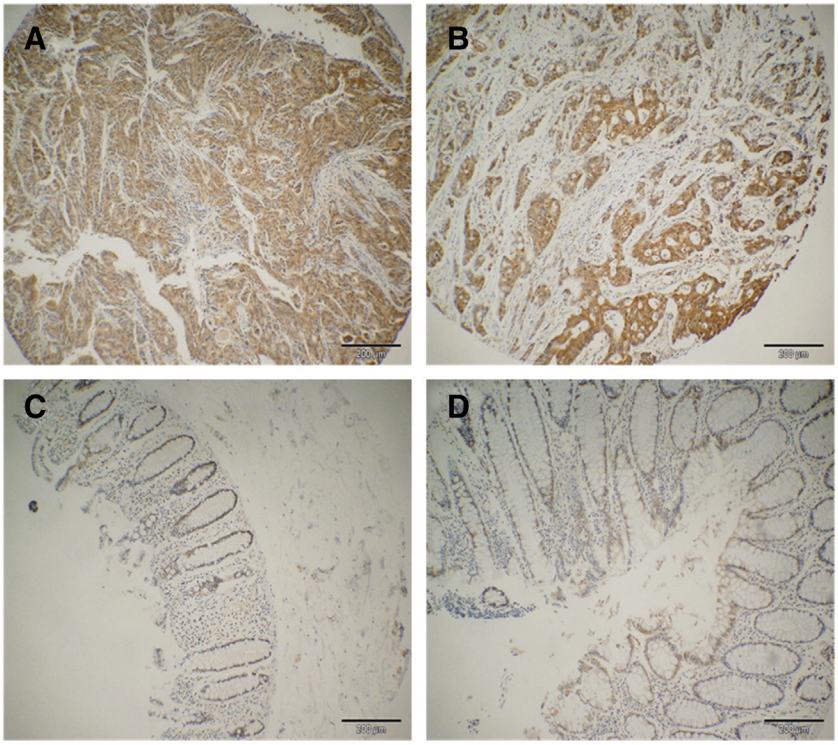

Fig. 2. Immunohistochemistry. Representative immunohistochemical analysis of 14-3-3 ε using a tissue microarray in A. Adenocarcinoma of the colon (Grade: 2, Stage: II, TNM:T3N1M0). B. Adenocarcinoma of the colon (Grade: 2, Stage: II, TNM:T3N1M0). C. Normal adjacent colon tissue (for A). D. Normal adjacent colon tissue (for B). The staining intensity of 14-3-3 ε was significantly higher in the adenocarcinoma of the colon as compared with that of the normal adjacent tissue. The bar on each image represents 200 μm.

Research data from cell lines models and clinical samples support a close relationship between 14-3-3 proteins and many types of cancer [30–32]. Most of these investigations are based on measuring levels of individual members of the 14-3-3 family by immunohistochemistry in cell lines or clinical tissue biopsies and correlating their expression values with clinical outcome and their role in regulating signalling pathways in tumour cells [33,34].

With a focus on CRC, Wang and co-workers reported on the investigation of 14-3-3 ε expression and its prognostic significance in CRC using immunohistochemical analysis of 137 clinicopathologically characterised cases. The data points to poor overall survival in CRC patients correlating with a loss of nuclear 14-3-3 ε expression levels [35]. Of the seven family members for 14-3-3 proteins, Sakai et al., reported that 14-3-3 β, γ, and σ proteins were found to be up-regulated in a 5-fluorouracil (5-FU) resistant cell line model (DLD-1/5-FU cells) compared to the parental cell line [36]. These results suggest an important role for these three proteins as antiapoptotic regulators and conveyers of drug resistance.

Few reports exist with reference to circulating levels of specific 14-3-3 protein family members in relation to specific disease states. Numerous investigations have positively correlated increasing levels of 14-3-3 proteins with the diagnosis of Creutzfeldt-Jakob disease in cerebrospinal fluid (CSF) [37–40]. Hatzipetros and co-workers investigated the levels based on previous findings [41,42], but concluded that levels of 14-3-3 ζ protein were not found to reliably correlate with the clinical behaviour of epithelial ovarian cancer [43]. An enzyme-linked immunosorbent assay (ELISA) was recently developed to detect the 14-3-3 η protein and aid as a new diagnostic tool for the early identification of inflammatory arthritis specifically in synovial fluid [44], and urinary 14-3-3 β/α protein has been reported as a candidate biomarker for renal cell carcinoma [45].

Results considered in this study suggest a correlation with increased levels of serotonin and the development of pre-malignant and malignant stages of CRC, perhaps relating to the synthesis or storage by the enterochromaffin cells. Considering reports on serotonin's role in promoting tumour growth and survival, a possible autocrine-like signalling pathway could play a role in CRC tumourogenesis [46,47]. Serotonin is a widely studied neurotransmitter/growth factor and was found to be consistently statistically significant when comparing plasma levels in individuals diagnosed with polyps compared to adenoma, stage I/II and stage III/IV patients. The gastrointestinal (GI) tract is the largest producer of serotonin (5-hydroxytryptamine (5-HT)) in the body, and as such it is intimately connected with GI function and physiology. The majority of serotonin in the body, approximately 90%, is synthesised by the enterochromaffin cells of the gastrointestinal tract. Over 60 years ago, Lembeck reported increased plasma 5HT in a patient with carcinoid tumour [48]. Since then, other groups have also reported on measurement of serotonin in cancer patient blood samples [49].

Coogan et al. found an association of reduced risk of CRC with regular use of selective serotonin reuptake inhibitors (SSRIs) [50], however other groups have found no link with antidepressant use [51]. Overexpression of serotonin receptors have also been implicated in the development of tumours through analysis of colon cancer tissue and the HT29 (human colon adenocarcinoma) cell line [47]. In breast cancer, correlation analysis revealed a significant link between the serotonin receptor, 5-Hydroxytryptamine receptor 2B (5-HTR2B) and estrogen receptor-α (ER-α) as well as between another serotonin receptor, 5-hydroxytryptamine receptor 4 (5-HTR4) and ER-α/progesterone receptor (PR) [52].

Of the other metabolites, phenylacetylglutamine levels are associated with tumour cytostasis and differentiation [53], with abnormal levels of stearoylcarnitine and oleoylcarnitine involved in lipid metabolism, frequently found to be deregulated in cancer cells [54]. Fructose is associated with more aggressive cancer behaviour and may promote metastasis, with a recent publication revealing higher-than-normal blood levels of fructose in pancreatic cancer patients, a trend that was found in this investigation [55,56].

Two proteins that were investigated in more detail after discovery proteomics and metabolomics were gamma enolase and PKM2 (pyruvate kinase isozymes M1/M2 in the protein list). Both of these proteins were found to show statistically significant fold-changes and demonstrated very similar expression level profiles in the plasma proteome comparing non-malignant and malignant patient samples. Gamma enolase, also known as enolase 2 (ENO2) or neuron-specific enolase, is an enzyme that in humans is encoded by the ENO2 gene and is the neuronal isoform of the glycolytic enzyme enolase that is involved in glucose metabolism [57]. This dimeric enzyme is mainly expressed in neurons (γγ-enolase) and in neuroendocrine cells (αγ-enolase) and is found in CSF, correlating with brain injury after cardiopulmonary arrest [58]. Gamma enolase has been reported as upregulated in a number of cancers, including participating in the tumourogenesis of CRC [59–64].

Many reports in the literature have firmly established a link between PKM2 expression levels and various cancers, by IHC, biofluid and MS based analyses [65–67]. The synergy with CRC is also well documented [68–70], including elevated levels detected in patient faecal specimens [71,72]. Pyruvate kinase catalyzes the last step in the process of glycolysis (rate-limiting), transfer of a high-energy phosphate group from phosphoenolpyruvate (PEP) to ADP, producing ATP and pyruvate. Cancer cells predominantly express PKM2 (regulated by oncogenes and tumour suppressors), the basis for the tumour M2-PK test, which was used as part of this investigation [73,74].

Altered metabolism is a characteristic of most cancer cells, revolving around central themes especially ATP generation and biosynthesis of macromolecules [75,76]. Metabolic enzymes are a key component of these metabolic changes, including gamma enolase and PKM2, both found to be significantly increased in plasma samples from CRC patients compared to control groups. There is also increasing evidence for the role of fructose in cancer cell metabolism, a molecule found to be elevated in this study from CRC samples compared to healthy controls. Liu et al. presented data showing the contribution of fructose to nucleic acid synthesis in cancer cells via TKT-mediated metabolism to facilitate increased proliferative capacity [77].

5. Conclusion

In conclusion, proteomic and metabolomic analysis of plasma patient samples is a powerful strategy to discover candidate protein biomarkers for CRC. These biomarkers have the potential to be used for the development of blood-based tests to support clinical management of CRC in combination with established blood-based biomarkers and colonoscopy screening. The data presented in this investigation may warrant future analysis of a large number of clinical samples with in-depth patient information in order to establish an evidence-based assessment of clinical utility.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2014.12.005.

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
[2] Altobelli E, Lattanzi A, Paduano R, Varassi G, di Orio F. Colorectal cancer prevention in Europe: burden of disease and status of screening programs. Prev Med 2014;62:132–41.
[3] Fleming M, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: pathologic aspects. J Gastrointest Oncol 2012;3:153–73.

[4] Dahabre J, Vasilaki M, Stathopoulos GP, Kondaxis A, Iliadis K, Papadopoulos G, et al. Surgical management in lung metastases from colorectal cancer. Anticancer Res 2007;27:4387–90.
[5] de Wit M, Fijneman RJ, Verheul HM, Meijer GA, Jimenez CR. Proteomics in colorectal cancer translational research: biomarker discovery for clinical applications. Clin Biochem 2013;46:466–79.
[6] Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002;1:845–67.
[7] Hortin GL, Sviridov D, Anderson NL. High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. Clin Chem 2008;54:1608–16.
[8] Jin WH, Dai J, Li SJ, Xia QC, Zou HF, Zeng R. Human plasma proteome analysis by multidimensional chromatography prefractionation and linear ion trap mass spectrometry identification. J Proteome Res 2005;4:613–9.
[9] Rajpal R, Dowling P, Meiller J, Clarke C, Murphy WG, O'Connor R, et al. A novel panel of protein biomarkers for predicting response to thalidomide-based therapy in newly diagnosed multiple myeloma patients. Proteomics 2011;11:1391–402.
[10] Bosch J, Luchini A, Pichini S, Tamburro D, Fredolini C, Liotta L, et al. Analysis of urinary human growth hormone (hGH) using hydrogel nanoparticles and isoform differential immunoassays after short recombinant hGH treatment: preliminary results. J Pharm Biomed Anal 2013;85:194–7.
[11] Capriotti AL, Caruso G, Cavaliere C, Piovesana S, Samperi R, Laganà A. Comparison of three different enrichment strategies for serum low molecular weight protein identification using shotgun proteomics approach. Anal Chim Acta 2012;740:58–65.
[12] Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, et al. The human serum metabolome. PLoS One 2011;6:e16957.
[13] Bi X, Lin Q, Foo TW, Joshi S, You T, Shen HM, et al. Proteomic analysis of colorectal cancer reveals alterations in metabolic pathways: mechanism of tumorigenesis. Mol Cell Proteomics 2006;5:1119–30.
[14] Ohta T, Masutomi N, Tsutsui N, Sakairi T, Mitchell M, Milburn MV, et al. Untargeted metabolomic profiling as an evaluative tool of fenofibrate-induced toxicology in Fischer 344 male rats. Toxicol Pathol 2009;37:521–35.
[15] Suhre K, Meisinger C, Döring A, Altmaier E, Belcredi P, Gieger C, et al. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. PLoS One 2010;5:e13953.
[16] Meleady P, Gallagher M, Clarke C, Henry M, Sanchez N, Barron N, et al. Impact of miR-7 over-expression on the proteome of Chinese hamster ovary cells. J Biotechnol 2012;160:251–62.
[17] Boschetti E, Giorgio Righetti P. Hexapeptide combinatorial ligand libraries: the march for the detection of the low-abundance proteome continues. Biotechniques 2008;44:663–5.
[18] Boschetti E, Righetti PG. The ProteoMiner in the proteomic arena: a non-depleting tool for discovering low-abundance species. J Proteomics 2008;71:255–64.
[19] Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 2006;24:5313–27.
[20] Lin PC, Lin JK, Lin CC, Wang HS, Yang SH, Jiang JK, et al. Carbohydrate antigen 19-9 is a valuable prognostic factor in colorectal cancer patients with normal levels of carcinoembryonic antigen and may help predict lung metastasis. Int J Colorectal Dis 2012;27:1333–8.
[21] Newton KF, Newman W, Hill J. Review of biomarkers in colorectal cancer. Colorectal Dis 2012;14:3–17.
[22] Makridakis M, Vlahou A. Secretome proteomics for discovery of cancer biomarkers. J Proteomics 2010;73:2291–305.
[23] Antelmann H, Tjalsma H, Voigt B, Ohlmeier S, Bron S, van Dijl JM, et al. A proteomic view on genome-based signal peptide predictions. Genome Res 2001;11:1484–502.
[24] Ling C, Zuo D, Xue B, Muthuswamy S, Muller WJ. A novel role for 14-3-3sigma in regulating epithelial cell polarity. Genes Dev 2010;24:947–56.
[25] Li Z, Liu JY, Zhang JT. 14-3-3sigma, the double-edged sword of human cancers. Am J Transl Res 2009;1:326–40.
[26] Pei HP, Ge H, Jiang R, Zhu H. Expression and clinical significance of 14-3-3 sigma and heat shock protein 27 in colorectal cancer. Zhonghua Wei Chang Wai Ke Za Zhi 2010;13:213–5.
[27] Yaffe MB. How do 14–3–3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett 2002;513:53–7.
[28] Dougherty MK, Morrison DK. Unlocking the code of 14-3-3. J Cell Sci 2004;117:1875–84.
[29] Tzivion G, Dobson M, Ramakrishnan G. FoxO transcription factors; regulation by AKT and 14-3-3 proteins. Biochim Biophys Acta 1813;2011:1938–45.
[30] Morrison DK. The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. Trends Cell Biol 2009;19:16–23.
[31] Hermeking H. The 14-3-3 cancer connection. Nat Rev Cancer 2003;3:931–43.
[32] Tzivion G, Gupta VS, Kaplun L, Balan V. 14-3-3 proteins as potential oncogenes. Semin Cancer Biol 2006;16:203–13.
[33] Liang Y, Liu J, Feng Z. The regulation of cellular metabolism by tumor suppressor p53. Cell Biosci 2013;3:9.
[34] Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 2008;30:214–26.
[35] Wang H, Huang H, Li W, Jin X, Zeng J, Liu Y, et al. Nuclear localization of 14-3-3epsilon inversely correlates with poor long-term survival of patients with colorectal cancer. J Surg Oncol 2012;106:224–31.
[36] Sakai A, Otani M, Miyamoto A, Yoshida H, Furuya E, Tanigawa N. Identification of phosphorylated serine-15 and -82 residues of HSPB1 in 5-fluorouracil-resistant colorectal cancer cells by proteomics. J Proteomics 2012;75:806–18.
[37] Aksamit AJ, Preissner CM, Homburger HA. Quantitation of 14-3-3 and neuron-specific enolase proteins in CSF in Creutzfeldt-Jakob disease. Neurology 2001;57:728–30.
[38] Collins S, Boyd A, Fletcher A, Gonzales M, McLean CA, Byron K, et al. Creutzfeldt-Jakob disease: diagnostic utility of 14-3-3 protein immunodetection in cerebrospinal fluid. J Clin Neurosci 2000;7:203–8.
[39] Gmitterová K, Heinemann U, Bodemer M, Krasnianski A, Meissner B, Kretzschmar HA, et al. 14-3-3 CSF levels in sporadic Creutzfeldt-Jakob disease differ across molecular subtypes. Neurobiol Aging 2009;30:1842–50.
[40] Takahashi H, Iwata T, Kitagawa Y, Takahashi RH, Sato Y, Wakabayashi H, et al. Increased levels of epsilon and gamma isoforms of 14-3-3 proteins in cerebrospinal fluid in patients with Creutzfeldt-Jakob disease. Clin Diagn Lab Immunol 1999;6:983–5.
[41] Waldemarson S, Krogh M, Alaiya A, Kirik U, Schedvins K, Auer G, et al. Protein expression changes in ovarian cancer during the transition from benign to malignant. J Proteome Res 2012;11:2876–89.
[42] He Y, Wu X, Liu X, Yan G, Xu C. LC-MS/MS analysis of ovarian cancer metastasis-related proteins using a nude mouse model: 14-3-3 zeta as a candidate biomarker. J Proteome Res 2010;9:6180–90.
[43] Hatzipetros I, Gocze P, Koszegi T, Jaray A, Szereday L, Polgar B, et al. Investigating the clinical potential for 14-3-3 zeta protein to serve as a biomarker for epithelial ovarian cancer. J Ovarian Res 2013;6:79.
[44] Kilani RT, Maksymowych WP, Aitken A, Boire G, St-Pierre Y, Li Y, et al. Detection of high levels of 2 specific isoforms of 14-3-3 proteins in synovial fluid from patients with joint inflammation. J Rheumatol 2007;34:1650–7.
[45] Minamida S, Iwamura M, Kodera Y, Kawashima Y, Tabata K, Matsumoto K, et al. 14-3-3 protein beta/alpha as a urinary biomarker for renal cell carcinoma: proteomic analysis of cyst fluid. Anal Bioanal Chem 2011;401:245–52.
[46] Soll C, Jang JH, Riener MO, Moritz W, Wild PJ, Graf R, et al. Serotonin promotes tumor growth in human hepatocellular cancer. Hepatology 2010;51:1244–54.
[47] Ataee R, Ajdary S, Rezayat M, Shokrgozar MA, Shahriari S, Zarrindast MR. Study of 5HT3 and HT4 receptor expression in HT29 cell line and human colon adenocarcinoma tissues. Arch Iran Med 2010;13:120–5.
[48] Lembeck F. 5-hydroxy-tryptamine in carcinoid tumor. Nature 1953:910–1.
[49] Lee MS, Cheng FC, Yeh HZ, Liou TY, Liu JH. Determination of plasma serotonin and 5-hydroxyindoleacetic acid in healthy subjects and cancer patients. Clin Chem 2000;46:422–3.
[50] Coogan PF, Strom BL, Rosenberg L. Antidepressant use and colorectal cancer risk. Pharmacoepidemiol Drug Saf 2009;18:1111–4.
[51] Cronin-Fenton DP, Riis AH, Lash TL, Dalton SO, Friis S, Robertson D, et al. Antidepressant use and colorectal cancer risk: a Danish population-based case-control study. Br J Cancer 2011;104:188–92.
[52] Kopparapu PK, Tinzl M, Anagnostaki L, Persson JL, Dizeyi N. Expression and localization of serotonin receptors in human breast cancer. Anticancer Res 2013;33:363–70.
[53] Piscitelli SC, Thibault A, Figg WD, Tompkins A, Headlee D, Lieberman R, et al. Disposition of phenylbutyrate and its metabolites, phenylacetate and phenylacetylglutamine. J Clin Pharmacol 1995;35:368–73.
[54] Santos CR, Schulze A. Lipid metabolism in cancer. FEBS J 2012;279:2610–23.
[55] Port AM, Ruth MR, Istfan NW. Fructose consumption and cancer: is there a connection? Curr Opin Endocrinol Diabetes Obes 2012;19:367–74.
[56] Hui H, Huang D, McArthur D, Nissen N, Boros LG, Heaney AP. Direct spectrophotometric determination of serum fructose in pancreatic cancer patients. Pancreas 2009;38:706–12.
[57] Craig SP, Day IN, Thompson RJ, Craig IW. Localisation of neurone-specific enolase (ENO2) to 12p13. Cytogenet Cell Genet 1990;54:71–3.
[58] Roine RO, Somer H, Kaste M, Viinikka L, Karonen SL. Neurological outcome after out-of-hospital cardiac arrest. Prediction by cerebrospinal fluid enzyme analysis. Arch Neurol 1989;46:753–6.
[59] Yeh CS, Wang JY, Chung FY, Lee SC, Huang MY, Kuo CW, et al. Significance of the glycolytic pathway and glycolysis related-genes in tumorigenesis of human colorectal cancers. Oncol Rep 2008;19:81–91.
[60] Karnak D, Beder S, Kayacan O, Ibiş E, Oflaz G. Neuron-specific enolase and lung cancer. Am J Clin Oncol 2005;28:586–90.
[61] Fujiwara H, Arima N, Ohtsubo H, Matsumoto T, Kukita T, Kawada H, et al. Clinical significance of serum neuron-specific enolase in patients with adult T-cell leukemia. Am J Hematol 2002;71:80–4.
[62] Kitakata H, Yasumoto K, Sudo Y, Minato H, Takahashi Y. A case of primary small cell carcinoma of the breast. Breast Cancer 2007;14:414–9.
[63] Katayama M, Nakano H, Ishiuchi A, Wu W, Oshima R, Sakurai J, et al. Protein pattern difference in the colon cancer cell lines examined by two-dimensional differential in-gel electrophoresis and mass spectrometry. Surg Today 2006;36:1085–93.
[64] Selga E, Morales C, Noé V, Peinado MA, Ciudad CJ. Role of caveolin 1, E-cadherin, enolase 2 and PKCalpha on resistance to methotrexate in human HT29 colon cancer cells. BMC Med Genet 2008;1:35.
[65] Schneider J, Neu K, Grimm H, Velcovsky HG, Weisse G, Eigenbrodt E. Tumor M2-pyruvate kinase in lung cancer patients: immunohistochemical detection and disease monitoring. Anticancer Res 2002;22:311–8.
[66] Landt S, Jeschke S, Koeninger A, Thomas A, Heusner T, Korlach S, et al. Tumor-specific correlation of tumor M2 pyruvate kinase in pre-invasive, invasive and recurrent cervical cancer. Anticancer Res 2010;30:375–81.
[67] Planque C, Kulasingam V, Smith CR, Reckamp K, Goodglick L, Diamandis EP. Identification of five candidate lung cancer biomarkers by proteomics analysis of conditioned media of four lung cancer cell lines. Mol Cell Proteomics 2009;8:2746–58.
[68] Eigenbrodt E, Basenau D, Holthusen S, Mazurek S, Fischer G. Quantification of tumor type M2 pyruvate kinase (Tu M2-PK) in human carcinomas. Anticancer Res 1997;17:3153–6.

[69] Zhou CF, Li XB, Sun H, Zhang B, Han YS, Jiang Y, et al. Pyruvate kinase type M2 is up-regulated in colorectal cancer and promotes proliferation and migration of colon cancer cells. IUBMB Life 2012;64:775–82.
[70] Meng W, Zhu HH, Xu ZF, Cai SR, Dong Q, Pan QR, et al. Serum M2-pyruvate kinase: a promising non-invasive biomarker for colorectal cancer mass screening. World J Gastrointest Oncol 2012;4:145–51.
[71] Hardt PD, Toepler M, Ngoumou B, Rupp J, Kloer HU. Measurement of fecal pyruvate kinase type M2 (tumor M2-PK) concentrations in patients with gastric cancer, colorectal cancer, colorectal adenomas and controls. Anticancer Res 2003;23:851–3.
[72] Tonus C, Sellinger M, Koss K, Neupert G. Faecal pyruvate kinase isoenzyme type M2 for colorectal cancer screening: a meta-analysis. World J Gastroenterol 2012;18:4004–11.
[73] Wong N, Ojo D, Yan J, Tang D. PKM2 contributes to cancer metabolism. Cancer Lett 2014;356:184–91.
[74] Wong N, De Melo J, Tang D. PKM2, a central point of regulation in cancer metabolism. Int J Cell Biol 2013:242513.
[75] Zhou W, Capello M, Fredolini C, Racanicchi L, Piemonti L, Liotta LA, et al. Proteomic analysis reveals Warburg effect and anomalous metabolism of glutamine in pancreatic cancer cells. J Proteome Res 2012;11:554–63.
[76] Blum R, Kloog Y. Metabolism addiction in pancreatic cancer. Cell Death Dis 2014;5:e1065.
[77] Liu H, Huang D, McArthur DL, Boros LG, Nissen N, Heaney AP. Fructose induces transketolase flux to promote pancreatic cancer growth. Cancer Res 2010;70:6368–76.