

Proteomic and molecular analysis in colorectal cancer: validation of the biomarkers desmin, SET and CK8

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Table of Contents

Title Page.....	i
Table of Contents.....	iii
Declaration.....	x
Acknowledgments.....	xi
Abbreviations.....	xii
Abstract.....	xv
1 Literature Review	1
1.1 Introduction.....	2
1.1.1 Colorectal cancer development.....	3
1.1.2 Anatomy of the bowel wall	3
1.1.3 Staging.....	4
1.2 The Genetic Basis of CRC	6
1.2.1 Familial Adenomatous Polyposis (FAP).....	6
1.2.2 Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch Syndrome.....	6
1.2.3 MUTYH associated adenomatous polyposis (MAP).....	7
1.2.4 Peutz-Jeghers syndrome	7
1.2.5 Juvenile polyposis.....	7
1.2.6 Hyperplastic-serrated polyposis syndrome	7
1.2.7 Sporadic CRC	8
1.3 Diet and lifestyle.....	8
1.4 Wnt signalling.....	8
1.4.1 Canonical Wnt signalling.....	9
1.4.2 APC Mutations in CRC.....	13
1.4.3 β catenin mutations in CRC	13
1.5 CRC statistics and therapy.....	13
1.5.1 Australian statistics	13
1.5.2 Treatment.....	14
1.5.3 Surgery relapse rates.....	14
1.6 Disseminated tumour cells	15
1.7 Current CRC Biomarkers.....	16
1.7.1 Biomarker discovery work performed previously in the laboratory	17
1.7.2 The Effects of Inflammatory Bowel Disease and Benign Tumours on CRC Biomarker Discovery	18

1.8	Current detection methods for CRC.....	18
1.9	Better therapy	19
1.9.1	Current therapy.....	19
1.9.2	Targeted therapies	20
1.10	Detection of differentially expressed proteins in CRC	21
1.10.1	2D DIGE.....	21
1.10.2	2D DIGE and CRC	23
1.10.3	Laser microdissection.....	25
1.11	Aims.....	26
1.11.1	Biomarker discovery.....	26
1.11.2	Biomarker verification.....	26
1.12	Hypotheses.....	26
1.12.1	Expected outcomes.....	27
2	Materials and Methods	29
2.1	Materials	30
2.2	Solutions	33
2.3	Methods.....	35
2.4	Specimen collection	35
2.5	Laser microdissection (LMD)	35
2.5.1	Tissue preparation for LMD	35
2.5.2	LMD.....	36
2.5.3	Diff Quick staining.....	36
2.6	2D Difference gel electrophoresis (2D DIGE)	36
2.6.1	Cy Dye DIGE labelling.....	36
2.7	2 DE.....	37
2.7.1	1 st dimensional IEF	37
2.7.2	Isoelectric focusing	37
2.8	2 nd dimension SDS PAGE.....	37
2.8.1	Imaging and analysis.....	38
2.8.2	Preparative gels.....	38
2.8.3	Silver staining (MS compatible)	39
2.9	LC MS/MS	39
2.9.1	Trypsin digestion	39
2.9.2	HPLC linear ion trap MS.....	39
2.10	CK8 Phosphopeptide enrichment and MALDI TOF/TOF	40

2.10.1	CK8 sample preparation	40
2.10.2	Trypsin digestion.....	40
2.10.3	Phosphopeptide enrichment and MALDI TOF/TOF	41
2.11	1DE and western blotting	42
2.11.1	Sample preparation and 1DE	42
2.11.2	Western blotting.....	42
2.12	Immunofluorescence	43
2.12.1	Frozen tissue preparation	43
2.12.2	Paraffin embedded tissue preparation	43
2.12.3	Staining procedure.....	43
2.13	Real-time RT-PCR	44
2.13.1	Sample preparation	44
2.13.2	RNA check gel.....	44
2.13.3	Reverse Transcription.....	44
2.13.4	Real-time PCR.....	45
2.13.5	Expression ratio calculations	45
2.14	siRNA transfection.....	46
2.15	RT ² Profiler PCR Arrays.....	46
2.16	Flow cytometry	46
3	Identification of CRC biomarkers.....	49
3.1	Introduction.....	50
3.1.1	Scarce labelling DIGE Pilot study.....	50
3.1.2	Statistical analysis of pilot study data.....	52
3.1.3	Emphron Tools.....	53
3.1.4	Results from the scarce labelling DIGE pilot study.....	54
3.2	Materials and methods	55
3.2.1	Laser microdissection	55
3.2.2	DIGE labelling	55
3.2.3	Electrophoretic separation of proteins.....	55
3.2.4	Gel imaging and analysis.....	56
3.2.5	Protein identification.....	56
3.3	Results	57
3.3.1	LMD	57
3.3.2	Tumour-Normal proteome changes detected by DIGE	59
3.3.3	Identification of proteins increased in abundance in colorectal cancer epithelial cells	61

3.4	Discussion	65
3.4.1	LMD.....	65
3.4.2	2D DIGE and LC MS/MS.....	65
3.4.3	Proteins identified as significantly increased in abundance in tumour compared to normal >3 fold	66
3.4.4	Proteins identified as significantly increased in abundance in tumour compared to normal >2 fold	68
3.5	Proteins chosen for further analysis.....	70
3.5.1	Cytokeratin 8	70
3.5.2	Phosphatase 2A inhibitor protein, SET	71
3.5.3	Desmin	72
3.6	Repeat offenders—proteins commonly identified in 2DE MS studies	72
4	Desmin	75
4.1	Introduction.....	76
4.1.1	Identification of desmin as a potential biomarker.....	76
4.1.2	Desmin	78
4.1.3	Hypotheses for the cell type expressing desmin.....	79
4.1.4	Immunofluorescence experimental study design.....	80
4.1.5	Aims	81
4.1.6	Hypotheses.....	81
4.2	Materials and methods.....	82
4.2.1	Characterisation of the desmin antibody.....	82
4.2.2	Immunofluorescence on frozen tissue	82
4.2.3	IF of paraffin embedded tissue	82
4.2.4	IF Analysis.....	83
4.3	Results.....	84
4.3.1	Characterisation of the desmin antibody.....	84
4.3.2	Desmin IF.....	85
4.3.3	Desmin and Vimentin IF	87
4.3.4	Desmin and von Willebrand factor IF.....	88
4.4	Discussion	90
4.4.1	Desmin as a marker of pericytes in CRC.....	90
4.4.2	Desmin expression in the late stage normal tissues.....	90
4.4.3	Pericytes in cancer angiogenesis	91
4.4.4	Pericytes and anti-angiogenic cancer therapies	91

4.4.5	Origin of pericytes	93
4.4.6	Pericytes can originate from mural cells and vascular progenitor cells	93
4.4.7	Pericytes can originate from activated fibroblast cells.....	94
4.4.8	Fibroblasts in cancer	94
4.4.9	The origin of tumour associated fibroblasts.....	95
4.4.10	Desmin as a histopathology marker.....	98
4.4.11	Critique of techniques used	98
4.5	Conclusion.....	99
5	Cytokeratin 8 Phosphorylation in CRC.....	101
5.1	Introduction.....	102
5.1.1	Cytokeratin 8 and CRC	102
5.1.2	Cytokeratins as intermediate filament proteins	103
5.1.3	Cytokeratins in cancer.....	103
5.1.4	CK8 phosphorylation.....	104
5.1.5	Study Design.....	104
5.1.6	Aims	106
5.1.7	Hypotheses	106
5.2	Materials and methods	107
5.2.1	Real-time RT-PCR	107
5.2.2	Enrichment and identification of CK8 phosphorylated proteins	107
5.2.3	Western blotting	109
5.2.4	Blocking EGFR signalling in Caco2 cells	110
5.3	Results	111
5.3.1	Real-time RT-PCR	111
5.3.2	Phosphorylation site identification	112
5.3.3	Confirmation of phosphorylation sites by 2D western blot.....	115
5.3.4	Quantification of phospho-CK8 by western blotting.....	115
5.3.5	Blocking EGFR signalling in Caco2 cells decreases levels of PS73 and PS431.....	120
5.4	Discussion	123
5.4.1	CK8 as a panel marker in the immunobead RT-PCR technique	123
5.4.2	The phosphorylation of CK8 is significantly increased in tumour tissue compared to matched normal mucosa.....	124
5.4.3	CK8 phosphorylation results discussion.....	124
5.4.4	CK8 phosphophorylation and the EGFR/Ras/Raf/ERK pathway.....	126
5.5	Conclusion.....	128

6	The Protein SET	131
6.1	Introduction	132
6.1.1	The Protein SET	132
6.1.2	SET, PP2A and Wnt signalling	134
6.1.3	The PP2A holoenzyme	134
6.1.4	PP2A in Wnt signalling at the plasma membrane	136
6.1.5	PP2A in Wnt signalling in the GSK3 β complex	136
6.1.6	PP2A is a negative regulator of Wnt signalling during embryogenesis	137
6.1.7	The effect of knocking down SET on PP2A and Wnt signalling	138
6.1.8	The role of SET in other cancer signalling pathways	138
6.1.9	The study design	139
6.1.10	Aims	140
6.1.11	Hypotheses	140
6.2	Materials and methods	141
6.2.1	Real-time RT-PCR	141
6.2.2	SET knock down using siRNA	141
6.2.3	RNA isolation and real-time RT-PCR	142
6.2.4	Protein extraction and western blotting	142
6.2.5	RT ² Profiler PCR Arrays	143
6.3	Results	144
6.3.1	Real-time RT-PCR	144
6.3.2	SET siRNA knock down-SET expression at the transcript level	146
6.3.3	SET siRNA knock down—SET and β catenin expression at the protein level	147
6.3.4	SET siRNA treated SW480 lysates analysed using the RT ² Profiler PCR 'Human Cancer Pathway Finder' Array	150
6.4	Discussion	155
6.4.1	SET as a RT-PCR CRC marker	155
6.4.2	SET in the Wnt signalling pathway	156
6.4.3	Hypotheses for reduced β catenin levels in the SET knock down HEK 293 cells	156
6.4.4	SET as a therapeutic target of Wnt signalling in CRC	158
6.4.5	SET siRNA knock down and RT ² Profiler PCR Array analysis	158
6.4.6	SET as a therapeutic target of CRC	163
6.5	Conclusion	164
7	Final Discussion	165

7.1	The interactions of the proteins of interest.....	166
7.1.1	SET/ PP2A and ERK/ CK8.....	166
7.1.2	SET/ PP2A and desmin	167
7.2	Critical review of the biomarker discovery process.....	169
7.3	Future Directions	170
7.3.1	DIGE study.....	170
7.3.2	Desmin as a marker of staging and anti-angiogenic therapies.....	170
7.3.3	CK8.....	171
7.3.4	SET.....	171
7.4	Concluding remarks	172
8	References.....	173

Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Georgia Arentz except where due reference has been made in the text. To the best of my knowledge and belief this work contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Abbreviations

%:	percentage
x g:	x gravity
°C:	degrees Celsius
µg:	microgram
µl:	microliter
µM:	micromolar
1°:	primary
1-DE:	one-dimensional electrophoresis
2°:	secondary
2-DE:	two-dimensional electrophoresis
2D:	two-dimensional
aa:	amino acid
ACN:	acetonitrile
bp:	base pair
BSA:	bovine serum albumin
BVA:	biological variation module
CHAPS:	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
CID:	collision-induced dissociation
cm:	centimetre
Cy:	Cyanine
Da:	Dalton
DIGE:	direct in gel analysis
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleoside triphosphate
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
ESI:	electro spray ionisation
EtOH:	ethanol
FA:	formic acid
Fig:	figure
GE:	General Electric
H ₂ O:	water
HCCA:	α-cyano-4-hydroxycinnamic acid

HPLC:	high performance liquid chromatography
IEF:	isoelectric focusing
IF:	immunofluorescence
IPG:	immobilised pH gradient
KCl:	potassium chloride
kDa:	kilodalton
LC:	liquid chromatography
LMD:	laser microdissection
M:	molar
mA:	milliampere
MALDI:	matrix assisted laser desorption ionisation
ml:	millilitre
mm:	millimetre
mM:	millimolar
mRNA:	messenger RNA
MS:	mass spectrometry
MS/MS:	tandem mass spectrometry
m/z:	mass-to-charge
n:	number of replicates
NaCl:	sodium chloride
NaPO ₄ :	sodium phosphate
NCBI:	National Centre for Biotechnology Information
ng:	nanogram
nl:	nanolitre
p:	pico
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
pH:	hydrogen ion concentration
pI:	isoelectric point
ppm:	parts per million
PS:	phosphoserine
RNA:	ribonucleic acid
RO:	reverse osmosis
rpm:	revolutions per minute
RT:	reverse transcription

RT-PCR: reverse transcription polymerase chain reaction
SDS: sodium dodecyl sulphate
TBE: Tris/boric acid/EDTA buffer
TBST: Tris-buffered saline Tween-20
TFA: trifluoroacetic acid
TOF: time of flight
Tris: Tris (hydroxymethyl) aminomethane
U: units
V: volts
v/v: volume per volume
w/v: weight per volume
w/w: weight per weight

Abstract

There is potential for significant improvements to be made in the diagnosis, staging, treatment and monitoring of colorectal cancer (CRC). The elucidation of proteins and pathways involved in CRC would aid in the development of biomarkers for detection, identify patients at risk of relapse and potentially give rise to new molecular targets of treatment. Laser microdissection (LMD) was used with minimal labelling two-dimensional gel electrophoresis (2D DIGE) and mass spectrometry to identify proteins significantly increased in eight early stage paired tumour-normal tissues. Seventeen individual proteins were identified as upregulated by >2 fold ($P<0.05$). The role of the proteins cytokeratin 8 (CK8) and SET in CRC were chosen for further analysis. A third protein, desmin, was chosen from a pilot study performed using LMD and saturation labelling 2D DIGE for further analysis.

The protein desmin was identified as significantly upregulated in a pilot study that aimed to identify proteins with altered expression in the cancerous epithelium and surrounding microenvironment. Using immunofluorescence (IF), desmin expression levels in the tissue stroma of late stage tumours compared to early stage tumours was significantly increased, $P<0.0001$. The desmin expressing cells were identified to be pericytes, formed around mature vasculature as a result of angiogenic stimulation. From this work desmin appears to have potential use as a histopathology marker for the identification of late stage patients and may help identify patients who would not benefit from the current anti-angiogenic therapies due to the presence of mature tumour microvasculature.

Three isoforms of CK8 were found to be upregulated in the DIGE study, with each isoform containing the phosphoserine (PS) residues 23, 431 and 73. Western blotting showed significantly increased phosphorylation levels at these sites in tumour compared to normal tissues. The MAP kinase ERK is known to phosphorylate the 73 and 431 residues and 50% of CRC patients have mutations in the EGFR/Ras/Raf/MEK/ERK signalling pathway (KRAS or BRAF mutations) resulting in constitutive ERK activation. Inhibition of EGFR activation in Caco2 cells showed a significant decrease ($P<0.0001$) in PS73 and PS431 levels by 59% and 65% respectively, indicating that patients with KRAS or BRAF mutations may have significantly increased PS73 and PS431 levels. Previously it has been shown that high levels of CK8 phosphorylation may help to protect cells against caspase degradation and evasion of apoptosis. This is the first report of the differential expression of phospho-CK8 isoforms in CRC.

SET is a known inhibitor of the tumour suppressor PP2A, a component of the GSK3 β complex that targets β catenin for degradation in the Wnt signalling pathway. Ninety percent of CRC patients have Wnt signalling pathway mutations resulting in constitutive pathway activation. The effect of knocking down SET via siRNA on β catenin levels was analysed in SW480 with constitutive Wnt signalling and

HEK293 with low levels of Wnt signalling. No changes in β catenin levels were observed in the SW480 cells, however a 24.5% reduction was detected in the HEK293 cells. The role of SET in other cancer associated pathways was analysed in the SET knock down cells using the RT² Profiler PCR Array 'Human Cancer Pathway Finder' plates with the expression of genes involved in apoptosis, angiogenesis and adhesion found to be altered.