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The differential expression of omega-3 and omega-6 fatty acid metabolising enzymes in colorectal cancer and its prognostic significance

Abdo Alnabulsi^{1,2}, Rebecca Swan¹, Beatriz Cash², Ayham Alnabulsi² and Graeme I Murray^{*1}

¹Department of Pathology, School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Foresterhill, Aberdeen AB25, 2ZD, UK and ²Vertebrate Antibodies, Zoology Building, Tillydrone Avenue, Aberdeen AB24 2TZ, UK

Background: Colorectal cancer is a common malignancy and one of the leading causes of cancer-related deaths. The metabolism of omega fatty acids has been implicated in tumour growth and metastasis.

Methods: This study has characterised the expression of omega fatty acid metabolising enzymes CYP4A11, CYP4F11, CYP4V2 and CYP4Z1 using monoclonal antibodies we have developed. Immunohistochemistry was performed on a tissue microarray containing 650 primary colorectal cancers, 285 lymph node metastasis and 50 normal colonic mucosa.

Results: The differential expression of CYP4A11 and CYP4F11 showed a strong association with survival in both the whole patient cohort (hazard ratio (HR)=1.203, 95% CI=1.092–1.324, $\chi^2=14.968$, $P=0.001$) and in mismatch repair-proficient tumours (HR=1.276, 95% CI=1.095–1.488, $\chi^2=9.988$, $P=0.007$). Multivariate analysis revealed that the differential expression of CYP4A11 and CYP4F11 was independently prognostic in both the whole patient cohort ($P=0.019$) and in mismatch repair proficient tumours ($P=0.046$).

Conclusions: A significant and independent association has been identified between overall survival and the differential expression of CYP4A11 and CYP4F11 in the whole patient cohort and in mismatch repair-proficient tumours.

Colorectal cancer is one of the major contributors to cancer-related mortality in the developed world (Siegel *et al*, 2014, 2016). The introduction of screening programmes and the development of new drugs have improved the survival rate of colorectal cancer patients; however, the average 5-year survival rate remains poor at 55% (Brenner *et al*, 2014). The characterisation of novel biomarker targets can further improve the survival rate, as it provides a better understanding of the complex molecular events underpinning tumour development and, if clinically validated, these biomarkers have potential roles in screening, diagnosis, prognosis and monitoring disease progression (Coghlin and Murray, 2015; Alnabulsi and Murray, 2016).

The CYP4 cytochrome P450 family of enzymes metabolises omega-3 and omega-6 fatty acids to biologically active eicosanoids that are implicated in tumour initiation, development and progression (Yu *et al*, 2011; Johnson *et al*, 2015). Arachidonic acid, an omega-6 fatty acid, is converted by CYP4A11 to 20-hydroxyicosatetraenoic acid (20-HETE), which is considered a key modulator in tumour progression, angiogenesis and metastasis (Ljubimov and Grant, 2005; Guo *et al*, 2007). CYP4F11 is not an efficient metaboliser of arachidonic acid compared with CYP4A11; however, it is the predominant CYP4 enzyme involved in the metabolism of omega 3-fatty acids (Dhar *et al*, 2008). The substrate specificity is not yet fully characterised for CYP4V2 and CYP4Z1

*Correspondence: Professor GI Murray; E-mail: g.i.murray@abdn.ac.uk

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(Guengerich and Cheng, 2011). Despite the recognition of the involvement of omega fatty acids in tumourigenesis, the role of the cytochrome P450 enzymes involved in this pathway has received very limited attention in cancer biology (Panigrahy *et al*, 2010).

Using monoclonal antibodies we have developed the cytochrome P450 enzymes CYP4A11, CYP4F11, CYP4V2 and CYP4Z1; this study has profiled the expression of these enzymes by immunohistochemistry on a tissue microarray containing a large and well-characterised cohort of colorectal cancers. The expression profile of each enzyme was established by light microscopy using a semi-quantitative scoring system. The prognostic significance of each enzyme was determined by assessing the relationship between their expression in tumours and overall survival.

MATERIALS AND METHODS

Monoclonal antibody development. Monoclonal antibodies to CYP4A11, CYP4V2 and CYP4Z1 were developed using short synthetic peptides (Murray *et al*, 1998). Multiple sequence alignments of the amino acid sequences were performed on these enzymes and other CYP4 family members, to identify regions with the highest amino acid diversity. To avoid poorly antigenic sequences of amino acids (e.g., transmembrane region), a range of bioinformatics tools were used to predict and model hydrophilic, accessible and antigenic polypeptide sequences, as well as the secondary and tertiary structures of each enzyme (Supplementary Materials and Methods S1).

The amino acid sequences of peptides used to generate the antibodies and their location on each enzyme are specified in Supplementary Table S1. All peptides (Almac Sciences Ltd, Edinburgh, UK) were conjugated to ovalbumin for immunisations and to bovine serum albumin for the enzyme-linked immunosorbent assay (ELISA) screenings (Duncan *et al*, 1992). The immunisation via the subcutaneous route, the production of hybridomas and the ELISA screenings were carried out as previously described (Murray *et al*, 1996, 1998; Brown *et al*, 2014). The development of the monoclonal antibody to CYP4F11 has been described previously (Kumarakulasingham *et al*, 2005).

Immunoblotting. The specificity of the monoclonal antibodies was established by immunoblotting using whole-cell lysate (human embryonic kidney cells-HEK 293, Novus Biologicals, Cambridge, UK) overexpressing the relevant CYP as a positive control and lysates from cells containing empty vector as a negative control. Microsomal fractions prepared from human liver tissues (BD Gentest Human Liver Microsomes Pooled Male Donors 20 mg ml⁻¹ catalogue number 452172, BD Biosciences, Bedford, MA, USA) were also used to carry out immunoblotting validation for each antibody. The immunoblotting was carried out as described, except that the polyvinylidene difluoride membrane was incubated overnight at 4°C with undiluted monoclonal antibody (neat hybridoma tissue culture supernatant) and the secondary antibody, horseradish-peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, Dorset, UK), was diluted 1/3000 in phosphate-buffered saline-Tween-20 (Swan *et al*, 2016). When using liver microsomes, 30 µg of samples were loaded per lane compared with 5 µg when using overexpression lysate.

Patient cohort and colorectal cancer tissue microarray. The patient cohort was retrospectively acquired from the Grampian Biorepository (www.biorepository.nhsgrampian.org). The cohort is composed of tissue samples from 650 patients who had undergone surgery for primary colorectal cancers between 1994 and 2009, at Aberdeen Royal Infirmary (Aberdeen, UK), which is the principal teaching hospital of NHS Grampian. Patients who had received neoadjuvant chemotherapy and/or radiotherapy were excluded.

Survival time was defined to be the period in whole months from the date of surgery to the date of death from any cause (i.e., all-cause mortality). Survival data on a 6-monthly basis was updated from the NHS Grampian electronic patient management system and no patients were lost to follow-up. At the time (March 2012) of the censoring of patient outcome data, there had been 309 (47.5%) deaths and patients who were still alive were censored. The median survival was 103 months (95% CI = 86–120 months), the mean survival was 115 months (95% CI = 108–123 months) and the median follow-up time, calculated by the 'reverse Kaplan-Meier' method, was 88 months (95% CI = 79–97 months). The clinico-pathological characteristics of the patients and their association with survival are described in Table 1.

Histopathology reporting was in accordance with The Royal College of Pathologists UK guidelines for the histopathological

Table 1. Clinico-pathological characteristics of all patients, their tumours and the relationship of each variable with overall survival

Characteristic	Number patients	Percentage	Relationship with survival
Sex			
Male	340	52.3	$\chi^2 = 0.027$, $P = 0.870$
Female	310	47.7	
Age (years)			
<70	305	46.9	$\chi^2 = 29.213$, $P < 0.001$
≥70	345	53.1	
Screen detected			
Yes	52	8	$\chi^2 = 16.381$, $P < 0.001$
No	598	92	
Tumour site			
Proximal colon	261	40.2	Proximal vs distal, $\chi^2 = 8.418$, $P = 0.004$ Distal vs rectal, $\chi^2 = 0.906$, $P = 0.341$ Colon vs rectum, $\chi^2 = 0.098$, $P = 0.754$
Distal colon	245	37.7	
Rectum	144	22.2	
Tumour differentiation			
Well/moderate	600	92.3	$\chi^2 = 0.976$, $P = 0.323$
Poor	50	7.7	
Extra-mural venous invasion			
Present	140	21.5	$\chi^2 = 100.946$, $P < 0.001$
Absent	510	78.5	
Microsatellite instability status			
Defective	96	15.2	$\chi^2 = 2.848$, $P = 0.091$
Intact	536	84.8	
pT stage			
T1	30	4.6	T1 vs T2, $\chi^2 = 0.382$, $P = 0.536$ T2 vs T3, $\chi^2 = 24.739$, $P < 0.001$ T3 vs T4, $\chi^2 = 30.159$, $P < 0.001$
T2	114	17.5	
T3	411	63.2	
T4	95	14.6	
pN stage			
N0	364	56	N0 vs N1, $\chi^2 = 54.071$, $P < 0.001$ N1 vs N2, $\chi^2 = 17.636$, $P < 0.001$
N1	177	27.2	
N2	109	16.8	
Dukes stage			
A	120	18.5	A vs B, $\chi^2 = 5.059$, $P = 0.025$ B vs C, $\chi^2 = 65.510$, $P < 0.001$
B	244	37.5	
C	286	44	

Significant values are highlighted in bold.

reporting of resection specimens of colorectal cancer, which includes guidance from version 5 of the tumour, node, metastasis staging system (Williams *et al*, 2007).

Blocks of formalin-fixed, paraffin-embedded tissue specimens were used to construct the tissue microarray as previously described (O'Dwyer *et al*, 2011; Brown *et al*, 2014; Swan *et al*, 2016). The histopathological processing of tissue specimens and the construction of the tissue microarray are described in Supplementary Materials and Methods S1

Immunohistochemistry. A Dako autostainer (Dako, Ely, UK) was used to perform the immunohistochemistry for each antibody using the Dako EnVision system (Dako; Kumarakulasingham *et al*, 2005, Brown *et al*, 2014). Antigen retrieval (microwaving in 10 mM citrate buffer pH 6.0 for 20 min) was performed for all antibodies, except CYP4A11. The immunohistochemistry procedure and the antigen retrieval are described in Supplementary Materials and Methods S1. A semi-quantitative scoring system was used to evaluate the intensity of immunostaining of each antibody (Kumarakulasingham *et al*, 2005; O'Dwyer *et al*, 2011; Brown *et al*, 2016; Swan *et al*, 2016). The scoring was conducted independently by two observers (RS and GIM) who were unaware of the clinical data and outcome. The assessment of cores was performed using light microscopy (Olympus BX 51, Olympus, Southend-on-Sea, Essex, UK). Simultaneous re-evaluation of the cores by both investigators was used to resolve any discrepancies in the scores (<5% of cases).

Assessment of MMR status. The status of mismatch repair protein (MMR) in the patient cohort was classified as either defective or proficient, based on the immunohistochemical assessment of MLH1 and MSH2 proteins (Brown *et al*, 2014).

Data analysis and statistics. The data were entered into an Excel 2013 spreadsheet before being analysed using IBM SPSS version 24 for Windows 7 (IBM, Portsmouth, UK). The following statistical tests were used: Mann–Whitney *U*-test, Wilcoxon signed-rank test, χ^2 -test, Kaplan–Meier survival analysis, log-rank test and Cox multivariate analysis (variables entered as categorical variables) including the calculation of hazard ratios (HRs) and 95% CIs. A probability value of $P \leq 0.05$ was regarded as statistically significant. The survival analysis of the different patients groups was conducted using the log-rank test. The scores for each protein were dichotomised using the following cutoff points; negative staining vs positive staining, negative and weak staining vs moderate and strong staining, and strong staining vs negative/weak/moderate staining. Further details of data analysis and statistics are provided in Supplementary Materials and Methods S1.

Ethics. The use of colorectal tissue samples in this study was approved by the Grampian Biorepository scientific access group committee (Tissue request number 0002). No written consent was required from patients for the use of formalin fixed wax embedded tissue samples in the colorectal cancer tissue microarray.

RESULTS

Monoclonal antibodies. During the hybridoma production, sequential ELISA screenings (immunogenic peptide specific to each enzyme) were used to determine the specificity of the monoclonal antibodies towards CYP4A11, CYP4V2 and CYP4Z1 (Duncan *et al*, 1992). Furthermore, immunoblotting showed a band migrating at the expected molecular weight for each antibody, whereas no band was detected in the negative controls (Supplementary Figure S1). The specificity of the antibody to CYP4F11 was confirmed previously (Kumarakulasingham *et al*, 2005).

Immunohistochemistry. CYP4A11, CYP4F11 and CYP4V2 antibodies showed immunoreactivity in normal colonic epithelium, primary colorectal tumours and lymph node metastasis, whereas CYP4Z1 showed immunoreactivity only in a very small proportion of primary tumours. The immunostaining was exclusively localised to the cytoplasm of the cells (Supplementary Figure S2). Intra-tumour heterogeneity was not observed in either primary or metastatic colorectal tumours.

There was a significant increase in the intensity of immunostaining in primary tumours compared with normal colonic mucosa for CYP4A11 ($P < 0.001$), CYP4F11 ($P < 0.001$) and CYP4V2 ($P < 0.001$) (Table 2 and Supplementary Figure S3). In contrast, a significant decrease in the expression of CYP4A11 ($P = 0.007$), CYP4F11 ($P < 0.001$) and CYP4V2 ($P < 0.001$) was observed in lymph node metastasis compared with all primary tumours. There was also a significant decrease in the expression of CYP4A11 ($P = 0.002$), CYP4F11 ($P < 0.001$) and CYP4V2 ($P < 0.001$) in lymph node metastasis compared with their corresponding primary Dukes C tumours.

Relationship with pathological parameters. The relationships between the main pathological parameters and the expression of CYP4A11, CYP4F11, CYP4V2 and CYP4Z1 are summarised in Supplementary Tables S2A–C. Both CYP4A11 ($\chi^2 = 13.148$, $P = 0.041$) and CYP4V2 ($\chi^2 = 24.474$, $P < 0.001$) showed significant associations with Dukes stage, but only CYP4V2 displayed a significant relationship with tumour stage ($\chi^2 = 17.837$, $P = 0.037$). The expression of CYP4A11 was significantly associated with tumour site ($\chi^2 = 15.703$, $P = 0.015$). CYP4F11 also showed significant associations with tumour site ($\chi^2 = 20.947$, $P = 0.002$), tumour differentiation ($\chi^2 = 8.5552$, $P = 0.036$) and MMR status ($\chi^2 = 13.441$, $P = 0.004$).

Survival analysis

Whole patient cohort. Different cutoff points of the immunostaining scores were used to investigate the association between the expression of CYP4A11, CYP4F11 and CYP4V2, and overall survival (Supplementary Table S3). The expression of CYP4A11 showed a consistent and significant association with overall survival (Figure 1). Overall, increasing intensity of CYP4A11 immunostaining was significantly associated with poorer outcome

Table 2. Comparison of the expression of CYP4's in normal colonic mucosa, primary colorectal cancer and lymph node metastasis

	Immunoreactivity (P-value, normal vs primary tumour)	Change in expression in tumour	Immunoreactivity (P-value, primary tumour vs lymph node metastasis)	Change in expression in lymph node	Immunoreactivity (P-value, paired primary Dukes C tumour vs lymph node metastasis)	Change in expression in lymph node
CYP4A11	$P < 0.001$	↑	$P = 0.007$	↓	$P = 0.002$	↓
CYP4F11	$P < 0.001$	↑	$P < 0.001$	↓	$P < 0.001$	↓
CYP4V2	$P < 0.001$	↑	$P < 0.001$	↓	$P < 0.001$	↓
CYP4Z1	$P = 0.303$	-	$P = 0.028$	↓	$P = 0.083$	-

Evaluation of normal colonic epithelium vs primary tumour samples for immunoreactivity (Mann–Whitney *U*-test, ↑ = increased in tumour, ↓ = decreased in tumour, - = no change between tumour and normal), and evaluation of primary Dukes C colorectal tumour samples and their corresponding metastasis samples for immunoreactivity (Wilcoxon signed-rank sum test, ↑ = increased in lymph node metastasis, ↓ = decreased in lymph node metastasis, - = no change between primary and metastatic tumour). Significant values are highlighted in bold.

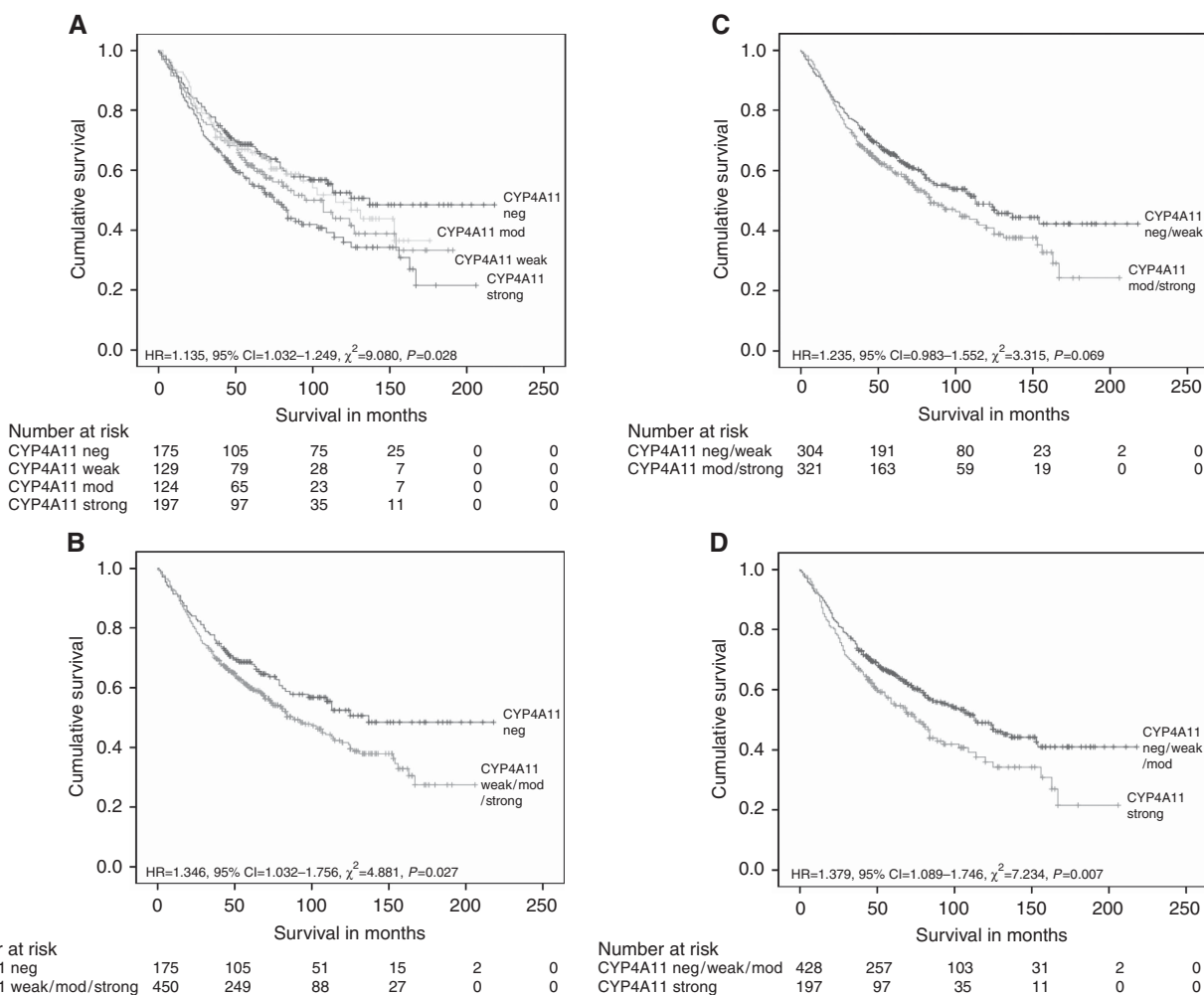


Figure 1. The overall relationship. The overall relationship between the expression of CYP4A11 and survival in the whole patient cohort using different cutoff points: negative vs weak vs moderate vs strong (A), further details of median survival times of individual groups, P-values and HRs are found in Supplementary Table S4), strong vs negative/weak/moderate (B), positive expression vs negative expression (C), and negative and weak vs moderate and strong (D).

(HR = 1.135, 95% CI = 1.032–1.249, $\chi^2 = 9.080$, $P = 0.028$). When each level of the intensity groups of CYP4A11 expression was considered independently using one reference group (negative expression), strong CYP4A11 immunostaining was significantly associated with poorer outcome (HR = 1.541, 95% CI = 1.144–2.077, $\chi^2 = 8.006$, $P = 0.005$) (Supplementary Table S4). The median survival was 137 months (95% CI undefined) and the mean was 132 months (95% CI = 117–147 months) for CYP4A11-negative tumours ($n = 175$), whereas the median survival was 75 months (95% CI = 58–91 months) and the mean was 96 months (95% CI = 84–109 months) for CYP4A11 strong expression tumours ($n = 197$).

Immunoreactivity for CYP4A11 was significantly associated with poorer prognosis (HR = 1.346, 95% CI = 1.032–1.756, $\chi^2 = 4.881$, $P = 0.027$) when compared with CYP4A11-negative tumours. For CYP4A11-positive tumours ($n = 450$), the median survival was 88 months (95% CI = 71–104 months) and the mean was 105 months (95% CI = 96–114 months), compared with a median of 137 (95% CI undefined) and a mean of 132 months (95% CI = 117–147 months) for CYP4A11-negative tumours ($n = 175$). Comparing strong CYP4A11-expressing tumours with negative/weak/moderate-expressing tumours also showed a significant association with survival (HR = 1.379, 95% CI = 1.089–1.746, $\chi^2 = 7.234$, $P = 0.007$). The median survival was 113 months (95% CI = 89–136 months) and the mean was 124 months (95%

CI = 114–134 months) for negative/weak/moderate CYP4A11-immunostaining tumours ($n = 428$), whereas the median survival was 75 months (95% CI = 58–91 months) and the mean was 96 months (95% CI = 84–109 months) for strong CYP4A11-immunostaining tumours ($n = 197$).

Exploratory analysis of CYP4 enzyme expression showed there was a significant association between the differential (combined) expression of CYP4A11 and CYP4F11, and survival (Supplementary Table S5). Therefore, a new variable, based on the differential expression of CYP4A11 and CYP4F11, was created to stratify tumours into three groups; CYP4A11 > CYP4F11, CYP4A11 = CYP4F11 and CYP4A11 < CYP4F11. Overall survival was significantly associated with the expression profiles of CYP4A11 > CYP4F11, CYP4A11 = CYP4F11 and CYP4A11 < CYP4F11 groups (HR = 1.311, 95% CI = 1.140–1.506, $\chi^2 = 14.968$, $P = 0.001$) (Figure 2). When each level of the differential expression groups was considered independently using pairwise comparisons and one reference group (CYP4A11 < CYP4F11), both CYP4A11 > CYP4F11 (HR = 1.733, 95% CI = 1.306–2.300, $\chi^2 = 14.405$, $P < 0.001$) and CYP4A11 = CYP4F11 (HR = 1.432, 95% CI = 1.064–1.928, $\chi^2 = 5.425$, $P = 0.020$) were significantly associated with poorer outcome (Supplementary Table S6). The mean survival was 137 months (95% CI = 124–151 months) (median survival undefined) for the CYP4A11 < CYP4F11 group ($n = 214$), the median survival was 95 months (95% CI = 72–117

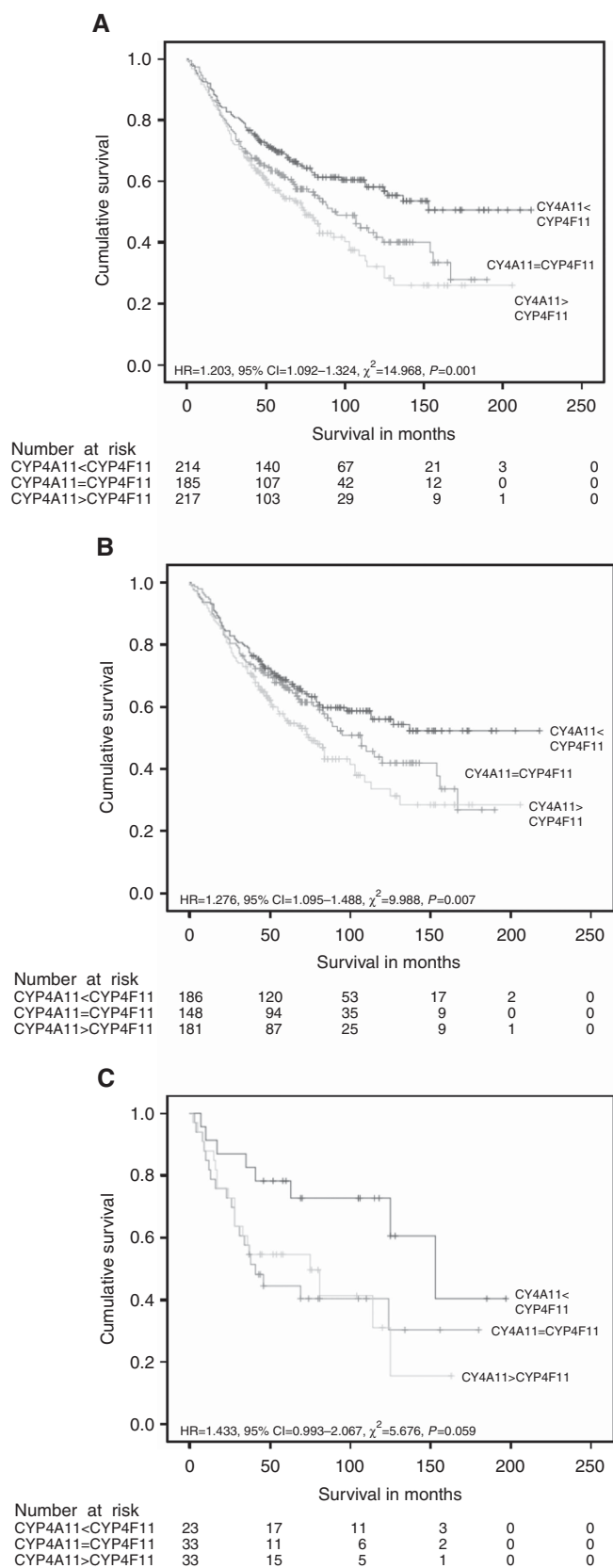


Figure 2. The overall relationship. The overall relationship between the differential expression of CYP4A11 and CYP4F11, and survival in the whole patient cohort (**A**), in MMR-proficient tumours (**B**) and in MMR-deficient tumours (**C**). Further details of median survival times of individual groups, *P*-values and HRs are found in Supplementary Table S6.

months) and the mean was 102 months (95% CI = 90–114 months) for the CYP4A11 = CYP4F11 group ($n = 185$), whereas the median survival was 75 months (95% CI = 60–89 months) and the mean survival was 94 months (95% CI = 81–106 months) for CYP4A11 > CYP4F11 group ($n = 217$).

The associations between the expression of CYP4A11, CYP4F11, CYP4V2 and CYP4Z1, and overall survival in relation to different tumour sites, Dukes stage and extramural venous invasion are shown in Supplementary Tables S7–S10.

Mismatch repair protein-proficient cohort. There was a significant association between the expression of CYP4A11 and overall survival in MMR-proficient tumours (HR = 1.156, 95% CI = 1.040–1.286, $\chi^2 = 11.221$, $P = 0.011$) (Figure 3 and Supplementary Table S11). When each level of the intensity groups of CYP4A11 expression was considered separately using pairwise comparisons and one reference group (negative expression), strong CYP4A11 immunoreactivity was significantly associated with poorer prognosis (HR = 1.644, 95% CI = 1.183–2.284, $\chi^2 = 8.626$, $P = 0.003$) (Supplementary Table S4). When comparing strong CYP4A11-expressing tumours with negative/weak/moderate-expressing tumours, the strong expression of CYP4A11 showed a significant association with worse survival (HR = 1.491, 95% CI = 1.152–1.929, $\chi^2 = 9.404$, $P = 0.002$). The positive expression of CYP4A11 was also significantly associated with poorer outcome when positive CYP4A11-expressing tumours were compared with negative CYP4A11-expressing tumours (HR = 1.375, 95% CI = 1.022–1.851, $\chi^2 = 4.485$, $P = 0.034$).

There was also a significant association between the differential expression of CYP4A11 and CYP4F11, and survival in MMR-proficient tumours (HR = 1.276, 95% CI = 1.05–1.488, $\chi^2 = 9.988$, $P = 0.007$) (Figure 2). When each level of the intensity groups was considered independently using pairwise comparisons and one reference group (CYP4A11 < CYP4F11), CYP4A11 > CYP4F11-expressing tumours were significantly associated with poorer outcome (HR = 1.629, 95% CI = 1.199–2.214, $\chi^2 = 9.261$, $P = 0.002$) (Supplementary Table S6). The median survival was 75 months (95% CI = 85–121 months) and the mean was 97 months (95% CI = 83–111 months) for CYP4A11 > CYP4F11-expressing tumours ($n = 181$), whereas the mean survival was 137 months (95% CI = 123–151 months) (median survival undefined) for CYP4A11 < CYP4F11-expressing tumours ($n = 186$).

Mismatch repair protein-deficient cohort. The lack of expression of CYP4F11 was significantly associated with worse overall survival compared with CYP4F11-positive tumours (HR = 0.479, 95% CI = 0.241–0.952, $\chi^2 = 4.682$, $P = 0.03$) (Supplementary Table S11 and Supplementary Figure S4). The median survival was 28 (95% CI = 21–34 months) and the mean was 49 months (95% CI = 28–70 months) for CYP4F11-negative tumours ($n = 16$) compared with a median of 114 (95% CI = 78–149 months) and a mean of 104 months (95% CI = 84–123 months) for CYP4F11-positive tumours ($n = 77$).

Overall, the association between survival and the differential expression of CYP4A11 and CYP4F11 just failed to reach the threshold for statistical significance in MMR-deficient cohort (HR = 1.433, 95% CI = 0.993–2.067, $\chi^2 = 5.676$, $P = 0.059$) (Figure 2). When each level of the intensity groups was considered independently using pairwise comparisons and one reference group (CYP4A11 < CYP4F11), both CYP4A11 > CYP4F11-expressing tumours (HR = 1.733, 95% CI = 1.306–2.300, $\chi^2 = 14.405$, $P < 0.001$) and CYP4A11 = CYP4F11-expressing tumours (HR = 1.432, 95% CI = 1.064–1.928, $\chi^2 = 5.425$, $P = 0.020$) were significantly associated with poorer outcome (Supplementary Table S6).

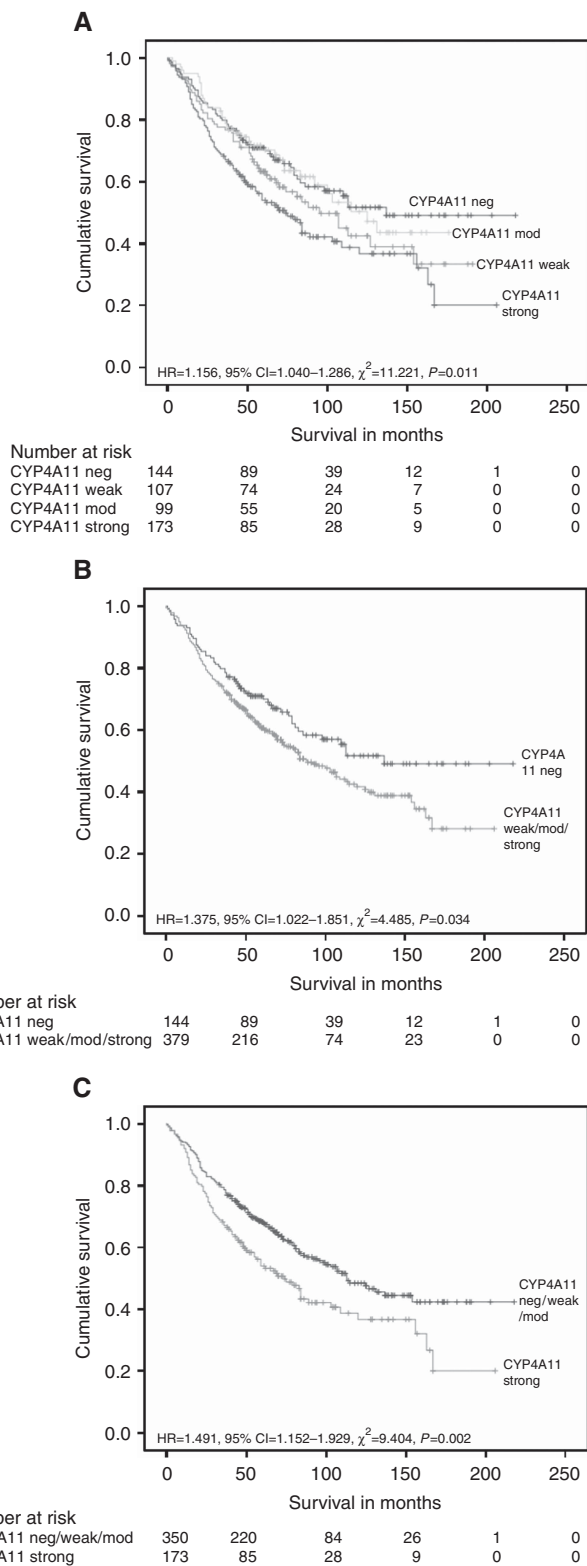


Figure 3. The relationship between the expression of CYP4A11 and survival in MMR-proficient tumours. The relationship between the expression of CYP4A11 and survival in MMR-proficient tumours using different cutoff points: negative vs weak vs moderate vs strong (A), further details of median survival times of individual groups, P-values and HRs are found in Supplementary Table S4), strong vs negative/weak/moderate (B) and positive expression vs negative expression (C).

Multivariate analysis. To evaluate the prognostic value of the differential expression of CYP4A11 and CYP4F11 (as a single variable) in relation to established prognostic parameters, multivariate analysis was performed using ‘Forward Stepwise: Conditional LR’ Cox regression method. The model showed there was a significant and independent prognostic value of using the differential expression of CYP4A11 and CYP4F11 in the whole patient cohort ($P=0.019$) and in MMR-proficient tumours ($P=0.046$) (Table 3 and Supplementary Tables S12 and S13). The differential expression was also a significant independent prognostic indicator in a multivariate analysis using only parameters that would be available at the time of biopsy in both the whole patient cohort ($P=0.001$) and in MMR-proficient tumours ($P=0.006$) (Supplementary Table S14).

DISCUSSION

The rise in incidence and the poor survival rate makes colorectal cancer a major health burden in the developed world (Brenner *et al*, 2014; Siegel *et al*, 2014, 2016). There is still urgent need to identify and validate biomarkers of colorectal cancer that can play a role in clinical practice (Alnabulsi and Murray, 2016).

In this study, we have produced monoclonal antibodies to P450 enzymes CYP4A11, CYP4V2 and CYP4Z1 using short synthetic peptides that are specific to the targets of interest. The antibody for CYP4F11 was generated in a previous study (Kumarakulasingham *et al*, 2005). The antibodies were used to profile the expression of each enzyme by immunohistochemistry, which was performed on a well-characterised colorectal cancer tissue microarray.

The cytochrome P450 superfamily is classified into families, subfamilies and individual forms according to sequence homology and substrate specificity (Spector, 2009; Almira Correia *et al*, 2011; Fleming, 2011). Members of CYP1, CYP2 and CYP3 families are the major xenobiotic metabolising enzymes whose roles in cancer have been extensively studied (Murray *et al*, 1991, 1993, 1999, 2001, 2010; Rodriguez-Antona *et al*, 2010; Stenstedt *et al*, 2012; Xu *et al*, 2012). The CYP4 and higher numbered families are involved in the metabolism of a diverse range of endogenous compounds including eicosanoids, fatty acids, steroids and vitamins (Spector, 2009; Arnold *et al*, 2010; Panigrahy *et al*, 2010; Fleming, 2011; Guengerich and Cheng, 2011; Niwa *et al*, 2011). The role of CYP4 family and higher numbered families is not well studied in tumour biology with the exception of those CYPs involved in sex hormone metabolism in relation to breast and prostate cancer (Brueggemeier *et al*, 2005; Leroux, 2005; Stein *et al*, 2012). Therefore, this study aimed to examine the role of the main CYP4 family enzymes in colorectal cancer by characterising the expression of these enzymes using a large and well-characterised patient cohort.

This study revealed there was a significant increase in the expression of CYP4A11 in primary colorectal tumours compared with normal colonic mucosa and the increased expression was significantly associated with poorer prognosis. Consistent with our finding, an upregulation of CYP4A11 was demonstrated by a cDNA microarray-bioinformatics analysis of 10 colorectal tumours and their corresponding normal tissues (Yeh *et al*, 2006). Furthermore, the overexpression of CYP4A11 has been linked to rise in 20-HETE levels and upregulation of vascular endothelial growth factor (VEGF) and matrix metalloproteinases-9 (MMP-9) in non-small cell lung cancer (Yu *et al*, 2011). Both VEGF and MMP-9 are strong promoters of tumour invasion and metastasis (Yu *et al*, 2011; Goel and Mercurio, 2013; Brown and Murray, 2015). Previous research also showed that using selective inhibitors to downregulate the expression of CYP4A11 in cell lines and animal models resulted in a decrease in tumour growth,

Table 3. The final multivariate model showing the significance of the differential expression of CYP4A11 and CYP4F11 in multivariate analysis for the whole patient cohort and MMR-proficient tumours

	Whole patient cohort			MMR-proficient tumours		
	Wald value	P-value	HR (95% CI)	Wald value	P-value	HR (95% CI)
Age (<70 vs ≥70)	31.115	<0.001	1.982 (1.554–2.529)	25.568	<0.001	1.993 (1.526–2.604)
EMVI (present vs absent)	38.825	<0.001	2.278 (1.758–2.951)	29.637	<0.001	2.245 (1.678–3.004)
Dukes stage (A vs B vs C)	53.435	<0.001	2.826 (0.762–4.191)	35.144	<0.001	2.622 (0.785–3.961)
Differential expression of CYP4A11 and CYP4F11 (CYP4A11 > CYP4F11 vs CYP4A11 = CYP4F11 vs CYP4A11 < CYP4F11)	5.515	0.019	1.186 (1.029–1.368)	3.983	0.046	1.173 (1.003–1.371)

Abbreviations: CI = confidence interval; EMVI = extramural venous invasion; HR = hazard ratio; MMR = mismatch repair protein. Significant values are highlighted in bold. Details of the intermediate steps and omnibus tests of model coefficients are shown in Supplementary Tables S12 and S13.

angiogenesis and metastasis of non-small cell lung cancer, renal adenocarcinoma and glioma (Guo *et al*, 2008, Alexanian *et al*, 2009, Yu *et al*, 2011). Our data have shown CYP4A11 is overexpressed in colorectal cancer; therefore, CYP4A11 may be a relevant therapeutic target in this type of cancer.

Comparing primary colorectal tumours to normal colonic mucosa also showed there was a significant increase in the expression of CYP4F11, which is a novel finding. In recent research, CYP4F11 expressed in cell lines (non-small cell lung cancer) converted oxalamides and benzothiazoles into stearoyl CoA desaturase (SCD) inhibitors (Theodoropoulos *et al*, 2016). Stearoyl CoA desaturase is emerging as a therapeutic target in cancer and therefore colorectal tumours with high CYP4F11 expression may be a valid target for SCD-targeted therapy.

The differential expression of CYP4A11 and CYP4F11 emerged as the best prognostic marker in this study. The distinct prognostic impact of the differential expression of CYP4A11 and CYP4F11 may be explained by differences in the enzymes substrates (Supplementary Figure S5). CYP4A11 converts arachidonic acid to metabolites that promote tumour growth and metastasis, whereas CYP4F11 metabolises omega 3-fatty acids to eicosanoids that inhibit tumour development and progression (Larsson *et al*, 2004; Kalsotra and Strobel, 2006; Dhar *et al*, 2008; Gelsomino *et al*, 2013; Barone *et al*, 2014). The differential expression of CYP4A11 and CYP4F11 was independently prognostic in multivariate analysis using the main prognostic parameters and also when only using information available at the time of biopsy diagnosis of colorectal cancer. Therefore, this biomarker combination could be a useful risk stratification tool especially if only tumour biopsies are available at the time of initial treatment decisions, which is a likely scenario considering colorectal cancer, especially rectal cancer, is moving towards neoadjuvant therapy followed by either observational follow-up or salvage surgery (Garcia-Aguilar *et al*, 2015).

The expression of each enzyme based on MMR status was also evaluated in this study, as this represents a major pathway in colorectal cancer (Boland and Goel, 2010; Geiersbach and Samowitz, 2011; Kim and Kim, 2014). Tumours lacking MMR proteins are already considered a distinct subgroup when dealing with prognosis and treatment of colorectal cancer (Hewish *et al*, 2010). Mismatch repair protein-proficient tumours represent the majority of colorectal cancer cases with a significantly worse prognosis than MMR-deficient tumours. Furthermore, novel promising treatments such as those targeting immune checkpoints have shown that MMR-proficient tumours are less responsive compared with MMR-deficient tumours (Le *et al*, 2015). Therefore, it is of particular interest to identify biomarkers for MMR-proficient tumours. In this study, the differential expression of CYP4A11 and CYP4F11 was significantly associated with prognosis in MMR-proficient tumours and, more importantly, both enzymes are actionable targets.

This study also found the expression of CYP4A11, CYP4F11 and CYP4V2 were significantly reduced in lymph node metastasis

compared with their corresponding primary tumours. This provides further evidence to the concept that the phenotype of cancer cells is defined by their exposure to/and interaction with different micro-environment factors during their migration and within the metastatic site (Witz, 2008; Klein *et al*, 2012; Maman and Witz, 2013; Brown and Murray, 2015). The interrelationship between cancer cells and non-cancer cells within the microenvironment is increasingly acknowledged as a major factor in determining and understanding metastasis (McKay *et al*, 2000; Coghlin and Murray, 2010, 2014). The variation in the phenotypic expression between primary and metastatic tumours raises further doubts over the effectiveness of existing metastatic treatment models that is based on phenotypic assessment of primary tumour specimens.

In summary, CYP4A11, CYP4F11 and CYP4V2 are over-expressed in colorectal cancer, the increased expression of CYP4A11 is associated with poorer prognosis in both the total patient cohort and in MMR-proficient tumours, whereas the expression of CYP4F11 is associated with better outcome in MMR-deficient tumours. The differential expression of CYP4A11 and CYP4F11, which was independently prognostic in both the whole patient cohort and in MMR-proficient tumours, could provide the basis for a risk stratification tool in colorectal cancer. Furthermore, both enzymes are actionable drug targets and therefore could have therapeutic applications in colorectal cancer.

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CONFLICT OF INTEREST

AA is a PhD student supported by Vertebrate Antibodies. BC and AA are employees of Vertebrate Antibodies. GM is a scientific advisor to Vertebrate Antibodies. RS declares no conflict of interest.

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