

CRP Present within Colorectal Cancer Cells is Taken Up from the Circulation and not Produced by the Tumour; Evidence of a Complex Relationship Between Host and Tumour

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RESULTS: This study demonstrates that colorectal tumours are not a site of CRP production. Tumours do however, express CRP and high cytoplasmic CRP was associated with necrosis ($P=0.028$) and high nuclear CRP was associated with increasing T-stage ($P=0.022$). Tumoural CRP was not associated with disease free or cancer-specific survival.

CONCLUSION: This study suggests that despite colorectal tumours expressing CRP, there is no evidence of CRP production at this site. Understanding the mechanism whereby CRP enters the cancer cells may offer a novel therapeutic target for patients presenting with a systemic inflammatory response.

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Key words: Colorectal cancer; Recurrence; Systemic inflammation; Survival; CRP

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ABSTRACT

AIM: Preoperative raised serum CRP (sCRP) is now widely accepted as being an independent predictor of poor survival. Although CRP is known to be made by the liver, whether the tumour is a site of CRP production remains unknown. The aim of this study was to determine, in colorectal cancer, if the tumour or liver is responsible for CRP production and what is the relationship between tumoural CRP and survival.

MATERIALS AND METHODS: To establish if the tumour produces CRP, reverse transcriptase PCR for CRP mRNA expression was performed on 30 malignant colorectal specimens, 19 non-malignant colorectal specimens from the resection margin and 30 healthy liver specimens from patients with no radiological evidence of liver metastasis. Tumour protein expression of CRP was assessed in a cohort of 147 paraffin embedded colorectal tumours.

INTRODUCTION

Colorectal cancer is the second most common cause of cancer death in Western Europe and North America. Each year in the UK, there are approximately 35,000 new cases and 16,000 deaths attributable to this disease^[1]. Surgical resection remains the primary treatment option with adjuvant chemotherapy given to those with regional lymph node metastasis. Unfortunately despite improvements in surgical techniques and the introduction of adjuvant chemotherapy, there are still a significant proportion of patients who develop recurrence and die of their disease^[2].

In non-cancer states, serum CRP becomes elevated in response to a complex interaction between hormones, inflammatory cytokines

and glucocorticoids. In colorectal cancer, it remains unknown if circulating CRP is produced by the tumour, liver or both^[3]. Possible mechanisms for serum CRP elevation include tumour growth and specific immune responses to tumour antigens^[4-8]. Cancer cells can increase the production of pro-inflammatory cytokines, which in turn stimulate the production of CRP in the liver^[9-10]. CRP production was initially thought to be restricted to the liver; however, recent studies have demonstrated expression of the acute phase protein in numerous extrahepatic tissues including the healthy kidney^[11-12]. CRP expression has also been observed in malignant tissue including; squamous cell carcinoma of the oesophagus, renal cell carcinoma and bone marrow biopsies from myeloma patients, however, its expression in colorectal cancer remains unknown^[13-15]. The role of CRP within the tumour cell remains unclear however, Yang and colleagues observed, in myeloma cells, that CRP activates numerous signaling pathways including the PI3K/Akt and Ras/Erk pathways through membrane bound Fcγ receptors. This resulted in increased cancer cell numbers via inhibition of apoptosis and increased proliferation^[15]. However, the role of cellular CRP in predicting survival and its relationship with clinicopathological factors remain unknown.

Therefore, the aims of the present study were to establish if colorectal tumour was a site of CRP production and what is the relationship between tumoural CRP, clinicopathological factors and survival in patients undergoing potentially curative resection for colorectal cancer.

METHODS

Patient characteristics

Fresh frozen tissue cohort: The cohort consisted of 30 malignant samples taken from patients with histologically proven colorectal cancer and 19 non-malignant colorectal tissue samples taken from the specimens' resection margin. Thirty healthy liver samples were taken from the above patients who had no histological evidence of metastatic disease at presentation. All patients were diagnosed with primary operable colorectal adenocarcinoma between January 1991 and August 1996 in a single surgical unit at Glasgow Royal Infirmary and underwent potentially curative resection following CT staging.

Immunohistochemistry cohort: Patients with histologically proven colorectal cancer who, on the basis of pre-operative staging CT scans and findings at laparotomy, were considered to have undergone potentially curative resection for colorectal cancer (Stages I-III) between 1997 and 2005 in a single surgical unit were included in the study. This cohort was identified from a prospectively maintained database and included elective and emergency patients. Patients with conditions known to acutely or chronically evoke an acute or chronic systemic inflammatory response were excluded. These included those patients with (1) pre-operative chemoradiotherapy; (2) clinical evidence of infection or (3) active chronic inflammatory disease such as active rheumatoid arthritis. Patients who died within 30 days of surgery were excluded from the survival analysis. The tumours were staged according to conventional TNM classification and additional pathological characteristics; (4) the presence of vascular invasion, peritoneal involvement (Ptl), margin involvement (MI) and tumour perforation (TP) were recorded from pathology reports issued at the time of resection. Routine laboratory measurements of haemoglobin (Hb), white-cell counts (WBC), neutrophils (Neut), lymphocytes (lymph), platelet counts (Plt), albumin (Alb) and C-reactive protein (sCRP) concentrations prior to surgery were recorded. Lymph node ratio (LNR), neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), Klintrup

score, Peterson Index and Ki67 were already available and described previously^[16-17].

Adjuvant therapy was given to patients at the discretion of the clinical oncologist or after multidisciplinary team assessment. Patient's clinical and pathological information was available to the oncologist making these decisions and the treatment offered was based on treatment guidelines for colorectal cancer at that time. This study was approved by the Research Ethics Committee, Glasgow Royal Infirmary, Glasgow.

RESEARCH METHODS

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Tissue processing: After resection of the primary tumour representative parts of malignant colorectal tissue were identified by a pathologist, Snap frozen and stored in liquid nitrogen.

RNA isolation: 50-75 mg of tissue was homogenised in a mortar and pestle in liquid nitrogen. 1 mL of TRIZOL[®] (Invitrogen, UK) was used to extract total RNA according to manufacturer's protocol. RNA quantity and quality was assessed by UV spectrometry (GeneQuant machine) and by examining rRNA bands after agarose gel electrophoresis. Only samples, that expressed both 18S band and a stronger 28S band, were included in this study.

cDNA synthesis: To guarantee no other DNA was present, samples were incubated with RNase-free DNase treatment and removal reagent kit (Applera, UK) and incubated for 30 minutes at 37°C. To ensure the same amount of cDNA being used for quantification of mRNA, a starting concentration of 1,000 ng of tRNA was used for each sample. Random hexamer primers (50 ng) were used for First Strand cDNA Synthesis using SuperScript TM II RT (Invitrogen, UK) according to manufacturer's instructions. Before using cDNA for PCR amplification, 2 units of RNase H (Applera, UK) were added to samples and incubated for 20 minutes at 37°C. Quality of cDNA was assessed by using a PCR control run with human β-actin. Product bands were assessed by examination of agarose gel electrophoresis. Only samples, which showed equal product bands at 330 bp, were included in this study.

Quantification of CRP gene expression: Real-time quantitative PCR was performed in an ABI Prism 7,900 Sequence Detection System (Applied Biosystems, UK) using CRP TaqMan[®] Gene Expression Assay (HS HS00265044-M1, Applied Biosystems, UK). For the TaqMan[®] Gene Expression Assays the manufacturer's protocol with recommended 40 rounds of amplification was used. Thermal cycler condition were 50°C for 2 min, 95°C for 10 min followed by 40×95°C for 15 sec and 60°C for 1 min. Product melting curve analysis and gel electrophoresis experiments were used to ensure that only one product of the expected size was amplified.

On each PCR plate negative controls were included. Quantitative values were obtained from the threshold cycle (Ct value) at which the increase TaqMan[®] probe fluorescent signal associated with an exponential increase of each individual PCR product reaching a fixed threshold value. Each individual primer had a fixed threshold Ct value. These fixed threshold values were used for every cDNA sample.

To enable comparison of different mRNA expression levels, their relation to the average expression level of the housekeeping gene HPRT (hypoxanthine-guanine phosphoribosyltransferase, Hs 4310890E, Applied Biosystems, UK) were evaluated. Data were analysed using the Sequence Detection Software, which calculates the threshold cycle (Ct) value. The expression of the target assay was normalised by subtracting the Ct value of the house keeping

gene from the Ct value of the relevant target assay. The fold increase, relative to the control, was obtained by using the formula $2^{-\Delta Ct}$, and then expressed as a percentage ($\times 100$). All samples were measured in triplicate.

Immunohistochemistry: Blocks from the primary tumour were fixed in 10% buffered formalin and embedded in paraffin wax. One representative block of tumour was selected for each patient. Sections (4 μ m) were cut and mounted on slides coated with aminopropyltriethoxysilane. Prior to use, the specificity of all antibodies were stringently test and have been reported previously^[18]. Sections were dewaxed and rehydrated through a series of xylene and graded alcohols. Antigen retrieval was performed by immersing slides in Tris/EDTA buffer (10 mM Trizma Base, 0.25 mM EDTA, pH 9.0) for 20 minutes at 96°C and then cooled for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ for 5 minutes. After transfer to a humidified chamber, sections were blocked by incubation with casein solution (Vector Laboratories, Peterborough, England) at a 1:10 dilution with Tris-buffered saline (TBS) for 20 minutes and then primary antibody overnight at 4°C. CRP (rabbit polyclonal, Abcam plc. Cambridgeshire, UK) 1:100 Antibody antigen binding was detected using the peroxidase-based Envision (Dako, Cambridgeshire, UK) technique using a diaminobenzidine chromogenic substrate system. Sections were then counter stained using Haematoxylin, dehydrated, and mounted in DPX. Appropriate positive controls were included in each run. Negative controls were omission of the primary antibody.

Scoring method: Tissue staining intensity was scored blind by 2 independent observers using a weighted histoscore method also known as the Hscore^[19]. Histoscores were calculated from the sum of (1 \times % cells staining weakly positive) + (2 \times % cell staining moderately positive) + (3 \times % cells staining strongly positive) with a maximum of 300. Histoscore analysis, therefore, provides a semi-quantitative classification of staining intensity. The inter-class correlation coefficient (ICCC) for each protein was calculated to confirm consistency between observers and the mean of the two observers' scores were used for analysis. The ICC values were consistently above 0.8 for the weighted histoscore (ICCC values ≥ 0.7 were considered acceptable). The observers were blinded to the clinical outcome of the patients.

Statistical Analysis: Differences in protein or gene expression levels were analysed using the Mann-Whitney U test or Kruskal-Wallis test when appropriate. Where no standard thresholds existed, continuous variables of protein/gene expression levels were categorised according to quartiles or dichotomised at the median (high/low expression) as indicated in results tables. Inter-relationships between the various clinical parameters were calculated using Cochran-Mantel-Haenszel Chi square test., a *P*-value of <0.05 was considered to be significant.

Cancer specific survival rates were generated using the Kaplan-Meier method. The log rank test was used to compare significant differences between subgroups using univariate analysis. Multivariate stepwise Cox-regression analysis was performed to identify factors that were independently associated with disease specific death. A stepwise backward procedure was used to derive a final model of the variables that had a significant independent relationship with survival. To remove a variable from the model the corresponding *P*-value had to be greater than 0.05.

RESULTS

CRP mRNA expression levels in liver and colorectal tissue

Thirty colorectal cancer patients were studied, 23 patients with colon

cancer and 7 patients with rectal cancer. There was matched non-malignant tissue available for analysis in 19 cases. Twenty two males and eight women, the median age of patients was 74 years (IQR 61-79 years). Sixteen patients were classified as node negative and 14 patients had node positive disease.

Expression of CRP from the liver samples had a median value of 6600 (IQR 4185.25-23670.90). mRNA was successfully extracted from all 30 tumour samples and 19 normal mucosal tissue samples, however CRP expression was not detected in any of these samples (Table 1).

Table 1 Liver and tumoral CRP mRNA expression.

Tissue	HPRT	Median CRP	IQR
Colorectal cancer	1	0	(0- 1.45 $\times 10^3$)
Non-malignant colon	1	0	(0- 8.09 $\times 10^5$)
Normal Liver	1	6600.03	(4185.25-23670.90)

Protein expression and correlation with clinical parameters

Clinicopathological Details: One hundred and forty seven patients who underwent elective surgery with curative intent for colorectal cancer were studied. The majority were under 75 years of age (61%), female (53%), were node negative (63%), with a low Peterson Index (93%). 20% received post-operative adjuvant chemotherapy. Forty two percent had right sided cancer, 30% had left sided cancer and 28% had rectal cancer. Most patients had a CRP (63%) and albumin (71%) concentration in the normal range. The minimum follow-up was 120 months; the mean follow-up of the survivors was 153 months. During this period, 41 patients had a cancer related death and 52 patients had a non-colorectal cancer death

Tumour CRP expression levels: Each cellular location was independently assessed for CRP expression levels in the tumour cell. Forty-seven percent of tumours exhibited nuclear expression, 50% cytoplasmic, and 0% membrane (Figure 1). Tumours were subdivided into those with high (upper tertile) or low (lower 2 tertiles) expression. On univariate analysis, neither cytoplasmic nor nuclear CRP expression was associated with disease free (*P*=0.241 and *P*=0.965 respectively) or cancer-specific survival (*P*=0.268 and *P*=0.703 respectively) (Table 2 and 3). Chi squared analysis demonstrated that cytoplasmic CRP only correlated with tumour necrosis (*P*=0.028) and nuclear CRP correlated with advanced T stage (*P*=0.022) (Table 4). Serum CRP was not associated with tumoural CRP expression at either nuclear (*P*=0.328) or cytoplasmic (*P*=0.653) localization.

DISCUSSION

This study demonstrates that despite expressing CRP, colorectal tumours are not a site of CRP production and possibly take this up from the circulation. Although CRP at different cellular locations was associated with increasing T stage and tumour necrosis, there was no association with survival.

CRP expression has been observed in malignant tissue before; however, its exact role remains unclear. In myeloma cells, CRP appeared to activate numerous signaling pathways resulting in increased proliferation and inhibition of apoptosis^[15]. This is particularly interesting given our finding that nuclear CRP was associated with increasing T stage. Furthermore, our findings support those of Contu and colleagues who saw that CRP was associated with tumour progression but not survival in rectal cancer patients^[20]. It is possible that tumoural CRP may drive colorectal cancer growth without affecting its potential for metastasis.

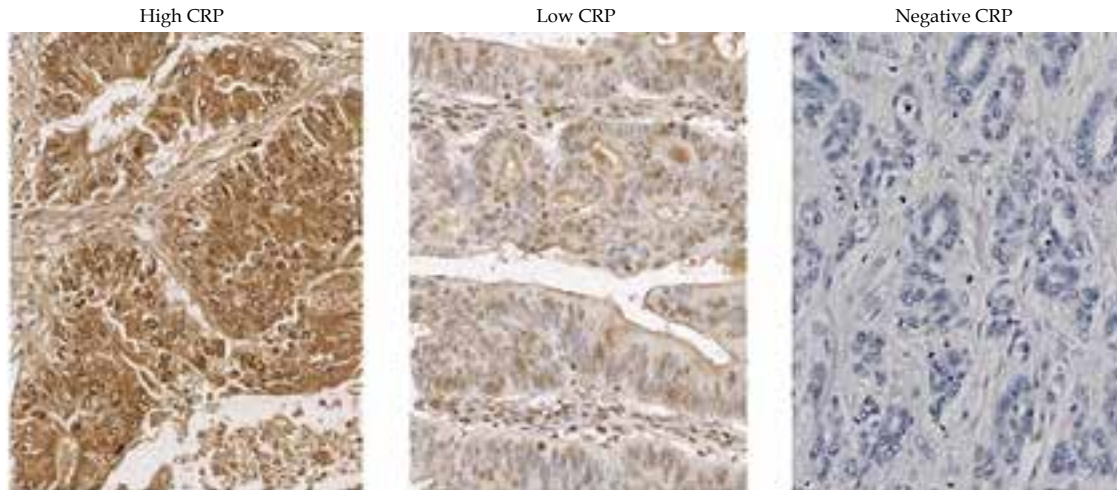


Figure 1 Variations in intensity of staining for CRP at different cellular locations.

Table 2 The relationship between clinicopathological characteristics and cancer specific survival in patients undergoing surgery for colorectal cancer: Univariate and multivariate analysis.

	Patients (n= 137)	P-value	Univariate analysis		Multivariate analysis
			Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)
Age (<75 / ≥75 years)	83 / 54	0.001	3.08 (1.60 - 5.90)	0.036	2.24 (1.06 - 4.76)
Sex (Female/ Male)	72 / 65	0.315	1.39 (0.77 - 2.62)		
Site (Right/ Left/ Rectum)	59 / 40 / 38	0.891	1.03 (0.70 - 1.51)		
T stage (1 / 2 / 3 / 4)	1 / 3 / 93 / 40	0.095	1.67 (0.91 - 3.06)		
Lymph node ratio (0 / 0.01-0.24 / 0.25-0.49 / >0.5)	85 / 32 / 14 / 6	<0.001	2.27 (1.67 - 3.09)	0.003	1.64 (1.18 - 2.27)
Lymph node sample (≥ 12 / <12)	86 / 51	0.495	1.25 (0.66 - 2.40)		
Differentiation (Mod/ Poor)	125 / 9	0.605	1.31 (0.47 - 3.70)		
Peterson Index (Low/ High)	128 / 9	0.138	2.20 (0.78 - 6.23)		
Klintrup Score (High/ Low)	38 / 99	0.004	8.17 (1.96 - 34.02)	0.024	5.33 (1.25 - 22.81)
Adjuvant therapy (Low/ High)	109 / 28	0.328	1.43 (0.70 - 2.95)		
Ki67 (Low / Intermediate / High)	49 / 41 / 47	0.001	2.05 (1.35 - 3.12)	0.062	
sCRP (Low / High)	87 / 50	<0.001	4.82 (2.45 - 9.47)	0.004	3.07 (1.43 - 6.59)
Albumin (Normal / low)	99 / 38	0.003	2.63 (1.38 - 4.99)	0.175	
Neutrophil lymphocyte ratio (Low / High)	88 / 49	0.809	0.92 (0.46- 1.82)		
Platelet lymphocyte ratio (Low / High)	36 / 101	0.347	0.73 (0.37- 1.42)		
nCRP (Low / High)	106 / 31	0.703	0.86 (0.39 - 1.88)		
cCRP (Low / High)	107 / 30	0.268	1.49 (0.74 - 3.00)		

Table 3 The relationship between clinicopathological characteristics and disease free survival in patients undergoing surgery for colorectal cancer: Univariate and multivariate analysis.

	Patients (n= 137)	P-value	Univariate analysis		Multivariate analysis
			Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)
Age (<75 / ≥75 years)	83 / 54	0.024	2.24 (1.11 - 4.50)	0.189	
Sex (Female/ Male)	72 / 65	0.699	0.87 (0.43 - 1.76)		
Site (Right/ Left/ Rectum)	59 / 40 / 38	0.723	1.08 (0.71 - 1.64)		
T stage (1 / 2 / 3 / 4)	1 / 3 / 93 / 40	0.296	1.42 (0.74 - 2.75)		
Lymph node ratio (0 / 0.01-0.24 / 0.25-0.49 / >0.5)	85 / 32 / 14 / 6	<0.001	2.05 (1.45 - 2.89)	0.022	1.53 (1.06 - 2.19)
Lymph node sample (≥ 12 / <12)	86 / 51	0.287	1.46 (0.73 - 2.93)		
Differentiation (Mod/ Poor)	125 / 9	0.851	1.12 (0.34 - 3.68)		
Peterson Index (Low/ High)	128 / 9	0.072	2.62 (0.92 - 7.47)		
Klintrup Score (High/ Low)	38 / 99	0.007	7.20 (1.71 - 30.25)	0.030	5.00 (1.17 - 21.44)
Adjuvant therapy (Low/ High)	109 / 28	0.205	1.65 (0.76 - 3.56)		
Ki67 (Low / Intermediate / High)	49 / 41 / 47	<0.001	2.34 (1.45 - 3.76)	0.046	1.69 (1.01 - 2.82)
sCRP (Normal / High)	87 / 50	0.001	3.26 (1.61 - 6.59)	0.090	
Albumin (Normal / low)	99 / 38	0.003	2.88 (1.43- 5.76)	0.044	2.22 (1.02 - 4.81)
Neutrophil lymphocyte ratio (Low / High)	88 / 49	0.456	1.31 (0.65- 2.65)		
Platelet lymphocyte ratio (Low / High)	36 / 101	0.334	0.70 (0.34- 1.45)		
nCRP (Low / High)	106 / 31	0.965	0.98 (0.42 - 2.27)		
cCRP (Low / High)	107 / 30	0.241	1.59 (0.73 - 3.43)		

Table 4 The relationship between clinicopathological factors and tumoural CRP in patients undergoing elective surgery for colorectal cancer.

Host factors													
	Age	Sex	Hb	Alb	sCRP	WBC	Neut	Lymph	Plt	NLR	PLR		
nCRP (Low / High) ^a	0.356	0.599	0.637	0.786	0.328	0.386	0.827	0.669	0.379	0.699	0.391		
cCRP (Low / High) ^a	0.621	0.611	0.541	0.286	0.653	0.746	0.236	0.442	0.825	0.458	0.322		
Tumour factors													
	Site	Dukes	Tstage	LNR	Differentiation	PI	VI	TP	MI	PH	Klintrup	Necrosis	Ki67
nCRP (Low / High)	0.243	0.062	0.022	0.107	0.607	0.094	0.729	0.345	0.345	0.203	0.476	0.454	0.551
cCRP (Low / High)	0.371	0.219	0.298	0.261	0.236	0.102	0.178	0.356	0.356	0.247	0.698	0.028	0.894

This is the first study to demonstrate that despite expressing CRP, the colorectal tumour is not a site of production. This suggests that a complex interaction between the host and the tumour exists with the tumour utilizing growth factors such as cytokines from the circulation. It is possible therefore, that CRP within the tumour acts as a growth factor and promoting malignant behavior. This is supported by our observation that nuclear CRP was associated with higher T stage. Grivennikov and colleagues observed that IL-6 via STAT3 had a profound impact on increasing tumour cell proliferation and reducing apoptosis^[21]. It is therefore plausible that, in a state of systemic inflammation, the tumour may utilize the abundant proinflammatory cytokines present in the host circulation to facilitate growth.

There is now a growing body of evidence to suggest that activation of the systemic inflammatory response is associated with poorer cancer-specific survival in patients undergoing potentially curative resection for colorectal cancer^[16-17]. Interestingly, this study supported by others demonstrates no association between tumoural CRP and survival despite a relationship with cancer progression^[20]. The systemic inflammatory response represents a complex biosystem of hormones, inflammatory cytokines and glucocorticoids. Serum CRP which is closely associated with IL-6 may represent a surrogate marker of systemic inflammatory activity whilst not, in itself representing an effector protein and further delineation of the inflammatory response is required^[9].

In conclusion, this study has demonstrated for the first time that despite colorectal tumours expressing CRP, the cancer cells and its microenvironment are not responsible for production. The results of this study suggest that a complex interaction between the tumour and the host exists. Identifying the mechanism of this interaction may offer a novel therapeutic target for high risk patients.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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