Src family kinases, HCK and FGR, associate with local inflammation and tumour progression in colorectal cancer

Running Title: HCK and FGR in colorectal cancer

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Conflicts of Interest Statement: The authors have no conflicts of interest to declare

Keywords: Src Family Kinase; HCK, FGR; Colorectal Cancer; Inflammation; Biomarker

ABSTRACT

Background: In colorectal cancer (CRC), inflammatory responses have been reported to associate with patient survival. However, the specific signalling pathways responsible for regulating inflammatory responses are not clear. Src family kinases (SFKs) impact tumourigenic processes, including inflammation.

Methods: The relationship between SFK expression, inflammatory responses and cancer specific survival (CSS) in stage I-III CRC patients was assessed using immunohistochemistry on a 272 patient discovery cohort and an extended 822 patient validation cohort.

Results: In the discovery cohort, cytoplasmic FGR associated with improved CSS (*P*=0.019), with membrane HCK (p=0.093) trending towards poorer CSS. In the validation cohort membrane FGR (p=0.016), membrane HCK (p=0.019), and cytoplasmic HCK (p=0.030) all associated with poorer CSS. Both markers also associated with decreased proliferation and cytotoxic T-lymphocytes (all p<0.05). Furthermore, cytoplasmic HCK was an independent prognostic marker compared to common clinical factors. To assess synergy a combine FGR+HCK score was assessed. The membrane FGR+HCK score strengthened associations with poor prognosis (p=0.006), decreased proliferation (p<0.001) and cytotoxic T-lymphocytes (p<0.001)

Conclusions: SFKs associate with prognosis and the local inflammatory response in patients with stage I-III CRC. Active membrane FGR and HCK work in parallel to promote tumour progression and down-regulation of the local inflammatory lymphocytic response.

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death in Europe[1]. Although outcomes have improved over the past decades, predominantly as a result of improvements in surgical technique and adjuvant/neo-adjuvant therapies, survival still remains poor, with 5-year survival of 60% across all stages of disease[2]. It is clear that the present TNM-based staging of CRC is suboptimal, with a need to identify characteristics pertaining to both the tumour and the host which may not only guide prognosis, but also novel adjuvant therapies.

Local and systemic inflammatory responses have been widely demonstrated to play an active role in tumour development across a wide range of cancers including CRC[3-5]. This is now an area of intense research producing inflammatory-based scoring systems such as the Galon's immunoscore[6], Klintrup-Makinen grade[7] or Glasgow Microenvironment score (GMS)[8] for local inflammation and for systemic inflammation the modified Glasgow prognostic score (mGPS)[9-11] or neutrophil-lymphocyte ratio (NLR)[12]. Of note all of these local and systemic inflammatory scoring algorithms have prognostic value independent of TNM staging[13]. However, the signalling pathways driving these local and systemic inflammatory responses in CRC are not clear. A better understanding of the mechanisms underlying the link between the tumour and inflammation by identifying key signalling pathways and their prognostic value may provide novel therapeutic targets for CRC.

One plausible candidate is the Src family kinases (SFKs). Deregulation of SFK activation is found in many cancers such as pancreatic, breast, ovarian, prostate, renal and CRC[14-19]. SFKs are known to regulate inflammatory responses and have a role in promoting metastasis. In CRC, SFK expression is increased in 80% of CRC as compared with normal colonic epithelium and has been shown to correlate with an increase in CRC metastases[19, 20]. Furthermore, expression of SFKs on myeloid cells is associated with poor prognosis and a

pro-tumour M2-like macrophage endotype[21]. However, there is little data regarding individual SFK expression within the tumour cells and their impact on patient survival and clinical response in CRC.

SFKs comprise eight members expressed in mammalian cells (Src kinase, BLK, FGR, FYN YES, HCK, LCK & LYN). All SFKs reside in an inactive state until dephosphorylated at Y^{527}, and in turn auto-phosphorylated at Y^{419}, following which phosphorylate their downstream targets such as focal adhesion kinase (FAK). As this mechanism is common to all family members, antibodies that recognise Y^{419} alone cannot be employed to determine which SFK is activated in a patient's tumour. However, cellular location can be employed as a surrogate of SFK activation, when inactive family members reside in the cytoplasm and once activated they translocate to the membrane, enabling each SFK member to be analysed individually.

The current study aims to assess tumour cell SFK expression at the membrane (active) and cytoplasm (inactive) to establish the effect of individual SFK members on survival, clinicopathological characteristics and inflammatory responses in patients with CRC.

METHODS

Patients

Discovery cohort patients were identified from a prospectively collected and maintained database of CRC resections performed in a single surgical unit in Glasgow Royal Infirmary. 271 patients who between 1997 and 2007 had undergone an elective, potentially curative resection for stage I-III CRC and were contained within a previously constructed tissue microarray (TMA) were included. The discovery cohort was extended to a larger validation cohort by the inclusion of retrospectively identified patients from CRC resections performed with the Western General Hospital, Glasgow. The validation cohort contained 937 patients who between 2000 and 2007 had undergone an elective, potentially curative resection for stage I-III CRC and were contained within previously constructed TMAs. Resection was considered curative on the basis of pre-operative computed tomography and intra-operative findings. Patients who had died within 30 days of surgery were excluded. Ethical approval was obtained from the West of Scotland Research Ethics Committee.

Clinicopathological Characteristics

Tumours were staged using the fifth edition of the AJCC/UICC-TNM staging system[22]. The presence of venous invasion was assessed using elastica staining. Following surgery, patients with stage III or high-risk stage II disease and without significant co-morbid disease precluding adjuvant treatment were considered for 5-fluorouracil-based chemotherapy. Patients were followed up and date and cause of death were crosschecked with the cancer registration system and the Registrar General (Scotland). Cancer-specific survival (CSS) was measured from date of surgery until date of death from CRC.

The presence of tumour necrosis and tumour stroma percentage (TSP) were assessed as previously described[23]. Mismatch repair (MMR) status was assessed as previously described[8]. Ki67 proliferation index and BRAF status were previously established for both cohorts.

The local inflammatory cell infiltrate was assessed using the Klintrup-Mäkinen (KM) grade as previously described on full sections taken at the deepest point of invasion[24]. Tumour infiltrating lymphocytes (TILs) were established from patient reports. CD3, CD8 and FoxP3 cell counts were established using immunohistochemistry on full sections as previously described[24]. Briefly, cell counts were measure separately at the invasive margin, within the stroma and within the cancer cell nests using a semi-quantitative method as absent, low, moderate or high. Absent and low were then grouped as low and moderate and high grouped as high.

Serum C-reactive protein (CRP) and albumin were recorded prospectively and measured within 30 days prior to surgery. The pre-operative systemic inflammatory response was defined using the mGPS. The mGPS was calculated as previously described[13]. Neutrophil, platelet and lymphocyte counts were previously established in this cohort and used to generate the NLR.

Immunohistochemistry

Immunohistochemical expression of SFK members and downstream target, FAK^{861} was carried out using a previously constructed CRC TMA (Figure S1)[25-27]. Sections were dewaxed in histoclear then rehydrated using graded alcohols. Antigen retrieval was performed under pressure for 5 minutes using either citrate buffer pH6 (Src kinase, FAK^{861}, FYN, HCK, LCK, YES) or EDTA buffer pH9 (SFK^{419}, LYN, FGR) before cooling for 20 minutes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 minutes. 5% normal horse serum was applied for 20 minutes at room temperature as a

blocking solution. TMA sections were incubated overnight at 4ºC with SFK^{419} (1:25; Millipore), FAK^{861} (1:200, Invitrogen), FYN (1:1500), LYN (1:25), HCK (1:1000), LCK (1:200) and YES (1:150, Cell Signaling) or for 60 minutes at room temperature for Src kinase (1:200) and FGR (1:4000, Cell Signaling) before washing the sections in TBS. Envision (Dako) was added to the sections for 30 minutes at room temperature before washing in TBS. DAB substrate was added for five minutes until colour developed before washing in running water for ten minutes. Slides were then counterstained in haematoxylin for 60 seconds and blued with Scotts' tap water before being dehydrated through a series of graded alcohols. Cover slips were applied using distrene, plasticizer, xylene (DPX).

Scoring

Stained TMA sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualized on SlidePath Digital Image Hub (Leica Biosystems, Milton Keynes, UK). Assessment of SFKs and FAK^{861} expression was performed by a single examiner (A.P or S.H) blinded to clinical data at x20 magnification (total magnification x400) using the weighted histoscore (H-score)[28-30]. The weighted histoscore is calculated using the following equation: 0 x (% cells not stained) + 1 x (% cells weakly stained) + 2 x (% cells moderately stained) + 3 x (% cells strongly stained). This gives a range of scores from 0 to 300 and is calculated individually for membrane and cytoplasmic staining. To ensure reproducibility, 10% of tumours were co-scored by a co-investigator (J.E or A.K.R).

Statistical Analysis

Within the discovery cohort, patient scores were analyzed by ROC analysis to determine the appropriate cut-off values for low and high expression (Table S1). These were then verified for significant factors using the validation cohort. The relationship between

clinicopathological characteristics and protein expression was examined using the chi-square test for linear trend. The relationship between expression and CSS was examined using Kaplan-Meier method. The log rank test was utilized to compare significant differences between subset groups using univariate analysis. Multivariate cox regression analysis was performed to identify those factors that were independently associated with CSS. A *P*-value <0.05 was considered statistically significant. All analyses were performed using SPSS version 22.0 (IBM SPSS) and conformed to the REMARK criteria.

RESULTS

SFKs and cancer-specific survival in a discovery cohort of 271 patients with CRC

A total of 271 patients who underwent an elective, potentially curative resection of stage I-III CRC (Table S2) were included in the study. Almost two thirds of patients were 65 or older at the time of surgery and just over half were male. Two thirds of patients underwent resection for colon cancer. Twenty patients (7%) had pathological confirmation of stage I disease, whereas 132 (49%) and 120 (44%) patients had stage II and stage III disease respectively. Thirty-five patients (13%) had MMR deficient CRC, and ninety-nine patients (36%) showed venous invasion. The median follow-up of survivors was 11.3 years (range 6.2-16.2 years) with 95 cancer-associated deaths and 68 non-cancer deaths.

Associations between tumour cell SFK expression and CSS are shown in Table 1. Src kinase, FYN, LYN and FAK^{861} were not associated with CSS at any cellular location. However, cytoplasmic FGR was significantly associated with improved CSS (HR 0.54 95% CI 0.31-0.91, p=0.019). Membrane HCK also trended towards an association with decreased CSS (HR 1.46 95%CI 0.93-2.29, p=0.093).

	Membrane			**Cytoplasmic**		
	N (%)	**10yr CSS (SEM)**	***P***	***N (%)***	**10yr CSS (SEM)**	***P***
SFK^{419} (n=260)			0.407			0.416
Low expression	179 (69)	65 (4)		142 (55)	65 (4)	
High expression	81 (31)	59 (6)		118 (45)	61 (5)	
Src kinase (n=268)			0.787			0.649
Low expression	30 (11)	55 (1)		63 (24)	65 (7)	
High expression	238 (89)	64 (3)		205 (76)	63 (4)	
FGR (n=225)			0.855			**0.019**
Low expression	70 (31)	66 (6)		150 (67)	59 (04)	
High expression	155 (69)	63 (4)		75 (33)	75 (5)	
FYN (n=244)			0.419			0.598
Low expression	187 (77)	67 (4)		93 (38)	63 (5)	
High expression	56 (23)	56 (8)		151 (62)	64 (4)	
HCK (n=232)			**0.093**			0.393

Low expression	112 (48)	71 (5)		87 (38)	68 (5)	
High expression	120 (52)	57 (5)		145 (62)	61 (4)	
LYN (n=246)			0.196			0.789
Low expression	190 (77)	61 (4)		176 (72)	63 (4)	
High expression	56 (23)	74 (6)		70 (28)	68 (6)	
FAK^{861} (n=252)						0.233
Low expression	-	-	-	218 (87)	66 (3)	
High expression				34 (13)	52 (9)	

Table 1. SFK expression and survival in discovery cohort patients with colorectal cancer (n=272)

SFKs and cancer-specific survival in a 822 validation cohort of patients with CRC

As FGR was associated with CSS and a trend was observed for HCK, these were taken forward for investigation in the validation cohort along with activation site SFK^{419}. Only patients with a valid score for all three SFK members were included in the analysis. A total of 822 patients who underwent an elective, potentially curative resection of stage I-III CRC (Table S2) were included in the study. Two thirds of patients were 65 or older at the time of surgery and just over half were male. Three quarter of patients underwent resection for colon cancer. 114 patients (14%) had pathological confirmation of stage I disease, whereas 396 (48%) and 312 (38%) patients had stage II and stage III disease respectively. One hundred and thirty-eight patients (17%) had MMR deficient CRC, and 268 patients (33%) had venous invasion. The median follow-up of survivors was 12.1 years (range 6.2-17.0 years) with 231 cancer-associated deaths and 270 non-cancer deaths.

Associations between tumour cell SFK expression and CSS are shown in Table 2. SFK^{416} did not associate with CSS at any cellular location. Similarly, associations between cytoplasmic FGR and CSS were not observed. However, membrane FGR associated with poorer CSS (HR 1.38 95% CI 1.06-1.80, p=0.016, Figure 1A). Similarly, HCK associated with poorer CSS at both cellular locations (membrane – HR 1.48 95% CI 1.06-2.06, p=0.019, Figure 1B; cytoplasmic – HR 1.34 95% CI 1.03-1.75, p=0.030).

	Membrane	**Cytoplasmic**

	N (%)	10yr CSS (SEM)	*P*	*N (%)*	10yr CSS (SEM)	*P*
SFK[419]			0.341			0.941
Low expression	746 (91)	70 (2)		156 (19)	68 (4)	
High expression	69 (9)	64 (6)		652 (81)	70 (2)	
FGR			**0.016**			0.195
Low expression	368 (45)	74 (2)		228 (29)	65 (3)	
High expression	447 (55)	66 (2)		571 (71)	71 (2)	
HCK			**0.019**			**0.030**
Low expression	704 (86)	71 (2)		460 (60)	73 (2)	
High expression	111 (14)	59 (5)		313 (40)	65 (3)	
FGR+HCK			**0.006**			0.721
Both low	335 (41)	76 (2)		136 (18)	72 (4)	
One high	402 (49)	66 (3)		408 (53)	69 (2)	
Both high	78 (10)	60 (6)		225 (29)	69 (3)	

Table 2. SFK expression and survival in validation cohort patients undergoing potentially curative resection of colorectal cancer (n=822)

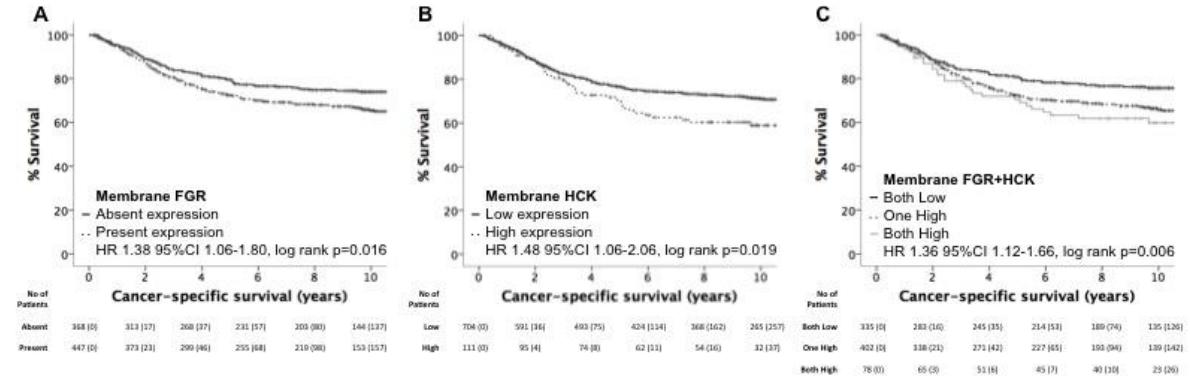

Figure 1. Activated FGR and HCK associates with poor prognosis in patients undergoing potentially curative resection of colorectal cancer (n=822). Kaplan Meier curves showing association of CSS and (A) membrane FGR, (B) membrane HCK and (C) combined membrane FGR+HCK in 822 patients with CRC.

HCK and FGR differentially associate with clinicopathological factors and markers of inflammation

Associations between FGR, clinicopathological characteristics and inflammatory markers as shown in Table 3. Activated membrane FGR showed significant associations with poor prognostic markers including higher TNM-stage (p=0,041), poorer differentiation (p=0.006), decreased necrosis (p=0.028), T-lymphocytes (p=0.022), cytotoxic T-lymphocytes (p=0.010) and increased mGPS (p=0.013). However, inactive cytoplasmic FGR significantly associated

with increased age (p=0.023), colon cancer (p=0.009), decreased peritoneal involvement (p=0.007), increased proliferation rate (p<0.001), deceased TSP (p=0.046), increased T-lymphocytes (p=0.032), cytotoxic T-lymphocytes (p=0.001) and regulatory T-lymphocytes (p=0.001) as well as increased PD-L1 TILs (p=0.019) and decreased PD-L1 tumour expression (p=0.013).

Associations between HCK, clinicopathological characteristics and inflammatory markers are shown in Table 3. Activated membrane HCK showed significant associations with younger age (p=0.013), rectal cancer (p=0.002), higher TNM-stage (p=0.014), lower proliferation rate (p<0.001), lower mGPS (p=0.014), as well as decreased cytotoxic T-lymphocytes (p<0.001), PD1-TILs (p=0.026) and PD-L1 TILs (p=0.032) but higher PD-L1 tumour expression (p=0.009). However, inactive cytoplasmic HCK only associated with increased TNM-stage (p=0.001), increased margin involvement (p=0.033), decreased MMR deficiency (p=0.038), decreased cytotoxic lymphocytes (p=0.015) and increased PD-L1 tumour expression (p<0.001).

	Membrane FGR			**Cytoplasmic FGR**			**Membrane HCK**			**Cytoplasmic HCK**		
	Absent (n=368)	**Present (n=447)**	***P***	**Low (n=228)**	**High (n=571)**	***P***	**Low (n=704)**	**High (n=111)**	***P***	**Low (n=460)**	**High (n=313)**	***P***
Age			0.137			**0.023**			**0.013**			0.183
<65	109 (29)	153 (34)		86 (38)	169 (29)		215 (30)	47 (42)		137 (30)	107 (34)	
>65	264 (71)	296 (66)		143 (62)	408 (71)		496 (70)	64 (58)		328 (70)	208 (66)	
Sex			0.438			0.058			0.408			0.521
Female	166 (45)	212 (47)		93 (41)	277 (48)		331 (47)	47 (42)		219 (47)	141 (45)	
Male	207 (55)	237 (53)		136 (59)	300 (52)		380 (53)	64 (58)		256 (53)	174 (55)	
Tumour site			0.625			**0.009**			**0.002**			0.584
Colon	283 (76)	334 (74)		158 (69)	449 (78)		547 (77)	70 (63)		352 (76)	233 (74)	
Rectum	90 (24)	115 (26)		71 (31)	128 (22)		164 (23)	41 (37)		113 (24)	82 (26)	
TNM-stage			**0.041**			0.369			**0.014**			**0.001**
I	59 (16)	55 (12)		26 (11)	82 (14)		107 (15)	7 (6)		79 (17)	27 (9)	
II	185 (50)	211 (47)		113 (49)	279 (48)		342 (48)	54 (49)		227 (49)	154 (49)	
III	129 (34)	183 (41)		90 (39)	216 (37)		262 (37)	50 (45)		159 (34)	134 (42)	
Differentiation			**0.006**			0.149			0.219			**0.016**
Mod/well	349 (94)	395 (88)		202 (88)	528 (92)		640 (90)	104 (94)		430 (93)	275 (87)	
Poor	24 (6)	54 (12)		27 (12)	49 (8)		71 (10)	7 (6)		35 (7)	40 (13)	
Venous invasion			0.954			0.619			0.138			0.830
Absent	251 (67)	303 (68)		151 (66)	391 (69)		486 (68)	68 (61)		311 (67)	213 (68)	
Present	122 (33)	146 (32)		78 (34)	186 (32)		225 (32)	43 (39)		154 (33)	102 (32)	
Margin involvement			0.595			0.942			0.822			**0.033**
No	353 (95)	421 (94)		216 (94)	545 (95)		670 (94)	104 (94)		445 (96)	290 (92)	
Yes	20 (5)	28 (6)		13 (6)	32 (5)		41 (6)	7 (6)		20 (4)	25 (8)	
Peritoneal involvement			0.976			**0.007**			0.798			0.746
No	272 (73)	327 (73)		151 (66)	435 (75)		517 (73)	82 (74)		340 (73)	227 (72)	
Yes	101 (27)	122 (27)		78 (34)	142 (25)		194 (27)	29 (26)		125 (27)	88 (28)	
Mismatch repair status			0.510			0.947			0.707			**0.038**
Competent	312 (84)	369 (82)		188 (83)	479 (83)		589 (83)	92 (84)		374 (81)	271 (86)	
Deficient	59 (16)	79 (18)		39 (17)	98 (17)		121 (17)	17 (16)		90 (19)	43 (14)	
Proliferation Index			0.358			**<0.001**			**<0.001**			0.088

Low	164 (44)	213 (48)		142 (63)	222 (38)		298 (42)	79 (73)		199 (43)	154 (49)	
High	206 (56)	235 (52)		84 (37)	354 (62)		412 (58)	29 (30)		264 (57)	159 (51)	
Necrosis			**0.028**			0.706			0.942			0.236
Low	207 (57)	283 (64)		135 (59)	343 (61)		424 (61)	66 (61)		283 (62)	180 (58)	
High	158 (43)	157 (36)		92 (41)	220 (39)		272 (39)	43 (39)		171 (38)	130 (42)	
Tumour stroma percentage			0.069			**0.046**			0.348			0.126
Low	290 (80)	321 (74)		153 (72)	448 (79)		539 (77)	72 (73)		355 (78)	221 (73)	
High	75 (20)	113 (26)		60 (28)	122 (21)		161 (23)	27 (27)		100 (22)	82 (27)	
Klintrup-Makinen grade			0.569			0.956			0.879			0.448
Weak	249 (68)	291 (66)		153 (67)	379 (67)		467 (67)	73 (66)		299 (66)	213 (69)	
Strong	117 (32)	149 (34)		74 (33)	185 (33)		229 (33)	37 (34)		155 (34)	98 (31)	
CD3+ Lymphocytes			**0.022**			0.032			0.303			0.765
Low	101 (30)	165 (40)		93 (43)	172 (33)		224 (35)	42 (38)		151 (35)	107 (36)	
Moderate	110 (33)	117 (28)		53 (25)	168 (32)		194 (30)	33 (31)		126 (30)	88 (30)	
High	125 (37)	136 (32)		68 (32)	187 (35)		228 (35)	33 (31)		149 (35)	100 (34)	
CD8+ Lymphocytes			**0.010**			**0.001**			**<0.001**			**0.015**
Low	141 (43)	202 (49)		118 (57)	222 (43)		278 (44)	65 (62)		180 (43)	155 (53)	
Moderate	82 (25)	117 (28)		47 (22)	146 (28)		176 (28)	23 (22)		117 (28)	65 (22)	
High	107 (32)	92 (29)		44(21)	153 (29)		184 (29)	17 (16)		124 (29)	71 (24)	
FoxP3+ Lymphocytes			0.091			**0.001**			0.263			0.565
Low	83 (30)	135 (36)		90 (46)	123 (28)		177 (32)	41 (40)		114 (31)	94 (35)	
Moderate	106 (38)	131 (35)		53 (27)	181 (41)		220 (40)	17 (16)		142 (39)	90 (34)	
High	91 (32)	106 (29)		53 (27)	141 (32)		151 (28)	46 (44)		111 (30)	82 (31)	
PD1 – TILs			0.177			0.081			**0.026**			0.829
Low	222 (66)	300 (70)		158 (73)	354 (66)		442 (66)	83 (77)		296 (67)	202 (67)	
High	116 (34)	127 (30)		60 (27)	183 (34)		227 (34)	25 (23)		143 (33)	101 (33)	
PD-L1 – TILs			0.761			**0.019**			**0.013**			0.704
Low	259 (76)	318 (75)		183 (81)	385 (73)		215 (30)	47 (42)		331 (76)	226 (74)	
High	82 (24)	106 (25)		43 (19)	143 (27)		496 (70)	64 (58)		107 (24)	78 926)	
PD-L1 – tumour			0.262			**0.013**			**0.009**			**<0.001**
Low	194 (57)	231 (53)		108 (48)	306 (57)		380 (57)	51 (44)		261 (60)	141 (46)	
High	144 (43)	202 (47)		119 (52)	227 (43)		285 (43)	65 (56)		178 (40)	168 (54)	
mGPS			**0.013**			0.789			**0.014**	194 (55)		0.169
0	181 (61)	189 (52)		107 (53)	256 (57)		299 (54)	71 (65)			160 (59)	
1	77 (26)	105 (29)		66 (32)	115 (26)		153 (28)	29 (26)		96 (27)	75 (28)	
2	40 (13)	70 (19)		30 (15)	78 (17)		100 (18)	10 (9)		65 (18)	38 (14)	
NLR			0.097			0.131			0.815			0.184
<5	231 (75)	147 (76)		147 (76)	330 (70)		418 (72)	68 (73)		264 (70)	197 (75)	
>5	76 (25)	46 (24)		46 (24)	139 (30)		163 (28)	25 (27)		111 (30)	65 (25)	

Table 3. Relationship between FGR or HCK expression and clinicopathological characteristics in patients undergoing potentially curative resection of colorectal cancer (n=822).

Activated membrane FGR and HCK work in parallel to promote tumour progression and dampen lymphocytic infiltration

As FGR and HCK show similar associations with prognosis and lymphocytic infiltration, they were combined into a single score to assess if they work together or synergistically. FGR and HCK were combined as follows at both cellular locations: low FGR and low HCK = both low; low FGR or high HCK = one high; high FGR and high HCK = both high. When assessed for associations with CSS, a high membrane FGR+HCK score significantly associated with poor prognosis (HR 1.36 95% CI 1.12-1.66, p=0.006, Figure 1C), with patients with one high or both high having similar prognosis. No associations were seen for the cytoplasmic FGR+HCK score and CSS.

To assess the effects on lymphocytic infiltrate, associations with clinicopathological factors and inflammation were assessed as shown in Table 4. A both high membrane FGR+HCK score significantly associated with younger age (p=0.012), rectal cancer (p=0.039), higher TNM-stage (p=0.004), lower proliferation rate (p<0.001), decrease T-lymphocytes (p=0.020), cytotoxic T-lymphocytes (p<0.001) and PD1-TILs (p=0.045). Whereas a high cytoplasmic FGR+HCK score only associated with increased proliferation rate (p=0.007) and increased PD-L1 TILs (p=0.003).

	Membrane FGR+HCK				**Cytoplasmic FGR + HCK**			
	Both Low (n=335)	**One Low (n=402)**	**Both High (n=78)**	*P*	**Both Low (n=136)**	**One High (n=408)**	**Both High (n=225)**	*P*
Age				**0.012**				0.632
<65	95 (28)	134 (33)	33 (42)		51 (37)	117 (28)	75 (33)	
>65	245 (72)	270 (67)	45 (58)		86 (63)	295 (72)	152 (67)	
Sex				0.871				0.373
Female	156 (46)	185 (46)	37 (47)		60 (44)	189 (46)	110 (49)	
Male	184 (54)	219 (54)	41 (53)		77 (56)	224 (54)	117 (51)	
Tumour site				**0.039**				0.249
Colon	261 (77)	308 (76)	48 (62)		94 (69)	318 (77)	171 (75)	
Rectum	79 (23)	96 (24)	30 (38)		43 (31)	94 (22)	56 (25)	
TNM-stage				**0.004**				0.056
I	58 (17)	50 (12)	6 (8)		22 (16)	58 (14)	23 (10)	
II	163 (48)	201 (50)	32 (41)		70 (51)	199 (48)	112 (49)	
III	119 (35)	153 (38)	50 (51)		45 (33)	155 (38)	92 (41)	
Differentiation				0.143				0.340
Mod/well	316 (93)	357 (88)	71 (91)		125 (91)	375 (91)	201 (89)	
Poor	24 (7)	47 (12)	7 (9)		12 (9)	37 (9)	26 (11)	
Venous invasion				0.454				0.605
Absent	235 (69)	267 (66)	52 (67)		95 (69)	266 (65)	160 (70)	
Present	105 (31)	137 (34)	26 (33)		42 (31)	146 (35)	67 (30)	
Margin involvement				0.592				0.070
No	321 (94)	381 (94)	72 (92)		132 (96)	391 (95)	209 (92)	
Yes	19 (6)	23 (6)	6 (8)		5 (4)	21 (5)	18 (8)	
Peritoneal involvement				0.910				0.130
No	249 (73)	291 (72)	59 (76)		95 (69)	295 (72)	173 (76)	
Yes	91 (27)	113 (28)	19 (24)		42 (31)	117 (28)	54 (24)	
Mismatch repair status				0.753				0.133
Competent	284 (84)	333 (83)	64 (83)		109 (80)	337 (82)	195 (86)	
Deficient	55 (16)	70 (17)	13 (17)		27 (20)	74 (18)	32 (14)	
Proliferation Index				**<0.001**				**0.007**
Low	141 (42)	180 (43)	56 (72)		75 (56)	184 (45)	91 (40)	
High	199 (58)	220 (55)	22 (28)		60 (44)	227 (55)	135 (60)	
Necrosis				0.094				0.535
Low	189 (57)	253 (64)	48 (62)		88 (65)	237 (59)	135 (61)	
High	144 (43)	142 (36)	29 (38)		47 (35)	166 (41)	87 (39)	
Tumour stroma percentage				0.056				0.809
Low	269 (79)	291 (75)	51 (70)		94 (73)	317 (79)	163 (73)	
High	70 (21)	96 (25)	22 (30)		35 (27)	85 (21)	59 (27)	
Klintrup-Makinen grade				0.599				0.646
Weak	228 (69)	260 (66)	52 (68)		92 (68)	263 (65)	155 (70)	
Strong	105 (31)	136 (34)	25 (32)		43 (32)	140 (35)	68 (30)	
CD3+ Lymphocytes				0.020				0.187
Low	91 (30)	143 (38)	32 (42)		50 (29)	142 (38)	65 (30)	
Moderate	99 (32)	106 (29)	22 (28)		35 (27)	108 (29)	70 (33)	
High	115 (38)	123 (33)	23 (30)		44 (34)	125 (33)	78 (37)	
CD8+ Lymphocytes				**<0.001**				0.615
Low	121 (41)	177 (48)	45 (61)		67 (54)	163 (44)	104 (50)	
Moderate	79 (26)	100 (27)	20 (27)		28 (22)	105 (28)	47 (22)	
High	99 (33)	93 (25)	9 (12)		30 (24)	106 (28)	58 (28)	
FoxP3+ Lymphocytes				0.513				0.051
Low	72 (29)	116 (35)	30 (41)		47 (42)	107 (32)	51 (28)	
Moderate	101 (40)	124 (38)	12 (16)		36 (32)	121 (36)	74 (41)	
High	77 (31)	88 (27)	32 (43)		29 (26)	106 (32)	58 (32)	
PD1 – TILs				**0.045**				0.327
Low	171 (65)	227 (68)	55 (79)		80 (72)	224 (66)	125 (66)	
High	94 (35)	109 (32)	15 (21)		31 (28)	116 (34)	65 (34)	
PD-L1 – TILs				0.567				**0.033**
Low	199 (75)	250 (75)	59 (79)		94 (81)	254 (76)	135 (70)	

High	67 (25)	82 (25)	16 (21)		22 (19)	82 (24)	57 (30)	
PD-L1 – tumour				0.084				0.263
Low	150 (57)	187 (55)	33 (44)		58 (50)	196 (58)	90 (45)	
High	113 (43)	154 (45)	42 (56)		59 (50)	142 (42)	106 (55)	
mGPS				0.633				0.190
0	158 (59)	164 (52)	48 (62)		62 (53)	173 (54)	116 (61)	
1	73 (27)	84 (26)	25 (32)		35 (30)	90 (28)	46 (24)	
2	35 (13)	70 (22)	5 (6)		19 (17)	57 (18)	27 (14)	
NLR				0.247				0.987
<5	213 (76)	223 (68)	50 (75)		82 (73)	243 (72)	134 (73)	
>5	68 (24)	103 (32)	17 (25)		30 (27)	95 (28)	50 (27)	

Table 4. Relationship between combined FGR+HCK expression and clinicopathological characteristics in patients undergoing potentially curative resection of colorectal cancer (n=822).

Cytoplasmic HCK is an independent prognostic factor for patients with CRC

FGR, HCK and the combined score were then taken into cox regression multivariate analysis along with significant clinical, pathological and inflammatory markers as shown in Table 5. On multivariate analysis (n=406), TMN-stage (p<0.001), venous invasion (p=0.012), margin involvement (p=0.030), peritoneal involvement (p=0.001), KM grade (p=0.015), T-lymphocytes (p=0.012), mGPS (p<0.001) and cytoplasmic HCK (p=0.015) were independent prognostic factors. However, membrane FGR (P=0.590), membrane HCK (P=0.287) and the combined membrane FGR+HCK score (p=0.167) were not independently associated with CCS.

	n=822		**n=406**	
	Univariate HR (95% CI)	***P***	**Multivariate HR** (95% CI)	***P***
Clinicopathological Characteristics				
Age (<65/>65)	1.03 (0.78-1.35)	0.854	-	-
Sex (Female/Male)	1.12 (0.87-1.46)	0.386	-	-
Tumour Site (Colon/Rectum)	0.96 (0.71-1.29)	0.809	-	-
TNM-Stage (I/II/III)	2.41 (1.94-3.01)	**<0.001**	1.87 (1.33-2.64)	**<0.001**
Differentiation (Moderate or well/Poor)	1.97 (1.35-2.86)	**<0.001**	0.99 (0.58-1.70)	0.750
Venous Invasion (Absent/Present)	2.16 (1.67-2.80)	**<0.001**	1.62 (1.11-2.38)	**0.012**
Margin Involvement (No/Yes)	3.27 (2.18-4.88)	**<0.001**	1.87 (1.06-3.28)	**0.030**
Peritoneal Involvement (No/Yes)	2.76 (2.25-3.57)	**<0.001**	1.94 (1.33-2.83)	**0.001**
Mismatch Repair Status (Competent/Deficient)	0.77 (0.53-1.12)	0.168	-	-
Ki67 Proliferation (Low/high)	0.65 (0.50-0.85)	b	0.99 (0.67-1.47)	0.704
Necrosis (Low/High)	1.32 (1.02-1.72)	0.038	1.20 (0.85-1.70)	0.180
Tumour Stroma Percentage (<50%/>50%)	1.81 (1.37-2.38)	<0.001	1.42 (0.99-2.05)	0.227
Inflammatory Characteristics				
Klintrup-Makinen Grade (Weak/Strong)	0.38 (0.27-0.54)	**<0.001**	0.54 (0.33-0.89)	**0.015**
CD3+ Lymphocytes (low/moderate/high)	0.67 (0.56-0.79)	**<0.001**	0.75 (0.59-0.90)	**0.012**

CD8+ Lymphocytes (low/moderate/high)	0.63 (0.53-0.75)	**<0.001**	0.92 (0.71-1.19)	0.566
FoxP3+ Lymphocytes (low/moderate/high)	0.68 (0.56-0.82)	**<0.001**	1.03 (0.80-1.33)	0.942
PD1 – TILs (low/high)	0.55 (0.39-0.77)	**0.001**	0.71 (0.45-1.11)	0.211
PD-L1 – TILs (low/high)	0.86 (0.61-1.21)	0.392	-	-
PD-L1 – tumour (low/high)	1.08 (0.82-1.44)	0.559	-	-
mGPS (0/1/2)	1.74 (1.46-2.07)	**<0.001**	1.62 (1.27-2.07)	**<0.001**
NLR (<5/>5)	1.44 (1.06-1.96)	**0.018**	1.26 (0.87-1.82)	0.620
SFKs				
Membrane FGR (absent/present)	1.38 (1.06-1.80)	**0.017**	1.14 (0.80-1.64)	0.590
Membrane HCK (low/high)	1.48 (1.06-2.06)	**0.020**	1.38 (0.83-2.31)	0.287
Cytoplasmic HCK (low/high)	1.34 (1.03-1.75)	**0.031**	1.58 (1.09-2.07)	**0.015**
Membrane FGR+HCK (both low/one high/both high)	1.36 (1.12-1.66)	**0.002**	1.20 (0.93-1.57)	0.167

Table 5. Clinicopathological characteristics of patients undergoing elective, potentially curative resection of colorectal cancer and survival

DISCUSSION

The results of this study provide evidence that FGR and HCK are highly expressed in CRC tumours and are associated with poorer patient prognosis. FGR and HCK are important within both the tumour, were they associated with increased TNM-stage and decreased proliferation, and within the microenvironment, were they showed strong associations with decreased local inflammation and PD1/PD-L1 expression on lymphocytes. Therefore, HCK and FGR may work synergistically to promote tumour progression and dampen the local lymphocytic inflammatory infiltrate.

The results within the present study are consistent with other studies that have suggested that HCK is overexpressed in CRC and correlates with poor patient prognosis. This poor prognosis is suggested to be due to effects on proliferation and facilitation of an alternative M2-like macrophage polarisation[15, 31]. This is similar to the results seen in the present study, HCK associates with decreased proliferation and poorer differentiation suggesting HCK overexpression promotes tumourigenesis. HCK has also been associated with tumour progression in other malignancies. In Chronic Myeloid leukaemia (CLL) increased expression of HCK associated with increased cell survival. This increase was due to direct interactions of HCK with BCR/ABL and STAT5 to up-regulate the Akt pathway, which is also known to regulate inflammation[15]. However, in renal cancer, active membrane HCK associated with increased CSS, in contrast to the results seen in the present study, suggesting the tumour origin and microenvironment may be important[18]. Previous literature on FGR in colorectal cancer is lacking, the current study suggests that FGR may be a tumour promoter that works by dampening T-lymphocyte infiltration in to the tumour and microenvironment. The data further suggests that FGR and HCK work in synergy as when assessed together as part of a combined membrane score, a both high score and one high score show similar

prognostic value, suggesting that they both work independently towards the same goal rather than together.

HCK and FGR also play an important role in the innate immune response via modulation of neutrophil phagocytosis, macrophage proliferation and migration[32-34]. However, HCK and FGR mainly exploit the innate immune response through regulating of the production of cytokines. When HCK and FGR are knocked out in mice in conjunction with LYN, the mice are completely protected from inflammatory effects due to defects in cytokine production, suggesting they work together[35]. HCK can also promote IL-6 secretion to up-regulate adaptive inflammation, yet HCK is likewise activated by IL-6 via direct interactions with GP130 to promote cell proliferation[15]. However, HCK has also been shown to be a negative regulator of neutrophil chemokine signalling to dampen local inflammatory responses[36]. This may be the case in the present study, when FGR and/or HCK are activated, they can then work in synergy to negatively regulate important cytokines, ordinarily secreted by neutrophils for T-lymphocyte recruitment within the tumour, which may explain why T-lymphocyte numbers decrease in our patients. However, another explanation may be that HCK and FGRs aberrant activation of innate immune cells in the tumour microenvironment facilitates tumourigenesis and enables progression in CRC. In a small subset of 100 patients from the study, membrane FGR but not HCK significantly shifted macrophage polarisation towards an M2-like phenotype, which has been shown to promote tumourigenesis and dampen lymphocytic inflitration (data not shown). Furthermore, this was supported by the results of the combined membrane score, were a both high score showed a greater effect on cytotoxic T-lymphocyte infiltration than a one high or both low score. This suggest although they can both work independently to regulate tumour progression and local inflammation, this effect is increased when acting synergistically towards a common goal.

In conclusion, the results of this study support HCK and FGR as active SFKs in patients with CRC that act in synergy to promote tumour progression and dampen local lymphocytic inflammation. Therefore, these two SFKs may help predict the prognosis of patients with CRC if incorporated into routine pathology alongside TNM-staging. They may also provide a therapeutic target and biomarker, with clinical inhibitors available, that may show value if targeted in clinical trials in conjunction with current immunotherapies in patients with high membrane expression.

Acknowledgments: This work was supported by the Royal College of Physicians and Surgeons of Glasgow Ritchie Fellowship.

REFERENCES

1. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J *et al.* Cancer incidence and mortality patterns in europe: Estimates for 40 countries in 2012. *Eur J Cancer* 2013;**49**;1374-1403.

2. Oliphant R, Nicholson GA, Horgan PG *et al.* Deprivation and colorectal cancer surgery: Longer-term survival inequalities are due to differential postoperative mortality between socioeconomic groups. *Ann Surg Oncol* 2013;**20**;2132-2139.

3. McAllister SS, Weinberg RA. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. *Nat Cell Biol* 2014;**16**;717-727.

4. Diakos CI, Charles KA, McMillan DC, Clarke SJ. Cancer-related inflammation and treatment effectiveness. *Lancet Oncol* 2014;**15**;e493-503.

5. Hanahan D, Coussens LM. Accessories to the crime: Functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012;**21**;309-322.

6. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: A prognostic factor that should not be ignored. *Oncogene* 2010;**29**;1093-1102.

7. Klintrup K, Makinen JM, Kauppila S *et al.* Inflammation and prognosis in colorectal cancer. *Eur J Cancer* 2005;**41**;2645-2654.

8. Park JH, McMillan DC, Powell AG *et al.* Evaluation of a tumor microenvironment-based prognostic score in primary operable colorectal cancer. *Clin Cancer Res* 2014.

9. Buchanan FG, Wang D, Bargiacchi F, DuBois RN. Prostaglandin e2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem* 2003;**278**;35451-35457.

10. McMillan DC. The systemic inflammation-based glasgow prognostic score: A decade of experience in patients with cancer. *Cancer Treat Rev* 2013;**39**;534-540.

11. Guthrie GJ, Roxburgh CS, Farhan-Alanie OM, Horgan PG, McMillan DC. Comparison of the prognostic value of longitudinal measurements of systemic inflammation in patients undergoing curative resection of colorectal cancer. *Br J Cancer* 2013;**109**;24-28.

12. Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: Experience in patients with cancer. *Crit Rev Oncol Hematol* 2013;**88**;218-230.

13. Park JH, Watt DG, Roxburgh CS, Horgan PG, McMillan DC. Colorectal cancer, systemic inflammation, and outcome: Staging the tumor and staging the host. *Ann Surg* 2015.

14. Capurso G, Di Florio A, Sette C, Delle Fave G. Signalling pathways passing src in pancreatic endocrine tumours: Relevance for possible combined targeted therapies. *Neuroendocrinology* 2013;**97**;67-73.

15. Poh AR, O'Donoghue RJ, Ernst M. Hematopoietic cell kinase (hck) as a therapeutic target in immune and cancer cells. *Oncotarget* 2015;**6**;15752-15771.

16. Kim HS, Han HD, Armaiz-Pena GN *et al.* Functional roles of src and fgr in ovarian carcinoma. *Clin Cancer Res* 2011;**17**;1713-1721.

17. Le XF, Bast RC, Jr. Src family kinases and paclitaxel sensitivity. *Cancer Biol Ther* 2011;**12**;260-269.

18. Roseweir AK, Qayyum T, Lim Z *et al.* Nuclear expression of lyn, a src family kinase member, is associated with poor prognosis in renal cancer patients. *BMC Cancer* 2016;**16**;229.

19. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. *Cancer Metastasis Rev* 2003;**22**;337-358.

20. Lieu C, Kopetz S. The src family of protein tyrosine kinases: A new and promising target for colorectal cancer therapy. *Clin Colorectal Cancer* 2010;**9**;89-94.

21. Poh AR, Love CG, Masson F *et al.* Inhibition of hematopoietic cell kinase activity suppresses myeloid cell-mediated colon cancer progression. *Cancer Cell* 2017;**31**;563-575 e565.

22. Fleming ID, American Joint Committee on Cancer., American Cancer Society., American College of Surgeons. *Ajcc cancer staging manual*. 5th ed / ed. Philadelphia: Lippincott-Raven, 1997;xv, 294 p.

23. Richards CH, Roxburgh CSD, Anderson JH *et al.* Prognostic value of tumour necrosis and host inflammatory responses in colorectal cancer. *The British journal of surgery* 2011;**99**;n/a-n/a.

24. Richards CH, Roxburgh CS, Powell AG, Foulis AK, Horgan PG, McMillan DC. The clinical utility of the local inflammatory response in colorectal cancer. *Eur J Cancer* 2014;**50**;309-319.

25. Roxburgh CS, Richards CH, Macdonald AI *et al.* The in situ local immune response, tumour senescence and proliferation in colorectal cancer. *British Journal of Cancer* 2013;**109**;2207-2216.

26. Powell AG, Ferguson J, Al-Mulla F *et al.* The relationship between genetic profiling, clinicopathological factors and survival in patients undergoing surgery for node-negative colorectal cancer: 10-year follow-up. *J Cancer Res Clin Oncol* 2013;**139**;2013-2020.

27. Roseweir AK, Halcrow ES, Chichilo S *et al.* Erk and p38mapk combine to improve survival in patients with braf mutant colorectal cancer. *Br J Cancer* 2018.

28. Hirsch FR, Varella-Garcia M, Bunn PA, Jr. *et al.* Epidermal growth factor receptor in non-small-cell lung carcinomas: Correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol* 2003;**21**;3798-3807.

29. Rizzardi AE, Johnson AT, Vogel RI *et al.* Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. *Diagn Pathol* 2012;**7**;42.

30. Mazieres J, Brugger W, Cappuzzo F *et al.* Evaluation of egfr protein expression by immunohistochemistry using h-score and the magnification rule: Re-analysis of the saturn study. *Lung Cancer* 2013;**82**;231-237.

31. Poh AL, G.; Khakham, Y.; Masson, F.; Preaudet, A.; Putockzi, T.; O'Donoghue,; R. Ernst, M. Hck activation enhances colorectal tumourigenesis by facilitating alternative macrophage polarisation. In Gaffen S ed. *Cytokine*: Elsevier, 2015;73.

32. Suh HS, Kim MO, Lee SC. Inhibition of granulocyte-macrophage colony-stimulating factor signaling and microglial proliferation by anti-cd45ro: Role of hck tyrosine kinase and phosphatidylinositol 3-kinase/akt. *J Immunol* 2005;**174**;2712-2719.

33. Cougoule C, Le Cabec V, Poincloux R *et al.* Three-dimensional migration of macrophages requires hck for podosome organization and extracellular matrix proteolysis. *Blood* 2010;**115**;1444-1452.

34. English BK, Ihle JN, Myracle A, Yi T. Hck tyrosine kinase activity modulates tumor necrosis factor production by murine macrophages. *J Exp Med* 1993;**178**;1017-1022.

35. Ley K. Fueling the fire: Src family kinases drive inflammation. *J Exp Med* 2014;**211**;1922.

36. Zhang H, Meng F, Chu CL, Takai T, Lowell CA. The src family kinases hck and fgr negatively regulate neutrophil and dendritic cell chemokine signaling via pir-b. *Immunity* 2005;**22**;235-246.