Biochemical and Biophysical Research Communications 440 (2013) 364–370

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Proteomic profiling of a mouse model of acute intestinal *Apc* deletion leads to identification of potential novel biomarkers of human colorectal cancer (CRC)

Abeer Hammoudi [a,1], Fei Song [a,1,2], Karen R. Reed [b,1], Rosalind E. Jenkins [c], Valerie S. Meniel [b], Alastair J.M. Watson [d], D. Mark Pritchard [a], Alan R. Clarke [b], John R. Jenkins [a,*]

[a] Department of Gastroenterology, Institute of Translational Medicine, The University of Liverpool, Crown Street, Liverpool L69 3GE, UK
[b] European Cancer Stem Cell Research Institute, Cardiff University, Hadyn Ellis Building, Maindy Road, Cardiff CF24 4HQ, UK
[c] The MRC Centre for Drug Safety Science, Institute of Translational Medicine, University of Liverpool, L69 3BX, UK
[d] Norwich Medical School, Elizabeth Fry Building, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK

ARTICLE INFO

Article history:
Received 12 August 2013
Available online 31 August 2013

Keywords:
Adenomatous polyposis coli
Biomarkers
Colorectal cancer
Proteomics

ABSTRACT

Colorectal cancer (CRC) is the fourth most common cause of cancer-related death worldwide. Accurate non-invasive screening for CRC would greatly enhance a population's health. Adenomatous polyposis coli (*Apc*) gene mutations commonly occur in human colorectal adenomas and carcinomas, leading to *Wnt* signalling pathway activation. Acute conditional transgenic deletion of *Apc* in murine intestinal epithelium (*AhCre*$^{+}$*Apc*$^{fl/fl}$) causes phenotypic changes similar to those found during colorectal tumourigenesis. This study comprised a proteomic analysis of murine small intestinal epithelial cells following acute *Apc* deletion to identify proteins that show altered expression during human colorectal carcinogenesis, thus identifying proteins that may prove clinically useful as blood/serum biomarkers of colorectal neoplasia. Eighty-one proteins showed significantly increased expression following iTRAQ analysis, and validation of nine of these by Ingenuity Pathaway Analysis showed they could be detected in blood or serum. Expression was assessed in *AhCre*$^{+}$*Apc*$^{fl/fl}$ small intestinal epithelium by immunohistochemistry, western blot and quantitative real-time PCR; increased nucelolin concentrations were also detected in the serum of *AhCre*$^{+}$*Apc*$^{fl/fl}$ and *Apc*$^{Min/+}$ mice by ELISA. Six proteins; heat shock 60 kDa protein 1, Nucleolin, Prohibitin, Cytokeratin 18, Ribosomal protein L6 and DEAD (Asp-Glu-Ala-Asp) box polypeptide 5,were selected for further investigation. Increased expression of 4 of these was confirmed in human CRC by qPCR. In conclusion, several novel candidate biomarkers have been identified from analysis of transgenic mice in which the *Apc* gene was deleted in the intestinal epithelium that also showed increased expression in human CRC. Some of these warrant further investigation as potential serum-based biomarkers of human CRC.

Abbreviations: *AhCre*$^{+}$*Apc*$^{fl/fl}$, acute conditional transgenic deletion of the *Apc* gene in the murine intestinal epithelium; *AhCre*$^{+}$*Apc*$^{+/+}$, wild type control for acute conditional transgenic deletion of the *Apc* gene in the murine intestinal epithelium; *Apc*, adenomatous polyposis coli; Apc$^{Min/+}$, germline mutations in the *Apc* gene, spontaneously develop multiple intestinal adenomas; CTBRC, Cancer Tissue Bank Research Centre; CK18, Cytokeratin 18; CRC, Colorectal cancer; DDX5, DEAD (Asp-Glu-Ala-Asp) box polypeptide 5; ECE, epithelial cell extracts; FAP, familial adenomatous polyposis; FDR, false discovery rate; gFOBt, guaiac faecal blood test; HSPD1, heat shock 60 kDa protein 1 (chaperonin); IHC, immunohistochemistry; i.p., intra-peritoneal; IPA, ingenuity pathways analysis; iTRAQ, isobaric tags for relative and absolute quantification; LC MS/MS, liquid chromatography–mass spectrometry; MMTS, methylmethanethiosulfonate; NCL, Nucleolin; PCR, polymerase chain reaction; PHB, Prohibitin; ROC, receiver operating characteristic; RPL6, Ribosomal protein L6; SEM, standard error of mean; TCEP, tris(2-carboxyethyl)phosphine; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid; WB, western blotting.

* Corresponding author. Address: Department of Gastroenterology, Institute of Translational Medicine, The University of Liverpool, The Henry Wellcome Laboratory, Crown Street, Liverpool L69 3GE, UK. Fax: +44 151 794 6825.

E-mail addresses: abeerwitwit@gmail.com (A. Hammoudi), FEI.SONG@physiologie.med.uni-giessen.de (F. Song), ReedKR@cf.ac.uk (K.R. Reed), RJenkins@Liverpool.ac.uk (R.E. Jenkins), MenielVS@cf.ac.uk (V.S. Meniel), alastair.watson@uea.ac.uk (A.J.M. Watson), Mark.pritchard@liverpool.ac.uk (D.M. Pritchard), ClarkeAR@cf.ac.uk (A.R. Clarke), John.Jenkins@Liverpool.ac.uk (J.R. Jenkins).

[1] These three authors are equally contributed.

[2] Current address: European Cancer Stem Cell Research Institute, Cardiff University, Hadyn Ellis Building, Maindy Road, Cardiff CF24 4HQ, UK.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.08.076

1. Introduction

Colorectal cancer (CRC) is the fourth most common cause of cancer-related deaths worldwide, and the second leading cause of cancer-related death in the UK. Surgical excision of non-invasive premalignant adenomas is curative, but few effective treatments exist for patients suffering from advanced CRC and prognosis is often very poor. Currently, many patients present when they have developed symptoms, such as a change in bowel habit or rectal bleeding, but these frequently indicate the presence of advanced stage disease. Current screening methods in the UK comprise the guaiac faecal occult blood test (gFOBt) which is highly variable in accuracy with 6.2–83.3% sensitivity and low uptake (55%) combined with colonoscopy, which is invasive, expensive and has low yield (11.8% carcinoma, 41.5% adenoma) [1–3]. There is therefore a pressing clinical need to develop alternative non-invasive screening tests for colonic neoplasia which have higher specificities and sensitivities. Here we present a strategy for identifying novel serum-based biomarkers of colonic neoplasia.

Colorectal cancers frequently arise via the adenoma-carcinoma sequence following acquisition of a series of genetic mutations. Mutations in the *Wnt* pathway, particularly *Apc*, are regarded as key to sporadic CRC development. Humans with inherited germline mutations in this gene develop multiple colorectal adenomas and subsequently CRC at an early age (i.e. Familial Adenomatous Polyposis [FAP]) [4]. Mouse strains, such as $Apc^{Min/+}$, which have germline mutations in the *Apc* gene also spontaneously develop multiple intestinal adenomas [5]. *Apc* mutations occur in >80% of cases of sporadic CRC and such mutations are thought to frequently occur early in the carcinogenesis process [6].

Acute conditional transgenic deletion of the *Apc* gene in murine intestinal epithelium ($AhCre^{+}Apc^{fl/fl}$) is time and site-specific, with nearly 100% efficiency for the crypt epithelial cells of the small intestine [7]. This murine model shows altered *Wnt* signalling and dramatically altered crypt-villus architecture and function with increased cell proliferation, apoptosis, migration and disrupted differentiation [7]. Many of these cellular changes are also observed during tumour development in this tissue. Further analysis of the intestinal epithelium of $AhCre^{+}Apc^{fl/fl}$ mice may therefore lead to identification of proteins which show altered expression in this genetic and tissue-dependent context and may also show altered expression during early stage human CRC development. By concentrating on proteins detectable in serum, we anticipated that this strategy might lead to identification of potential novel serum-based CRC biomarkers.

2. Materials and methods

All experiments involving mouse colonies were performed with UK Home Office approval. To study changes in protein profiles after the early deletion of *Apc* in intestinal tissues, $AhCre^{+}Apc^{+/+}$ and $AhCre^{+}Apc^{fl/fl}$ mice were generated and maintained on an outbred background [7]. Cre-recombinase activity was induced from the Ah-Cre transgene by 3 intra-peritoneal (i.p.) injections of 80 mg/kg β-naphthoflavone within 24 h, with the mice then being sacrificed at day 4 or 5. The small intestines were quickly dissected and flushed with chelating agent (HBSS/EDTA) solution. Epithelial cells were harvested by centrifugation and pellets of total intestinal epithelial samples from the mice were prepared. To confirm the changes in protein profiles of any candidate genes in another model (serum), normal colonic tissue and colonic polyp tissues were collected from 6 month old $Apc^{Min/+}$ mice and aged matched wild-type littermates ($Apc^{+/+}$).

Blood was collected from the chest cavity directly following cervical dislocation and immediately transferred into anti-clotting tubes (Sarstedt). Serum and cell pellets were separated by centrifugation for 2 min at maximum speed. Serum was transferred into fresh tubes and snap-frozen in liquid nitrogen. Serum samples from 3 to 4 mice in each cohort were equally pooled for ELISA assays. RNA and protein were extracted from enriched epithelial cell samples prepared from mouse small intestine. Epithelial cell pellets were homogenised and RNA was extracted using a RNeasy Mini Kit (Qiagen). Protein was extracted from epithelial-enriched samples using modified RIPA buffer for western blot or 0.5 M triethylammonium bicarbonate/0.1% SDS (TEAB/SDS) buffer for iTRAQ labelling as follows:

2.1. Epithelial cell preparation

Following β-naphthoflavone injections as described above, equal quantities of ECE from 3 mice of each genotype ($AhCre^{+}Apc^{+/+}$ and $AhCre^{+}Apc^{fl/fl}$) were pooled and each pool was then equally split to generate 2 $Apc^{+/+}$ ($AhCre^{+}Apc^{+/+}$A and $AhCre^{+}Apc^{+/+}$B) and 2 $Apc^{fl/fl}$ ($AhCre^{+}Apc^{fl/fl}$A and $AhCre^{+}Apc^{fl/fl}$B) samples in order to increase statistical confidence in the quantification of peptides. Each of the 4 samples was then labelled with a separate iTRAQ® reagent and processed according to the iTRAQ® manufacturer's specifications.

2.2. iTRAQ labelling of epithelial extracts

The cell pellet was lysed in TEAB/SDS with sonication and freeze–thawing to help disrupt membranes and DNA. Insoluble cell debris was removed by further centrifugation at 14,000*g* and 4 °C for 10 min. The supernatants were assessed for protein content by Bradford assay and aliquots were prepared at 3.75 mg/mL in lysis buffer. Aliquots of 75 μg protein were prepared in duplicate for the $AhCre^{+}Apc^{+/+}$ and $AhCre^{+}Apc^{fl/fl}$ mice, and these were reduced with TCEP, alkylated with MMTS and digested with trypsin (which cuts C-terminal to Lys and Arg, except when adjacent to Pro) before labelling with iTRAQ 4plex reagent (AB Sciex, Foster City, MA, USA) [8]. The labelled samples were mixed and subjected to strong cation exchange chromatography [9,10] prior to LC-MS analysis. Fractions of 2 mL were collected at a flow rate of 1 mL/min and were evaporated to dryness in a SpeedVac (Eppendorf). The samples were then combined and the mixture was analysed by LC-MS/MS.

2.3. LC-MS

Each cation exchange fraction was re-suspended in 120 μL 5% ACN/0.05% trifluoroacetic acid (TFA) and 60 μL was loaded onto the column. Samples were analysed on a QSTAR® Pulsar i hybrid mass spectrometer (AB Sciex) and delivered into the instrument by automated in-line liquid chromatography (integrated LCPackings System, 5 mm C18 nano-precolumn and 75 μm × 15 cm C18 PepMap column; Dionex, California, USA) via a nano-electrospray source head and 10 μm inner diameter PicoTip (New Objective, Massachusetts, USA) [9]. The precolumn was washed for 30 min at 30 μL/min with 5% ACN/0.05% TFA prior to initiation of the solvent gradient in order to reduce the level of salt in the sample. A gradient from 5% ACN/0.05% TFA (v/v) to 60% ACN/0.05% TFA (v/v) in 70 min was applied at a flow rate of 300 mL/min. Data were acquired using Analyst QS1.1 (build 9865). The MS was operated in positive ion mode with survey scans of 1 s, and with MS/MS automatically triggered for the 3 most intense ions. Collision energies were increased 3–5 V above standard values to improve the reporter ion detection and reproducibility. Data were searched using ProteinPilot version 4 (AB Sciex) against the SwissProt database (November 2011), with mouse as the species (32,752 entries), MMTS as a fixed modification of cysteine and biological modifications allowed [11]. Embedded in the ProteinPilot software are

Table 1
The table contains: Protein name; Description; Location; Family; Entrez Gene (LocusLink)/UniProt/Swiss-Prot Accession number; Fold Change by iTRAQ; (protein expression changes that fall within 1% global quantification FDR ($P < 0.01$) are marked with #); p-value (t-test) for iTRAQ identification analysis; whether detected in blood; whether detected in plasma/serum; our reasons for Inclusion/Exclusion (U = Too ubiquitous; R = reagent difficulties, no suitable antibodies or functioning PCR probes; V = Further validation undertaken) * within 1% global FDR for quant ($P < 0.01$).

Name	Description	Location	Family	Entrez gene (LocusLInk)/uni prot/swiss-prot accession	Av fold Change	p-value	Blood	Plasma/ serum	Inclusion/ Exclusion
ALB	Albumin	Extracellular Space	Transporter	P07724	1.717	0.000	X	X	U
NCL	Nucleolin	Nucleus	Other	P09405	1.514	0.002	X	X	V
CK18	Keratin 18	Cytoplasm	Other	P05784	1.487	0.001	X	X	V
RPL6	Ribosomal protein L6	Cytoplasm	Other	P47911	1.296	0.003	X		V
DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	Nucleus	Enzyme	Q61656	1.368	0.002	X		V
HBA	Hemoglobin, alpha	Cytoplasm	Transporter	P01942	1.386	0.004	X		U
HBB1	Hemoglobin, beta	Cytoplasm	Transporter	P02088	1.380	0.011	X		U
H2AFX	H2A histone family, member X	Nucleus	Other	P27661	1.419	0.000	X		V
CPS1	Carbamoyl-phosphate synthetase 1, mitochondrial	Cytoplasm	Enzyme	Q8C196	1.383	0.001	X	X	V
RPL35	Ribosomal protein L35	Cytoplasm	Other	Q6ZWV7	1.323	0.031	X	X	R
HSPD1 (includes EG:3329)	Heat shock 60kD a protein 1 (chaperonin)	Cytoplasm	Other	P63038	1.668	0.029	X	X	V
CTIIIIA1	Catenin (cadherin-associated protein), alpha 1, 102 kDa	Plasma Membrane	Other	P26231	1.517	0.001	X	X	V
PHB	Prohibitin	Mitochondria	Other	P67778	1.375	0.033	X	X	V

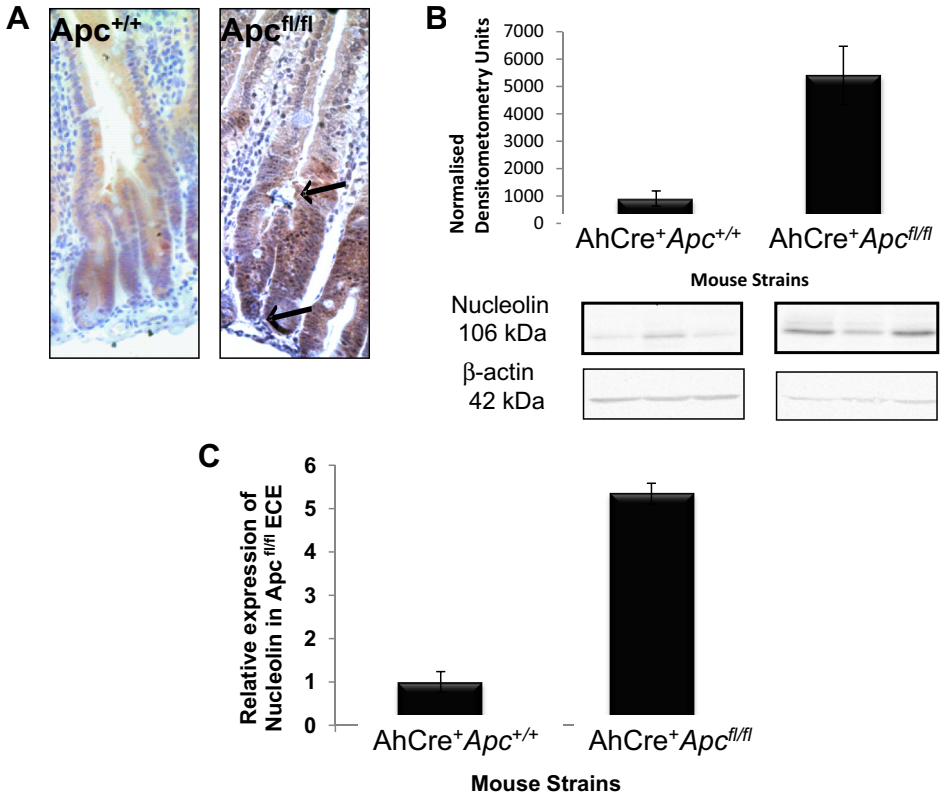

Fig. 1. An example of the validation of proteomic data for nucleolin. (A) Immunohistochemical staining of Nucleolin (NCL) in *AhCre*$^{+}$*Apc*$^{+/+}$ and *AhCre*$^{+}$*Apc*$^{fl/fl}$ small intestinal tissue sections. The area between the arrows shows the zone where *Apc* has been deleted. Magnification ×40. (B) Densitometric analysis and representative images of WB of intestinal epithelial cellular extracts from *AhCre*$^{+}$*Apc*$^{+/+}$ and *AhCre*$^{+}$*Apc*$^{fl/fl}$ mice showing the expression of NCL in 3 mice for each group. β-Actin levels were used for normalisation. NCL showed a 5.94-fold increase in *AhCre*$^{+}$*Apc*$^{fl/fl}$ mice compared to the wild type counterparts. Error bars represent SEM, * P value equal to 0.045 (t-test). (C) Relative expression of NCL mRNA in *AhCre*$^{+}$*Apc*$^{fl/fl}$ mouse intestinal samples compared to *AhCre*$^{+}$*Apc*$^{+/+}$ mice (5 from each group) using qRT-PCR. A 5.35-fold increased expression was observed in *Apc*$^{fl/fl}$ samples. Error bars represent SEM. * P value equal to 0.029 (Mann–Whitney U test).

settings for mass tolerance and resolution based on the instrument used, in this case a QSTAR, and therefore these were approximately 50 ppm and 10,000 for the precursor and fragment ions, respectively. For false discovery rate (FDR) analysis, the data were searched against the reversed database [12] (1). Proteins were included in the analysis if they fell within 1% global FDR and were identified using at least 2 peptides at >90% confidence, or 1 peptide at 99% confidence.

Table 2
Fold change observed in the iTRAQ analysis; apparent increase as detected by IHC in the "Apcfloxed zone"; WB; qRT PCR real-time PCR; (NS = non-specific antibody, ND = not done. * = statistically significant (qRT-PCR) observed for the initial 9 candidate proteins. # = protein expression changes fall within 1% global quantification FDR ($P < 0.01$). Six proteins (highlighted in bold) identified as potential biomarkers requiring further study. This selection was based on finding increased expression using 2 independent methodologies and evidence in the scientific literature suggesting a potential role for the protein during tumourigenesis.

Description	AhCre^{+}*Apc*$^{fl/fl}$			
	iTRAQ fold change	IHC location	WB fold change	qRT-PCR fold change
Nucleolin	**2.735**#	**Yes**	**5.94**	**5.35***
Keratin 18	**2.226**#	**Yes**	**5.41**	**1.95**
Ribosomal protein L6	**1.318**	**Yes**	**2.16**	**1.22**
DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	**2.432**#	**Yes**	**2.66**	**1.63***
H2A histone family, member X	**3.746**	Yes	ND	ND
Carbamoyl-phosphate synthetase 1, mitochondrial	**2.972**#	ND	2.13	ND
Heat shock 60 kDa protein 1 (chaperonin)	**1.668**	**Yes**	**2.95**	**ND**
Catenin (cadherin-associated protein), alpha 1, 102 kDa	**1.517**#	No	1.38	1.21
Prohibitin	**1.375**	**Yes**	**NS**	**3.19***

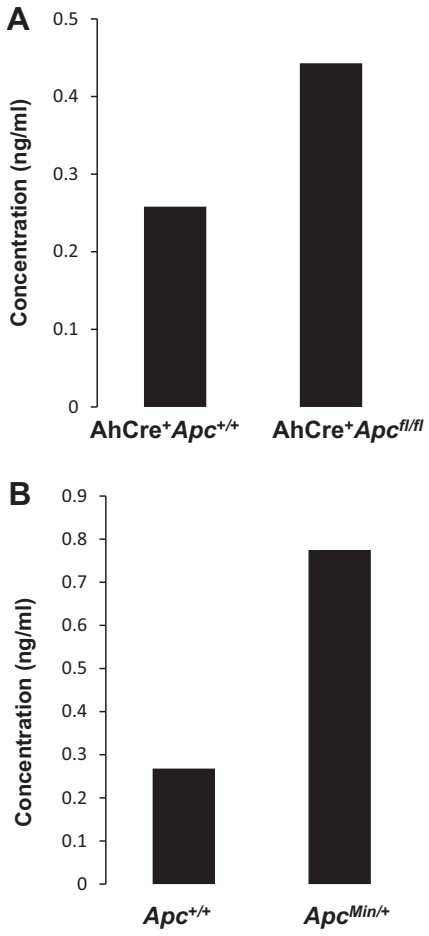

Fig. 2. Increased NCL protein concentrations in pooled ($n \geqslant 3$ per genotype) mouse serum samples. (A) *AhCre*$^{+}$*Apc*$^{fl/fl}$ mice showed serum NCL protein concentration increased by 1.7-fold compared to the wild type cohort (*AhCre*$^{+}$*Apc*$^{+/+}$). (B) Six month old *Apc*$^{Min/+}$ mice showed increased serum NCL concentration compared to age matched controls.

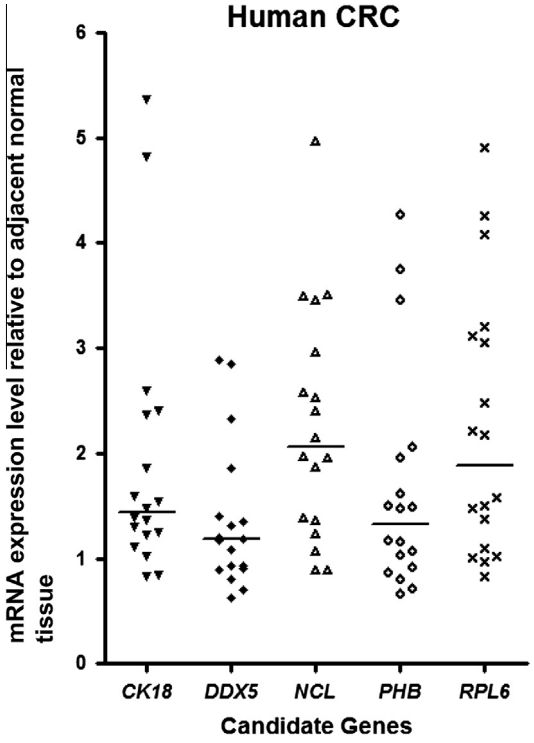

Fig. 3. Taqman quantitative real-time PCR analysis of NCL, CK18, RPL6, DDX5, and PHB expression in CRC tumour tissue presented as fold change relative to adjacent normal tissue. The horizontal line of each group of data shows the median fold change for each gene. Mann–Whitney U test $P < 0.05$ (18 paired samples in each set).

2.4. Ingenuity pathway analysis (IPA)

IPA was performed on the up-regulated proteins using the biomarkers programme to identify proteins which were up-regulated in serum or blood.

2.5. Immunohistochemistry (IHC)

IHC testing comprised fixing intestinal tissue in 10% neutral buffered formalin or in Methacarn (methanol:chloroform:acetic acid at 4:2:1 ratio). Tissue sections (5 μm) were used for IHC using the primary antibody dilutions shown in Supplementary Table 1.

2.6. Western blotting (WB)

WB was performed using antibodies initially at concentrations recommended by the manufacturer and subsequently refined [13] (Supplementary Table 1). Western blots were developed using ECL^{+} reagents (Amersham Biosciences) according to the manufacturer's instructions. Densitometry analysis was performed using Quantiscan software.

2.7. cDNA synthesis

Total RNA from human CRC and mouse tissue samples were used to synthesise first strand cDNA using a VersoTM cDNA Kit

(Thermo Scientific) and anchored oligo-dT primers (Thermo Scientific) according to the manufacturer's instructions.

2.8. qRT-PCR

Single-stranded cDNA samples were amplified in a Polymerase chain reaction (PCR) using sequence-specific primers (Eurogentec) and probes from the Universal Probe Library (Roche) that were designed using the Universal ProbeLibrary Assay Design Centre, using PCR Master mix (Roche) and a light cycler 480 (Roche), (see Supplementary Table 2 for primers and probes used).

2.9. Human samples

Total RNA samples from patient CRC tumour tissues (Dukes' stages A–C) were obtained from the Liverpool Cancer Tissue Bank Research Centre (CTBRC). All CRC cancer tissues and adjacent uninvolved colonic mucosa were obtained from surgically removed specimens with informed patient consent. Uninvolved colonic mucosa was generally taken 5–10 cm away from any malignant tissue.

2.10. ELISA

Sandwich enzyme immunoassays were purchased from Uscn Life Science Inc and assays were performed following the manufacturer's instructions.

2.11. Statistical analyses

For qRT-PCR experiments, comparisons of medians of delta Ct data sets between cohorts were carried out using the Mann–Whitney U test (GraphPad PRISM for Windows v15). For WB analyses, comparisons of means between the mouse cohorts were carried out using student *t*-test.

3. Results

Eighty-one proteins were identified (LC MS/MS) as being up-regulated, by at least 1.2-fold,in the small intestine of $AhCre^{+}Apc^{+/+}$ mice and these were subjected to IPA. This focused our candidates down to nine proteins whose expression changes we further investigated in the small intestine and serum of this mouse model using independent techniques. Six of the most promising proteins; heat shock 60 kDa protein 1 (chaperonin) (HSPD1), Nucleolin (NCL), Prohibitin (PHB), Cytokeratin 18 (CK18), Ribosomal protein L6 (RPL6) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5) were chosen for further assessment in human colorectal tissue samples.

An assessment of the quality of the iTRAQ data was performed in order to assess confidence in the analysis. Since the replica $AhCre^{+}Apc^{fl/fl}$ labelled samples ($Apc^{fl/fl}$A and $Apc^{fl/fl}$B) were duplicate $AhCre^{+}Apc^{fl/fl}$ pools, the A:B ratio for these samples should be 1 for each protein; the average ratio was in fact 0.988. Similarly the replica the $AhCre^{+}Apc^{+/+}$ labelled samples ($Apc^{+/+}$A and $Apc^{+/+}$B) were duplicate pools and the ratio for these samples should also be 1 for each protein: in this case the average was 1.017. Thus, we can have confidence in the quality of the data. The digestion and labelling efficiencies were assessed using PilotDescriptiveStats v2.62p (ABSciex). This revealed that 94.2% of peptide termini were as expected for tryptic digestion and 95.2% of the peptides exhibited no missed cleavage. In addition, 100% of C-terminal lysine residues and 97.2% of N-termini were iTRAQ labelled. Finally, there was a 2.38-fold redundancy in acquiring confident identifications. A simple statistical analysis was performed to determine which proteins to investigate further for their potential as biomarkers of colorectal carcinogenesis. Four ratios were calculated for $AhCre^{+}Apc^{fl/fl}$:$AhCre^{+}Apc^{+/+}$ ($Apc^{fl/fl}$A:$Apc^{+/+}$A, $Apc^{fl/fl}$A:$Apc^{+/+}$B $Apc^{fl/fl}$B:$Apc^{+/+}$A $Apc^{fl/fl}$B:$Apc^{+/+}$B) and the $AhCre^{+}Apc^{+/+}$A:$AhCre^{+}Apc^{+/+}$B ratio. Allthe ratios were converted into natural logarithms, set 1 was tested for normality using the Shapiro Wilk test (there were too few data points in set 2), and the variance between the two sets was determined by F-test (StatsDirect v2.6.8). For the normally distributed samples, a one-tailed, unpaired Student's *t*-test for equal or unequal variance, as appropriate, was applied that revealed whether the difference in mean ratios between set 1 and set 2 was significant. These results suggested that the dataset was of sufficiently high quality to be used as a first step in the biomarker discovery pipeline. (See Supplementary Table 3, proteins identified, the ratio of expression $AhCre^{+}Apc^{+/+}$ (WT) to $AhCre^{+}Apc^{fl/fl}$ and *p*-values). The data were also subjected to FDR analysis on quantification using the PilotDescriptiveStats tool (AB Sciex).

Following iTRAQ analysis, the 81 proteins that were up-regulated by at least 1.2-fold were processed using the IPA programmes that could identify proteins detectable in blood or serum. This identified 13 proteins, which represented potential serum based biomarkers for the detection of early CRC (Table 1); following exclusions for non-specificity to CRC, 9 potential candidate proteins remained.

A number of experimental methodologies including IHC, WB, ELISA and qRT-PCR were employed for further validation studies to assess these proteins as potential CRC biomarkers. Initial validation studies used intestinal samples from $AhCre^{+}Apc^{fl/fl}$ mice, with subsequent validation of promising candidates performed using tissue or serum from $Apc^{Min/+}$ mice [5]. This well-established mouse intestinal tumourigenesis model spontaneously develops numerous lesions throughout the large and small intestine, and combined with published literature supporting the role of these proteins in cancer development, was used to focus further evaluation of these proteins in human tissue samples.

Results obtained with one candidate, Nucleolin (NCL) are shown in Fig. 1. Similar analyses were undertaken for 9 candidate proteins (Table 2). Based on our data, selection criteria and a literature review, 6 proteins (Bold, in Table 2) were selected for further studies as potential biomarkers of human CRC.

We assessed the concentration of one of these proteins in the serum of $AhCre^{+}Apc^{fl/fl}$ animals well as $Apc^{Min/+}$ mice. Pooled samples from $n \geqslant 3$ mice per genotype were used in each case, hence statistical analysis could not be performed. Fig. 2A shows NCL protein concentration was increased 1.72-fold in the serum of $AhCre^{+}Apc^{fl/fl}$ animals. Fig. 2B demonstrates that serum NCL concentration was also elevated in $Apc^{Min/+}$ mice at the 6 month time-point when intestinal adenomas have developed. Thus, one of our candidate proteins showed increased serum expression in 2 mouse models following deletion of *Apc* expression.

The hypothesis underpinning this study was that the protein profiling of a mouse model can translate into meaningful analysis of human clinical samples. Indeed, heat shock 60 kDa protein 1 (chaperonin) (HSPD1) has recently been demonstrated to be up-regulated in tumour tissue relative to adjacent normal tissue in colon cancer patients [14].

Therefore, we assessed the relative expression of our candidate proteins Nucleolin (NCL), Prohibitin (PHB), Cytokeratin 18 (CK18), Ribosomal protein L6 (RPL6) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5) mRNA in matched normal and tumour tissue samples from a cohort of 15 human patients with CRC (Fig. 3). Four of these 5 mRNAs demonstrated statically significant (Mann–Whitney U) increases in CRC, with median values of NCL = 2.064

($p = 0.026$); PHB = 1.325 ($p = 0.020$); CK18 = 1.436 ($p = 0.041$); RPL6 = 1.881 ($p = 0.003$) and DDX5 = 1.183 ($p = 0.1371$).

4. Discussion

The involvement of *Apc* inactivation and consequential activation of *Wnt* signalling in the aetiology of intestinal epithelial tumourigenesis in both humans and mice is undisputed [15,16]. However, the precise and important molecular driving forces downstream of *Wnt* signalling have not yet been fully elucidated. We have therefore proposed that a proteomic analysis of the $AhCre^{+}Apc^{fl/fl}$ mouse model can potentially identify some of the protein changes taking place during adenoma initiation and subsequently the adenoma-carcinoma sequence. This strategy may therefore identify potential biomarkers of human colorectal carcinogenesis.

The studies were performed using samples obtained from a mouse model due to the tractability of this system [17]. We focused our proteomic studies on intestinal epithelial cells, which enabled avoidance of the potential problems of serum or plasma analysis [18,19]. As micro-array analysis of the $AhCre^{+}Apc^{fl/fl}$ model has already been published [7], the aim of this study was to provide a proteomic profile, specifically because mRNA levels are not always associated with changes in protein expression. Indeed, it has previously been suggested that mRNA changes represent at most 40% of the protein changes present [20,21].

The disease-associated profile generated by MetaCore™ analysis (Supplementary Fig. 1) strongly supported our iTRAQ data as being representative of the appropriate clinical area, with the 3 most common conditions identified being adenomatous polyps, intestinal polyposis, and adenomatous polyposis coli. Most of the other 20 most common diseases identified in this analysis were also variations of gastrointestinal neoplasia. Thus, we are confident that our approach using murine *Apc*-deficient epithelial tissue is valid and robust, allowing identification of proteins involved in colorectal carcinogenesis. MetaCore™ was also used to identify the network of interacting proteins based on our iTRAQ data focusing on; The global network of all the upregulated proteins identified (Supplementary Fig. 2A–D).

The study was designed to identify a number of proteins that could potentially be used as biomarkers for human CRC. We focused on the 81up-regulated proteins identified in the initial iTRAQ analysis. IPA identified 13 of these up-regulated proteins that could be detected in blood or serum. Nine of these proteins were further assessed using a variety of techniques including IHC, WB and qRT-PCR, using intestinal epithelial samples generated from the $AhCre^{+}$-$Apc^{fl/fl}$ mouse model. Of these, only 6 proteins (NCL [Nucleolin], PHB [Prohibitin], CK18 [Cytokeratin 18], RPL6 [Ribosomal protein L6], DDX5[DEAD (Asp-Glu-Ala-Asp) box polypeptide 5], HSPD1 [Heat shock 60 kDa protein 1 (chaperonin)]) met our criteria to be selected for further studies and we subsequently demonstrated that 4 of these (NCL, PHB, CK18 and RPL6) showed statistically significant increased expression in human colorectal tumour tissue relative to adjacent normal tissue from the same patients by qRT-PCR, with similar findings previously published for HSPD1 [14].

Thus in conclusion, we identified 6 proteins which showed increased expression following *Apc* deletion in mouse models and these potential biomarkers were also increased to varying extents in human CRC tissue. Of these proteins, 3 (CK18) [22], PHB [23] and HSPD1 [14], have previously been directly associated with CRC; 2 (NCL and DDX5) have previously been suggested as playing a role in carcinogenesis in general, andone, RPL6, has only previous limited published association with carcinogenesis, and none specifically with CRC. This association of our candidates with CRC and carcinogenesis strongly supports our strategy of using a murine model and protein profiling to generate potential novel biomarkers of a human disease. It is, however, very unlikely that any of these biomarkers will individually enable detection of the presence of any stage of colorectal neoplasia [24], but a panel, potentially containing one or more of our 6 candidate biomarkers, could have some proven clinical utility in the future. This highlights the need for further studies on these proteins and similar proteomic profile analysis of other mouse models of CRC.

Acknowledgments

We thank Mark Bishop and Derek Scarborough for providing technical assistance and Peter Skellorn for data table construction. Funding was provided by a North West Cancer Research Fund project grant to John R Jenkins, a Liverpool Cancer Centre Research Development Fund grant to D Mark Pritchard and John R Jenkins, a Cancer Research UK programme grant to Alan R Clarke, and an Iraqi government Ph.D studentship to Abeer Hammoudi.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.076.

References

[1] P. Hewitson, P. Glasziou, E. Watson, et al., Cochrane systematic review of colorectal cancer screening using the fecal occult blood test (hemoccult): an update, Am. J. Gastroenterol. 103 (2008) 1541–1549.
[2] C. Tonus, M. Sellinger, K. Koss, et al., Faecal pyruvate kinase isoenzyme type M2 for colorectal cancer screening: a meta-analysis, World. J. Gastroenterol. 18 (2012) 4004–4011.
[3] J.A. Burch, K. Soares-Weiser, D.J. St John, et al., Diagnostic accuracy of faecal occult blood tests used in screening for colorectal cancer: a systematic review, J. Med. Screen. 14 (2007) 132–137.
[4] K.W. Kinzler, M.C. Nilbert, L.K. Su, et al., Identification of FAP locus genes from chromosome 5q21, Science 253 (1991) 661–665.
[5] A.R. Moser, H.C. Pitot, W.F. Dove, A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse, Science 247 (1990) 322–324.
[6] P.M. Calvert, H. Frucht, The genetics of colorectal cancer, Ann. Intern. Med. 137 (2002) 603–612.
[7] O.J. Sansom, K.R. Reed, A.J. Hayes, et al., Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration, Genes Dev. 18 (2004) 1385–1390.
[8] R.D. Unwin, D.L. Smith, D. Blinco, et al., Quantitative proteomics reveals posttranslational control as a regulatory factor in primary hematopoietic stem cells, Blood 107 (2006) 4687–4694.
[9] N.R. Kitteringham, A. Abdullah, J. Walsh, et al., Proteomic analysis of Nrf2 deficient transgenic mice reveals cellular defence and lipid metabolism as primary Nrf2-dependent pathways in the liver, J. Proteomics 73 (2010) 1612–1631.
[10] C. Rowe, C.E. Goldring, N.R. Kitteringham, et al., Network analysis of primary hepatocyte dedifferentiation using a shotgun proteomics approach, J. Proteome Res. 9 (2010) 2658–2668.
[11] W.H. Tang, B.R. Halpern, I.V. Shilov, et al., Discovering known and unanticipated protein modifications using MS/MS database searching, Anal. Chem. 77 (2005) 3931–3946.
[12] W.H. Tang, I.V. Shilov, S.L. Seymour, Nonlinear fitting method for determining local false discovery rates from decoy database searches, J. Proteome Res. 7 (2008) 3661–3667.
[13] C.R. Barker, J. Hamlett, S.R. Pennington, et al., The topoisomerase II-Hsp90 complex: a new chemotherapeutic target?, Int J. Cancer 118 (2006) 2685–2693.
[14] Y. Jung, S. Lee, H.S. Choi, et al., Clinical validation of colorectal cancer biomarkers identified from bioinformatics analysis of public expression data, Clin. Cancer Res. 17 (2011) 700–709.
[15] G.M. Caldwell, C.E. Jones, A.M. Ashley, et al., Wnt signalling in adenomas of familial adenomatous polyposis patients, Br. J. Cancer 103 (2010) 910–917.
[16] N.S. Fearnhead, M.P. Britton, W.F. Bodmer, The ABC of APC, Hum. Mol. Genet. 10 (2001) 721–733.
[17] M.M. Taketo, Mouse models of gastrointestinal tumors, Cancer Sci. 97 (2006) 355–361.
[18] S.M. Hanash, S.J. Pitteri, V.M. Faca, Mining the plasma proteome for cancer biomarkers, Nature 452 (2008) 571–579.
[19] H. Wang, S. Hanash, Intact-protein analysis system for discovery of serum-based disease biomarkers, Methods Mol. Biol. 728 (2011) 69–85.

[20] Q. Tian, S.B. Stepaniants, M. Mao, et al., Integrated genomic and proteomic analyses of gene expression in Mammalian cells, Mol. Cell. Proteomics 3 (2004) 960–969.
[21] J.R. Jenkins, A proteomic approach to identifying new drug targets (potentiating topoisomerase II poisons), Br. J. Radiol. 81 (1) (2008) S69–S77.
[22] T. Makino, M. Yamasaki, A. Takeno, et al., Cytokeratins 18 and 8 are poor prognostic markers in patients with squamous cell carcinoma of the oesophagus, Br. J. Cancer 101 (2009) 1298–1306.
[23] D. Chen, F. Chen, X. Lu, et al., Identification of prohibitin as a potential biomarker for colorectal carcinoma based on proteomics technology, Int. J. Oncol. 37 (2010) 355–365.
[24] H. Tjalsma, Identification of biomarkers for colorectal cancer through proteomics-based approaches, Expert Rev. Proteomics 7 (2010) 879–895.