**PROTEOMIC PROFILING OF RECTAL CANCER REVEALS ACID CERAMIDASE IS IMPLICATED IN RADIATION RESPONSE**

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**Abstract**

**Background -** Neoadjuvant chemoradiotherapy (CRT) is used in locally advanced rectal cancer when tumours threaten the circumferential resection margin, with varying response to treatment. This experimental study aimed to identify significantly differentially expressed proteins between patients responding and not responding to CRT, and to validate any proteins of interest.

**Methods –** Mass spectrometry (with isobaric tagging for relative quantification) analysis of rectal cancers pre- and post-CRT, and at resection. Validation of proteins of interest was performed by assessing tissue microarray (TMA) immunohistochemistry expression in a further 111 patients with rectal cancer.

**Results** – Proteomic data are available via ProteomeXchange with identifier PXD008436. Reduced abundance of contributing peptide ions for acid ceramidase (AC) (log fold change -1.526, p=1.17E-02) was observed in CRT responders. Differential expression of AC was confirmed upon analysis of the TMAs. Cancer site expression of AC in stromal cells from post-CRT resection specimens was observed to be relatively low in pathological complete response (p=0.003), and relatively high with no response to CRT (p=0.017).

**Conclusion** – AC may be implicated in the response of rectal cancer to CRT. We propose its further assessment as a novel potential biomarker and therapeutic target.

**Significance**

There is a need for biomarkers to guide the use of chemoradiotherapy in rectal cancer, as none are in routine clinical use. We have determined acid ceramidase may have a role in radiation response, based on novel proteomic profiling and validation in a wider dataset using tissue microarrays. The ability to predict or improve response would positively select those patients who will derive benefit, prevent delays in the local and systemic management of disease in non-responders, and reduce morbidity associated with chemoradiotherapy.

**Keywords**

Rectal; cancer; chemoradiotherapy; response; proteomics; biomarker.

**Introduction**

Colorectal cancer is the second commonest cause of cancer related death in the UK(1). Outcomes and survival in rectal cancer have improved with the recognition that circumferential resection margin (CRM) involvement predicts a greater risk of local recurrence and poorer survival(2,3), and with widespread adoption of total mesorectal excision(4,5). In cases where the CRM is identified to be threatened on pre-treatment magnetic resonance imaging(6-8), neo-adjuvant treatment with chemoradiotherapy (CRT) is utilised to attempt to downstage these tumours, enhance R0 resection rates and reduce the risk of local recurrence(9-11).

In 10-15% of patients treated with CRT, a pathological complete response (pCR) is observed in the subsequent resection specimen. These patients not only have a lower risk of local recurrence but improved overall survival(12,13). Whilst it is possible to attempt to clinically and radiologically identify those patients achieving a complete response following CRT(14), and in whom the morbidity of surgical resection may be spared with a watch and wait approach(15), there are currently no biomarkers that can reliably predict response to CRT(16). A longer downstaging period (>8 weeks) subsequent to CRT completion has also been demonstrated to increase the rate of pCR(17), although temporal changes in tumour phenotype that may explain this phenomenon have not been assessed.

The current pCR rate of 10-15% remains quite low, and a small proportion of patients develop disease progression whilst undergoing CRT. Work to further improve this response rate is urgently needed. The ability to predict response would positively select those patients who will derive benefit, whilst sparing others the morbidity associated with CRT and a delay in the local and systemic management of their disease(18-21). The necessity for further research in this area has recently been highlighted by the Association of Coloproctology of Great Britain and Ireland, who have advocated the need for reliably predicting the response of rectal cancer to CRT, and for novel strategies for improving the sensitivity of rectal cancer to radiation therapy(22).

Proteomic analysis has the potential advantage of providing the most direct assessment of functional tumour biology(23). We aimed to undertake a novel temporal proteomic profiling of rectal cancers both prior to and following CRT, to determine differentially expressed proteins between responders and non-responders to CRT as defined by standard tumour regression grading (TRG). Any candidate proteins would be subsequently validated using a specially constructed tissue microarray (TMA) of rectal cancer specimens.

**Methods**

**Ethical Approval**

The work presented in this paper was performed under NHS Research Ethics Committee approval (12/NW/0011).

**Rectal Cancer Sampling**

Patients receiving a diagnosis of rectal cancer at The Countess of Chester Hospital and identified by the multidisciplinary team as requiring CRT were approached for inclusion in the study. Those giving informed consent to participate were invited back to the endoscopy unit for a limited flexible sigmoidoscopy and biopsy. Three ‘double bites’ with a standard endoscopic forcep (≈3mm3 tissue in total) of peripheral tumour was biopsied, with deliberate attempts to avoid the area of necrotic tissue centrally. Patients were contacted one week after CRT cessation and invited to return for a further limited flexible sigmoidoscopy and biopsy. On the day of resection, a sample of tumour and normal adjacent mucosa were obtained. Following delivery of the colorectal specimen, the proximal staple line was incised and a linear cut made down the antimesenteric border before excising a peripheral section of tumour using forceps and a scalpel. All samples were placed in individually labelled cryodorfs, snap frozen in liquid nitrogen and transferred to a -80°C storage facility.

**Sample Preparation and Labelling**

Protein was extracted from sampled tissue by mechanical dissolution and sonication in 500mM triethylammonium bicarbonate with 0.1% sodium dodecyl sulphate. After centrifugation at 4°C and 20000g for 15 minutes, the supernatant was recovered and protein concentration determined by Bradford assay. Labelling with iTRAQ reagents was carried out according to the Sciex protocol for an 8-plex procedure (Framingham, MA, USA). In brief, 100μg of protein from each sample was reduced with Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and capped with methylmethanethiosulfate (MMTS), before overnight digestion with trypsin (Promega, Southampton, UK). Peptides were then labelled with isobaric tags, pooled and diluted to 5mL with 10mM potassium dihydrogen phosphate/25% acetonitrile (ACN) and acidified to pH<3 with phosphoric acid.

**Cation Exchange**

Samples were fractionated on a Polysulfoethyl A strong cation-exchange column (200 × 4.6mm, 5µm, 300Å; Poly LC, Columbia, MD) at 2ml/min using a gradient from 10mM potassium dihydrogen phosphate/25% ACN (w/v) to 0.15M potassium chloride/10mM potassium dihydrogen phosphate/25% ACN (w/w/v) in 75 minutes. Fractions of 2mL were collected and dried by centrifugation under vacuum (SpeedVac, Eppendorf UK Ltd, Stevenage, UK). Fractions were reconstituted in 1mL of 0.1% trifluoroacetic acid (TFA) and were desalted using a mRP Hi Recovery protein column 4.6 x 50mm (Agilent, Berkshire UK) on a Vision Workstation (Applied Biosystems/Life Technologies, Paisley, UK) prior to mass spectrometry analysis.

**Mass Spectrometry**

Desalted fractions were reconstituted in 40µL 0.1% formic acid and 5µL aliquots were delivered into a Triple TOF 5600 (Sciex, Warrington, UK) via an Eksigent NanoUltra cHiPLC System (Sciex) mounted with a microfluidic trap and analytical column (15cm × 75μm) packed with ChromXP C18−CL 3μm. A NanoSpray III source was fitted with a 10μm inner diameter PicoTip emitter (New Objective, Woburn, USA). The trap column was washed with 2% ACN/0.1% formic acid for 10 minutes at 2μL/min before switching in-line with the analytical column. A gradient of 2−50% ACN/0.1% formic acid (v/v) over 90 minutes was applied to the column at a flow rate of 300nL/min. Spectra were acquired automatically in positive ion mode using information-dependent acquisition powered by Analyst TF 1.5.1. software (Sciex). Up to 25 MS/MS spectra were acquired per cycle (approximately 10Hz) using a threshold of 100 counts per second and with dynamic exclusion for 12 seconds. The rolling collision energy was increased automatically by selecting the isobaric tagging for relative and absolute quantification (iTRAQ) check box in Analyst, and manually by increasing the collision energy intercepts by 5.

**Protein Identification**

Data were searched using ProteinPilot 4.2 and the Paragon algorithm (Sciex) against the latest version of the SwissProt database (release 2013\_08: 20,266 human entries), with MMTS as a fixed modification of cysteine residues and biological modifications allowed. Mass tolerance for precursor and fragment ions was 10ppm. No missed cleavages or variable modifications were allowed for peptides used for quantification. The data were also searched against a reversed decoy database and only proteins lying within a 1% global false discovery rate were taken forward for analysis. Quantification of proteins was relative to a common pooled sample present in all experiments. iTRAQ data for proteins identified by 2 or more peptides with at least 90% confidence of correct sequence assignment, or by a single peptide with at least 99% confidence were log2 transformed, batch corrected and included in subsequent analyses.

**Differential Analysis and Pathway Analysis**

A number of direct two group comparisons were made using Partek® (St Louis, USA) to identify proteins significantly different between the groups. A two-way Analysis of Variance (ANOVA) was employed to identify the differential proteins whilst accounting for batch effect. In the case of paired samples, a 3-way ANOVA was performed with additional inclusion of the patient factor. With the aim of reducing the false discovery rate, the Benjamini-Hochberg multiple test correction was used. Those proteins identified as statistically significantly different were subjected to analysis with Ingenuity Pathway Analysis (Redwood City, USA).

**Tissue Microarray Construction**

Formalin-fixed paraffin-embedded tissue blocks were retrieved from archived storage for 111 consecutive patients who had undergone surgical resection for a rectal adenocarcinoma (following CRT) at The Countess of Chester Hospital between 2007 and 2015. Three sets of tissue microarrays were constructed comprising; a) tissue from the diagnostic biopsy specimen (n=106), b) ‘normal’ colonic epithelium obtained from the colonic end of the resection specimen outside the radiation field (n=111), and c) tissue from the cancer site, specifically targeting residual malignant cells where present (n=111). In the case of pCR, tissue was obtained from the scar in order to assess stromal expression. Triplicate 0.6mm cores were obtained from donor blocks and transferred into recipient blocks in a randomised fashion.

**Immunohistochemical Staining for Acid Ceramidase and Analysis**

IHC staining was performed using the EnVision™ FLEX system (Dako UK Ltd). Tissue microarray sections were dewaxed and antigen retrieval performed, prior to peroxidase block and then application of the AC primary antibody (BD612302, mouse monoclonal, BD Transduction Laboratories). A horseradish peroxidase conjugated secondary antibody was applied prior to development with diaminobezidine tetrahydrochloride, and counterstaining with haematoxylin.

Stained sections of the tissue microarrays were assessed under light microscopy by two individuals (MW and DB), and semi-quantitative analysis for each tissue core present was undertaken using a simplified H-score(24). The system applies a score to the percentage of positive staining cells (0-5) added to a score for the intensity of that positivity (0-3). The breakdown of scoring for the percentage of positive staining cells is defined as 0%=0, <1%=1, 1-10%=2, 10-33%=3, 33-66%=4, and >66%=5, with the intensity score defined as none=0, weak=1, intermediate=2, strong=3. Each tissue core was assigned an individual score for epithelial and stromal staining respectively, where the tissue subsets were present. Where any discrepancy in scoring was discovered, consensus score was achieved after both assessors reviewed samples together. The H-scores for each tissue subset were dichotomised about the median score for that tissue to determine relative high and low expression. Expression was correlated with regression grading using *X*2.

**Tumour regression grading**

Haematoxylin and eosin stained slides were reviewed by consultant histopathologist (MW) to confirm the diagnosis of adenocarcinoma, and slides from the resection specimen were assessed for tumour regression grading. The four-tier system recommended by The Royal College of Pathologists, and similar to that described by Ryan(25) was used.

* TRG 4: no viable tumour cells (fibrosis or mucous lakes only)
* TRG 3: single cells or scattered small groups of cancer cells
* TRG 2: residual cancer outgrown by fibrosis
* TRG 1: minimal or no regression (extensive residual tumour)

**Results**

**Proteomic Profiling**

Eight patients were studied, with clinical variables detailed in Table 1. All patients were male with a median (range) age of 74 (50-78) years at diagnosis. 3359 proteins were identified, all of which were present in at least half the samples, and were subjected to bioinformatics analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(26) partner repository with the dataset identifier PXD008436. A summary of the data set can also be found in Supplementary File A.

The abundance of contributing peptide ions for 18 proteins were significantly different between pre- and post-CRT samples, of which 16 were upregulated and 2 downregulated. There were 19 upregulated and 10 downregulated proteins when comparing pre-CRT and resection samples, and 9 upregulated and 30 downregulated between post-CRT and resection samples. These proteins are detailed in Supplementary File B. When comparing tumours between those patients subsequently dichotomised as relative responders or non-responders to CRT, the abundance of contributing peptide ions for 8 proteins were significantly different, 5 of which were upregulated in responders and 3 downregulated. These proteins are detailed in Table 2.

The ceramide degradation pathway was identified as the most significantly downregulated pathway in those tumours responding to CRT as demonstrated in Table 3, and is based on the significantly lower abundance of contributing peptide ions for AC. This protein was selected initially for further evaluation on the basis of literature evidence implicating it in radiotherapy response in prostate cancer, and for the availability of a commercially available chemotherapeutic inhibitor, as detailed in the discussion.

**Tissue Microarray Staining for Acid Ceramidase**

Clinical and pathological data of patients included for tissue microarray analysis are detailed in Supplementary File C. There were 82 males and 29 females with a median (range) age of 66 (26-86) years at diagnosis. A pCR (TRG4) was observed in 22 cases (19.8%), TRG3 = 15, TRG2 = 55 and TRG1 = 19. There were 37 relative responders to CRT (TRG 4-3), and 74 relative non-responders (TRG 2-1). Differential staining of the tissue microarrays for AC was observed across all tissue types (normal colon, diagnostic biopsy, and cancer site at resection) as depicted by the images in Figures 1, 2 and 3 respectively. Of the 111 cases, 86-107 were successfully represented in the TMAs (depending on the tissue component), as demonstrated in Table 4.

A relatively low stromal expression of AC at the cancer site from the resection specimen was associated with both a pathological complete response (TRG 4) ( p=0.003), and a good response to CRT (TRG 3-4) (p=0.048), whereas a relatively high expression of AC was associated with a poor response to CRT (TRG 1-2) (p=0.017).

A relatively high epithelial expression of AC in normal adjacent colon was also associated with a poor response to CRT (p=0.012). There were no statistically significant results from the diagnostic biopsy samples, although 9/11 cores representing patients with a poor response to CRT (TRG 1) had a relatively high, but non statistically significant, stromal expression of AC (p=0.075).

Relatively high AC expression in both epithelium and stroma at the cancer site from the resection specimen correlated with an increased incidence of local recurrence (p=0.031 and p=0.038 respectively), as detailed in Table 5. There was no association between AC expression and the incidence of metastatic disease but a poorer overall survival was correlated with AC expression in normal colonic epithelium. The mean survival in those with a relatively high expression (n=51) was 89.7 months (95% CI 80.7 - 98.8), which was significantly longer than those with a relatively low expression, where mean survival was 71.9 months (95% CI 61.5 - 82.3) ((p=0.013, Mantel-Cox).

**Table 1 - Proteomic Profiling Patient Characteristics**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Age | Gender | Neoadjuvant Treatment | Response(Y/N) | Procedure | Dukes’ Stage | Histology |
| 77 | M | Capecitabine plus 45Gy in 25 fractions | Y | Open low anterior resection | C1 | Moderately differentiated adenocarcinoma |
| 50 | M | Capecitabine plus 50.4Gy in 28 fractions | Y | Laparoscopic low anterior resection | B | Moderately differentiated adenocarcinoma |
| 77 | M | Capecitabine plus 45Gy in 25 fractions | N | Open low anterior resection | C1 | Moderately differentiated adenocarcinoma |
| 77 | M | Capecitabine plus 50.4Gy in 28 fractions | Y | Ultra-low Hartmann's | B | Moderately differentiated adenocarcinoma |
| 63 | M | Capecitabine plus 50.4Gy in 28 fractions | N | Laparoscopic low anterior resection | D | Moderately differentiated adenocarcinoma |
| 71 | M | Capecitabine plus 50.4Gy in 28 fractions | Y | Laparoscopic low anterior resection | B | Moderately differentiated adenocarcinoma |
| 57 | M | Capecitabine plus 50.4Gy in 28 fractions | N | Laparoscopic low anterior resection | B | Moderately differentiated adenocarcinoma |
| 78 | M | Capecitabine plus 45Gy in 25 fractions | N | Abdomino-perineal excision | B | Moderately differentiated adenocarcinoma |

Clinico-pathological characteristics of those patients included for proteomic profiling.

**Table 2 - Differentially Expressed Proteins**

|  |  |  |  |
| --- | --- | --- | --- |
| **Accession Number** | **Name** | **p Value** | **Log Fold-Change** |
| Q9NZM1 | Myoferlin | 4.35E-02 | -1.633 |
| Q13510 | Acid ceramidase | 1.17E-02 | -1.526 |
| P09525 | Annexin A4 | 1.93E-02 | -1.524 |
| P41219 | Peripherin | 2.13E-02 | 1.583 |
| P12109 | Collagen alpha-1(VI) chain | 3.61E-02 | 1.800 |
| P80748 | Ig lambda chain V-III region LOI | 4.82E-02 | 1.866 |
| P07602 | Proactivator polypeptide | 5.90E-04 | 1.943 |
| P01860 | Ig gamma-3 chain C region | 2.74E-02 | 2.549 |

Proteins identified from the differential abundance of contributing peptide ions between relative responders and non-responders to CRT. A negative log fold-change indicates reduced expression in relative responders, and a positive log fold-change increased expression.

**Table 3 - Associated Upregulated and Downregulated Pathways**

|  |  |  |
| --- | --- | --- |
| **Upregulated Pathways** | **p Value** | **Ratio** |
| Lipid antigen presentation by CD1 | 5.28E-03 | 1/24 (0.042) |
| Autoimmune thyroid disease signalling | 9.23E-03 | 1/42 (0.024) |
| Haematopoiesis from pleuripotent stem cells | 9.67E-03 | 1/44 (0.024) |
| Primary immunodeficiency signalling | 1.01E-02 | 1/46 (0.022) |
| Allograft rejection signalling | 1.05E-02 | 1/48 (0.021) |

|  |  |  |
| --- | --- | --- |
| **Downregulated Pathways** | **p Value** | **Ratio** |
| Ceramide degradation | 9.92E-04 | 1/6 (0.167) |
| Sphingosine and sphingosine-1phosphate metabolism | 1.32E-03 | 1/8 (0.125) |
| Sphingosine-1-phosphate signalling | 1.78E-02 | 1/108 (0.009) |

Upregulated and downregulated pathways identified from proteins with differential abundance of contributing peptide ions, when comparing relative responders and non-responders to CRT.

**Table 4 - Analysis of Tissue Microarray Acid Ceramidase Expression**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Tissue Microarray** | **Cellular Subset** | **Cases Represented** | **Median H Score** | **TRG Comparison** | **p Value (x2)** |
| Diagnostic Biopsy | Epithelium | 86/105 | 5.0 | pCR vs Others | 0.695 |
| Relative Response | 0.699 |
| Others vs Non-response | 0.375 |
| Stroma | 86/105 | 5.0 | pCR vs Others | 0.472 |
| Relative Response | 0.764 |
| Others vs Non-response | **0.075** |
| Cancer Site | Epithelium | 68/89 | 5.5 | pCR vs Others | N/A |
| Relative Response | 0.506 |
| Others vs Non-response | 0.487 |
| Stroma | 105/111 | 4.0 | pCR vs Others | **0.003** |
| Relative Response | **0.048** |
| Others vs Non-response | **0.017** |
| Normal Colon | Epithelium | 107/111 | 6.5 | pCR vs Others | 0.816 |
| Relative Response | 0.196 |
| Others vs Non-response | **0.012** |

Each tissue assessed (diagnostic biopsy, resection specimen cancer site or normal colon) and subset (epithelium or stroma) was analysed by correlating lower/higher IHC scoring for AC (dichotomised about the median score) against the respective TRG group (compared as pCR (TRG 4) vs. others (TRG 3-1), relative responders (TRG 4-3) vs. relative non-responders (TRG 2-1), and non-responders (TRG 1) vs. others (TRG 4-2)) using x2.

**Table 5 – Clinical Outcomes and Tissue Microarray Acid Ceramidase Expression**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Tissue** | **Relative Acid Ceramidase Expression** | **Local Recurrence** | **Metastases** | **Death** |
| **n=** | **p value** | **n=** | **p value** | **n=** | **p value** |
| Diagnostic Biopsy Epithelium | Lower n=42 | 1 | 0.518 | 17 | 0.061 | 11 | 0.263 |
| Higher n=44 | 2 | 10 | 8 |
| Diagnostic Biopsy Stroma | Lower n=37 | 0 | 0.180 | 12 | 0.520 | 8 | 0.570 |
| Higher n=49 | 3 | 15 | 11 |
| Cancer Site Epithelium | Lower n=33 | 0 | **0.031** | 11 | 0.376 | 7 | 0.338 |
| Higher n=35 | 5 | 14 | 10 |
| Cancer Site Stroma | Lower n=56 | 1 | **0.038** | 17 | 0.180 | 11 | 0.200 |
| Higher n=49 | 6 | 20 | 14 |
| Normal Colon Epithelium | Lower n=56 | 3 | 0.615 | 21 | 0.403 | 22 | **0.001** |
| Higher n=51 | 3 | 17 | 6 |

Relative tissue microarray acid ceramidase expression in each tissue subset was correlated with local disease recurrence, metastatic disease and death using Fisher’s exact test.

**Discussion**

We have undertaken novel proteomic profiling of rectal cancer, and identified that AC is implicated in radiation sensitivity, with lower expression observed in radiosensitive tumours. Further independent validation has been undertaken in 111 rectal cancer patients where stromal tumour expression of AC in post-CRT specimens is consistent with the original findings. A high epithelial expression of AC in normal colon has been associated with a poor response to CRT.

Whilst AC was one of eight proteins originally identified as differentially expressed between relative responders and non-responders to CRT, we have initially focussed on AC individually in the validation work on the basis of the evidence for its implication in prostate cancer response to radiotherapy, and the availability of a commercially available chemotherapeutic inhibitor. Our proteomic profiling also revealed myoferlin as another protein that may be implicated in response to CRT. Myoferlin is a muscle specific protein that has been identified in cancer cells and has been shown to confer a poor prognosis in oropharyngeal cancers(37), another tumour group often treated with radiotherapy. Further work is also needed to assess the role of this protein in rectal cancer.

Correlation of AC expression with outcome data has additionally demonstrated association between relatively high epithelial and stromal expression in post-CRT cancer site tissue and local recurrence, and poorer survival with relatively low epithelial expression in normal colon. These findings further implicate AC in radiation response, although the latter is contrary to relatively high expression in normal colon epithelium correlating with improved response to CRT. This may be a statistical anomaly, but requires further analysis in a prospective dataset.

We recognise the limitations of the initial proteomic profiling. The number of proteins with significant difference is small compared to the number of identified proteins, and the number of proteins used for Ingenuity Pathway Analysis is also small. This is a consequence of a small initial study and a desire to keep statistical selection stringent to avoid false discovery, and is why we looked to and would recommend validation. Tissues were also not micro-dissected in sample preparation, however this was intentional in order to mimic a clinical diagnostic biopsy and ensure translation of any results to clinical practice.

Previous individual biomarker studies have focussed on candidate proteins involved in DNA damage repair, cell proliferation, angiogenesis and apoptosis, most frequently evaluating p53, p21, KRAS, EGFR, VEGF, Bax, Bcl-2, thymidylate synthetase, and Ki-67(27). No single protein has been consistently demonstrated to be predictive of response to CRT in rectal cancer. Equally gene expression profiles investigating pCR have not been comparable between studies(16). Given the variable response to CRT, and the lack of reproducibility between studies, it is likely that multiple genes and cellular pathways play a role in the regulation of tumour response, notwithstanding post-translational modifications and interactions in the tumour micro-environment(28).

The stroma (or tumour micro-environment) is considered to play an essential role in both the development of malignancy and in the resistance to treatment. This environment, composed of extracellular matrix, fibroblasts, endothelial cells, and cells of the immune system regulates the behaviour of tumour cells and co-evolves(29). Cancer cells have been demonstrated to activate and recruit carcinoma-associated fibroblasts, which are able to both stimulate cell growth and invasion, as well as inflammation and angiogenesis but may also be tumour inhibiting(30). This process is considered to be under the regulation of transforming growth factor β (TGFβ) signalling, which itself is primarily activated by integrins secreted from both tumour and stromal cells. In normal tissues TGFβ suppresses epithelial cell division but tumour cells can develop escape mechanisms to become resistant to TGFβ growth suppression. In addition, TGFβ drives epithelial mesenchymal transition, increasing the potential for metastasis(31). The host response, through natural killer cells, has also been observed as a determinant of response to CRT(32). Particularly given the association of higher expression of AC in normal colon epithelium with a poor response to CRT, it may be AC is implicated in mediating radio-resistance through tumour-stroma interaction or host response, and could represent a therapeutic target.

AC is a sphingolipid - a group of biomolecules known to be responsible for important signalling functions in the control of cell growth, differentiation and apoptosis, and itself catalyses the cleavage of ceramide into sphingosine and free fatty acid(32,33). Ceramide has been observed to accumulate in radiation induced apoptosis, and is described as a tumour suppressor lipid(34). Lower levels of AC may therefore facilitate ceramide accumulation and radiation induced apoptosis, with higher levels facilitating ceramide cleavage, driving the cell away from apoptosis towards survival. Downregulation of the corresponding gene for AC with siRNA in a prostate cancer cell line has been convincingly demonstrated to confer radio-sensitivity(35). This *in vitro* study in PPC-1 cells assessed radiation response by clonogenic and cytotoxic assays, demonstrating that upregulation of AC decreased sensitivity to radiation and created cross-resistance to chemotherapy. The small molecule AC inhibitor LCL385 was also sufficient to sensitize PPC-1 cells to radiation. Carmofur is a fluorouracil analogue which has been used in the US in the adjuvant setting for colorectal and breast cancer. In addition to its presumed primary mode of action as a thymidylate synthase inhibitor it has also independently been shown to inhibit AC in the human tumour derived cell lines SW403 (colorectal) and LNCaP (prostate), a property not shared by fluorouracil or capecitabine(36). Considering our study, further *in vitro* work is needed to compare the radiosensitisation of the commonly used 5-fluorouracil (5FU, thymidylate synthase inhibition) with carmofur (thymidylate synthase and AC inhibition). Should *in vitro* work give further confirmation of the role of AC in rectal cancer response to CRT, a translation study comparing the radiosensitisation of carmofur against 5FU or capecitabine *in vivo* is possible.

Whilst our data has not suggested a role for AC as an upfront predictive biomarker of response to CRT, it does appear to be implicated in mediating radio-resistance, and further work to establish its role is urgently needed. The contribution, if any, of the established AC inhibitor carmofur also needs to be further defined in this patient population.

**Titles and Legends to Figures**

Figure 1 - Normal Colon TMA Cores.

The depicted cores were observed from one TMA section having been stained for AC (A&C), with the corresponding cores (B&D) from another section exposed to a mouse IgG isotype as a negative control. The core in image A demonstrates high epithelial expression of AC (H-score = 5+3 = 8) in a case where a poor response to CRT (TRG1) was assigned to the resection specimen. Low epithelial expression of AC is observed in image C (H-score = 2+1 = 3) in a case where a good response to CRT (TRG3) was assigned to the resection specimen. The rectangular bar in each image represents 300µm.

Figure 2 - Diagnostic Biopsy TMA Cores

The depicted cores were observed from the same TMA section having been stained for AC (A&C), with the corresponding cores (B&D) from another section exposed to a mouse IgG isotype as a negative control. The core in image A demonstrates high epithelial (H-score = 5+3 = 8) and stromal (H-score = 5+2 = 7) expression of AC, in a case where a minimal response to CRT (TRG2) was assigned to the resection specimen. Low epithelial (H-score = 1+1 = 2) and stromal expression (H-score = 0+0 = 0) of AC is demonstrated in image C in a case where a subsequent pCR (TRG4) was observed. The rectangular bar in each image represents 300µm.

Figure 3 - Cancer Site (Resection Specimen) TMA Cores

The depicted cores were observed from the same TMA section having been stained for AC (A&C), with the corresponding cores (B&D) from another section exposed to a mouse IgG isotype as a negative control. The core in image A demonstrates high epithelial (H-score = 5+3 = 8) and stromal (H-score = 5+2 = 7) expression of AC, in a case where a poor response to CRT (TRG1) was assigned. Low epithelial (H-score = 0+0 = 0) but higher stromal expression (H-score = 3+2 = 5) of AC is demonstrated in the core in image C in a case with a minimal response to CRT (TRG2). The rectangular bar in each image represents 300µm.

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