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Original research

A combined FAK, c-MET, and MST1R three-protein panel risk-stratifies colorectal cancer patients

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ABSTRACT

Focal adhesion kinase (FAK) is a key tyrosine kinase downstream of c-MET (or hepatocyte growth factor receptor, HGFR) and MST1R (macrophage-stimulating protein receptor or recepteur d'origine Nantais, RON) membrane receptors. The pathway plays an important role in cancer survival and invasion. In this study, we examined the protein expression of FAK, c-MET, and MST1R levels in a well-annotated cohort of 330 colorectal cancer patients. We found FAK to be overexpressed in colorectal adenocarcinomas ($p = 0.0002$), and FAK levels correlated positively with phospho-FAK levels ($R^2 = 0.81$). In comparison, MST1R levels were not significantly different, and c-MET levels were slightly higher in the normal samples. We then developed a combined 3-protein panel of FAK, c-MET, and MST1R expression signatures that can robustly risk-stratify colorectal cancer across all stages into three clusters that differ in progression-free survival. The colorectal cancer subgroup with high FAK, low c-MET, and low MST1R protein levels showed the worst progression-free survival with particularly early progression of disease ($p = 0.0053$). Combined FAK, c-MET, and MST1R were independently prognostic for progression-free survival in stage II colorectal cancers in a multivariate model. The 3-protein panel provides a potentially clinically attractive method for risk-stratification and adjuvant therapy guidance, especially in stage II disease.

Introduction

A number of signaling pathways have been described to contribute to colorectal cancer (CRC) pathogenesis, the aggregate result of which is a perturbed cell survival/death/proliferation balance. One contributing factor is the focal adhesion kinase (FAK, p125), a non-receptor tyrosine kinase that has been shown to be overexpressed in CRC [1]. FAK has been shown to prevent anoikis and prevent death receptor-induced cell death [2,3]. FAK also induces cell cycle progression through cyclin D1, through Krüppel-like factor 8, Src-ERK, or JUN N-terminal kinase (JNK) signaling [3]. Both kinase and non-kinase domains of FAK contribute; FAK has been described to form a complex with Src and Akt in colorectal cancer cell lines, mediating the interaction between Src and Akt, thereby facilitating CRC invasion *in vitro* and enhancing anchorage-independent cell growth. Thus, FAK is thought to be important for CRC invasion, including liver metastasis, where the expression level is particularly high [4]. Despite its well established role *in vitro*, its clinical, prognostic value of FAK is less clear. In a study of 80 CRCs, FAK expression level by immunohistochemistry (IHC) did not show FAK to be a significant prognostic factor [5]. This is in contrast with another study of 183 CRCs by IHC, which showed FAK positivity has been shown to be associated with poor overall survival (OS) in CRC patients [6]. Considering that FAK inhibition is pharmaceutically targetable using orally bioavailable agents (*e.g.*, defactinib), better understanding of the significance of FAK may help to guide future therapy.

FAK activation is achieved through a number of different pathways. FAK is a key component of the signal transduction pathways triggered by integrins, where FAK is recruited and activated in response to focal contacts [3,7]. Also, upstream of FAK, ligation of c-MET (also known as hepatocyte growth factor (HGF) receptor, HGFR) or the related recepteur d'origine Nantais (RON/macrophage-stimulating protein receptor (MST1R)) has been shown to activate FAK. c-MET been shown to be overexpressed in CRC compared to normal epithelium and adenomas, as well as being genetically amplified [8,9], with the level of genetic amplification even higher in liver metastases [10]. *c-MET* mRNA levels, which roughly correlated with c-MET protein levels by IHC in the study, increased with increasing T stage, and it was significantly higher with node-positive disease [8]. In contrast, strong c-MET staining by IHC was not associated with prognostic value with regards to disease-free interval in a study restricted to stage II patients [11]. Examining its distribution, however, the relative distribution of

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membranous and cytoplasmic c-MET was found to be prognostic in stage I and II CRCs [12].

c-MET and MST1R are not only closely related as homologs, but their co-(over)expression has been reported in multiple cancers, with co-expression being associated with worse prognosis in ovary, breast and bladder cancers [13–15]. c-MET and MST1R have been described to form a complex, thereby achieving cross-activation [16–18]. Indeed, in gastro-oesophageal cancer, c-MET, MST1R, and their respective ligands (HGF and HGF-like protein and, respectively), were found to be highly expressed, and co-expression of MST1R and c-MET was associated with poorer survival [19].

In this study, we found FAK levels to correlate positively with c-MET and MST1R levels in a cohort of well-characterized 330 CRC patients, suggesting a close relationship between the three proteins in CRC. While the levels of each protein were found to be non-prognostic for progression-free survival (PFS), it was the combined score that was most robustly prognostic for PFS, being especially prognostic in stage II cancers. Our finding has significant clinical utility, since stage II cancers pose a particular clinical challenge for selecting patients that need adjuvant therapy *vs.* those who do not, and our new combined FAK/MST1R/c-Met protein score may be a prognostic biomarker for that important clinical decision process.

Methods

Patient samples and tissue microarrays

Our cohort consisted of 330 total number of newly diagnosed cases of colorectal cancer between 1994 and 2008 at the University Health Network (UHN, Toronto). This study was approved by UHN's institutional Research Ethics Board (REB), and all methods were carried out with UHN's relevant institutional guidelines and regulations. All pathological diagnoses were confirmed by a board-certified pathologist (MHR). The study used only fully de-identified retrospective material that had been collected as part of routine clinical care. Thus, the UHN REB had determined that informed consent was waived. Tissue microarrays (TMAs) were constructed as described previously [20]. Briefly, for each case, four to eight 0.6-mm and 1.5-mm cores were obtained from formalin-fixed and paraffin-embedded tissue blocks and transferred into a receiving paraffin block using a manual tissue arrayer (Beecher Instruments). Hematoxylin and eosin (H&E) stained slides of these cases were also reviewed for the presence of normal colonic mucosa, adenomas, and adenocarcinomas. For The Cancer Genome Atlas (TCGA) cohort analysis, the data (gene expression levels and patient survival data) were downloaded from cBioPortal.org (*http://www.cbioportal.org/study/summary?id=coadread_tcga_pub*).

Immunohistochemistry (IHC)

IHC was performed as described previously [21]. Briefly, formalin-fixed and paraffin-embedded tissue core TMAs were used. The TMA contained 43 normal colonic tissue control samples (patient-matched), as well as 62 adenoma samples. Antibodies specific for FAK (Cell Signaling, catalogue number 3285, dilution of 1:40), phospho-FAK (phospho-Y861, Thermo Fisher, catalogue number 44-626G, dilution of 1:2000), c-MET (Ventana, catalogue number 790–4430, clone SP44, dilution 1:200) and RON beta (Santa Cruz, catalogue number sc-322, dilution 1:400) proteins were used. Expression was detected by peroxidase-diaminobenzidine chemistry using the NovoLink Polymer Detection System (Vision BioSystems) after microwave boiling in 5% (m/v) urea in Tris-buffered saline. A Ventana iView 3,3′-diaminobenzidine tetrahydrochloride (DAB) detection kit was used for visualization of the antibody signals.

IHC intensity was scored by digital image analysis using the Aperio Color Deconvolution algorithm (Leica BioSystems), with corrections applied. Different channels (DAB and blue) allowed for identification of nuclear and cytoplasmic areas. The raw values initially obtained were adjusted to account for the non-specific nuclear staining. The total weighted IHC score (IHC H-score) of a sample slide was calculated by multiplying the expression intensity of individual tumor areas (score, 0–3) by their relative contribution (0–100%) to total tumor area and adding these to yield a total weighted sum. The IHC H-scores thus have a theoretical range of 0 to 300. Tumor samples were categorized into high *vs.* low levels based on whether the H-score for each sample was higher or lower than the median H-score for the 330 tumor samples, respectively.

Statistics and survival analyses

Spearman correlations were computed between protein expression levels of the individual markers. Comparisons of expression levels of individual proteins or combined clusters were performed by Kruskal-Wallis or Mann-Whitney tests. Overall and progression-free survival were examined using the Kaplan-Meier method, and survival curves were generated using the JMP 11.0 software (SAS Institute). Log-rank tests were used to examine for differences in survival, and a Cox proportional hazard model was employed to examine survival in multivariate analyses. For all statistical analyses, a p value cut-off of 0.05 was used to define statistical significance.

Availability of materials and data

All data generated or analysed during this study are included in this published article (and its Supplementary Information files) or available from the authors upon reasonable request.

Results

FAK, c-MET, and MST1R expression levels correlate with one another in CRC tumors

Expression levels of FAK, c-MET, and MST1R were examined in our cohort of colorectal cancer patients by immunohistochemistry (IHC). FAK levels were widely variable within our CRC cohort, and the levels were higher in CRC patient samples in comparison to normal controls (Fig. 1). In contrast, MST1R levels were not significantly different between normal and CRC tissues, while c-MET levels appeared slightly higher in normal samples (Fig. 1C and D, respectively). FAK and phospho-FAK levels correlated strongly with each other (Fig. 1E), suggesting that a higher FAK protein level is associated with increased FAK activity.

Because of the known functional relationship between the three proteins, we next compared the H-scores between the three markers, and all three proteins were found to positively correlate with one another. Specifically, intermediate correlation was seen between FAK and MST1R ($\rho = 0.3736$, $p < 0.0001$) and also between MST1R and c-MET ($\rho = 0.4787$, $p < 0.0001$) (Table 1). A weaker, but still significant correlation was observed between FAK and c-MET ($\rho = 0.2691$, $p < 0.0001$), suggesting a close relationship between the three proteins in CRCs.

A combined FAK, MST1R, and c-MET proteomic profile is prognostic of progression-free survival (PFS) in CRC

We next examined the prognostic significance of each of the three proteins, as well as phospho-FAK, in our cohort as individual biomarkers. Examining progression-free survival (PFS), none of the markers alone were prognostic for PFS in our cohort when each of the three markers was dichotomized with respect to its respective median H-score (Supplementary Fig. 1). Because of the close expression relationship observed between the three proteins, we next examined the prognostic significance of a combined FAK, c-MET, and MST1R protein profile. Interestingly, when we examined PFS with respect to a combined FAK/MST1R/c-MET protein profile, the different profiles roughly converged into three different clusters (Fig. 2A). The best PFS was observed for cluster 1, which comprised of patients with $FAK^{Low}/MST1R^{High}/c\text{-}MET^{Low}$ and $FAK^{Low}/MST1R^{Low}/c\text{-}MET^{High}$ CRCs, and median PFS times were not reached for either of these combinations. The worst PFS was observed with $FAK^{High}/MST1R^{Low}/c\text{-}MET^{Low}$ CRCs, with a particularly short median PFS (22.4 months), and this combination was

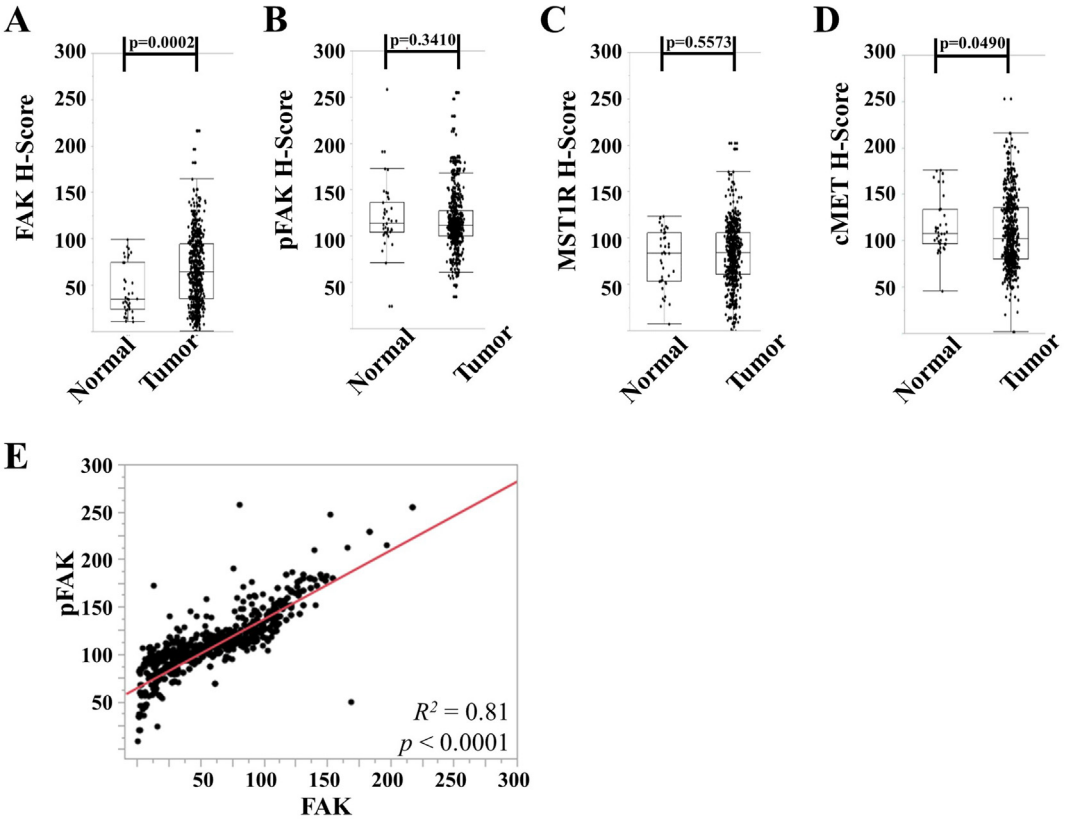


Fig. 1. Comparison of immunohistochemical H-score values for (A) FAK, (B) phospho-FAK, (C) MST1R, and (D) c-MET between cancer and benign mucosa from the tissue microarray. Boxes indicate 25th, 50th (median), and 75th percentiles of H-score distribution from bottom to top, respectively, and lower and upper whiskers denote 1st and 99th percentiles, respectively. (E) Correlation between phospho-FAK and FAK H-score values for tumor samples. For A–D, 330 tumor samples were compared against 43 normal colonic tissue samples.

designated as cluster 3. The remaining combinations, with intermediate PFS, were designated as cluster 2. Among the cluster 2 combinations, the shortest progression-free period was observed with $FAK^{High}/MST1R^{Low}/c\text{-}MET^{High}$, with median progression-free period of 44.3 months, followed by $FAK^{High}/MST1R^{High}/c\text{-}MET^{High}$ (114.8 months). Representative tumors from each cluster are shown in Fig. 3. When phospho-FAK was examined in combination with MST1R and c-MET, similar clustering patterns were observed for PFS, with phospho-$FAK^{High}/MST1R^{Low}/c\text{-}MET^{Low}$ CRCs also having the worst PFS (Supplementary Fig. 2). We next examined an independent cohort (TCGA; *http://www.cbioportal.org/study/summary?id=coadread_tcga_pub*), which was stratified into three cohorts based on the mRNA levels for *PTK2* (FAK), *MET* (c-MET), and *MST1R* (MST1R). While statistical significance was not reached, the OS curves for the three clusters showed a similar pattern, with Cluster 3 ($PTK2^{High}/MET^{Low}/MST1R^{Low}$) having worse OS (Supplementary Fig. 3). PFS data were not available for the TCGA cohort. Of note, direct comparison between the TCGA cohort and our cohort is limited by the fact that mRNA abundance (TCGA cohort) and protein abundance (our cohort) are not necessarily directly concordant.

When the converging profiles were combined into clusters, the three clusters retained their prognostic strength, being predictive for PFS for the whole cohort ($p = 0.0053$; Fig. 2B). We next performed sub-group analyses, examining the prognostic significance of the clusters within the AJCC stages. Interestingly, the difference in PFS with respect to the combined FAK/MST1R/c-MET proteomic profile was found to be driven largely by the difference observed in stage II disease. While the combined profile was not a significant prognostic factor in other stages, the combination was particularly robust in stage II disease ($p < 0.0001$; Fig. 2C).

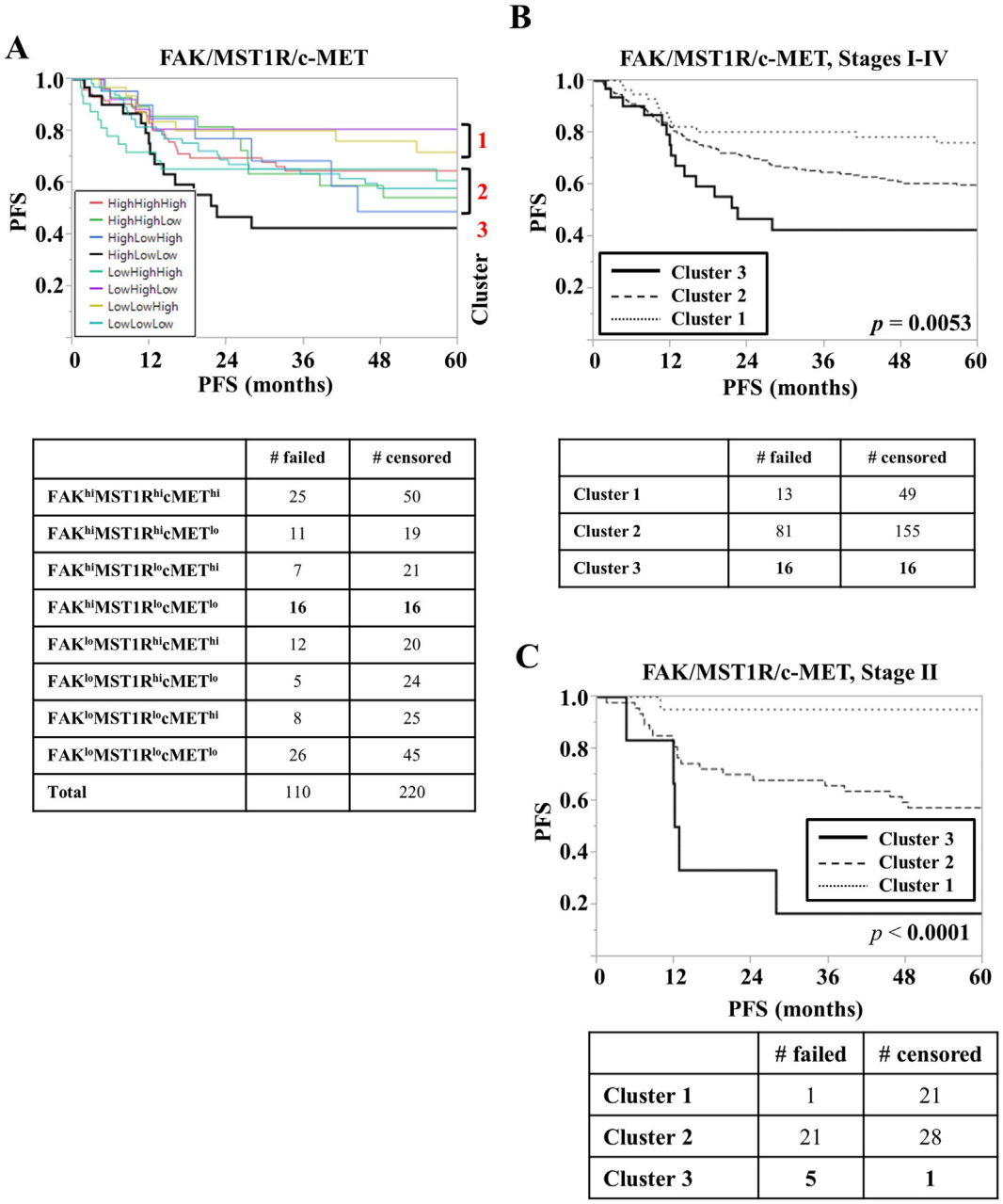
Table 1
Spearman correlations between proteins.

	FAK	MST1R
FAK		$\rho = 0.3736$ $p < 0.0001$
cMET	$\rho = 0.2691$ $p < 0.0001$	$\rho = 0.4787$ $p < 0.0001$

Characteristics of cluster 3 ($FAK^{High}/MST1R^{Low}/c\text{-}MET^{Low}$) CRCs

We next compared the patient and tumor characteristics between the three clusters. As expected, FAK levels were higher, while c-MET and MST1R levels were significantly lower in cluster 3 (Table 2). However, in terms of patient characteristics, no significant differences were observed regarding the patient gender and age at diagnosis. At presentation, there were no significant differences with respect to the AJCC, pT, or pN stages. There were also no significant differences with respect to tumor site (right *vs.* left colon), size, histologic grade, or mismatch repair status. Patients also did not differ significantly as to whether adjuvant systemic therapy was administered. In contrast, PFS showed significant differences: 50% of cluster 3 CRC patients progressed, compared to 34.3% of cluster 2 and 21.0% of cluster 1 patients, respectively ($p = 0.0143$). Cluster 3 also showed lower overall survival of 34.4% (compared to 48.4% and 45.8% for clusters 1 and 2, respectively), although the difference was not statistically significant.

We focused the subsequent analyses on stage II patients, where the clinical decision for adjuvant chemotherapy is most challenging. By multivariate analysis, the combined protein profile was a significant prognostic factor in a model that took into account patient age, gender, tumor size, tumor grade, tumor site, and T stage, with significant PFS differences between cluster 3 and cluster 1 (HR = 16.1, $p = 0.0196$), as well as between cluster 2 and cluster 1 (HR = 7.6, $p = 0.0107$) (Table 3). Taken together,



	# failed	# censored
FAK^{hi}MST1R^{hi}cMET^{hi}	25	50
FAK^{hi}MST1R^{hi}cMET^{lo}	11	19
FAK^{hi}MST1R^{lo}cMET^{hi}	7	21
FAK^{hi}MST1R^{lo}cMET^{lo}	**16**	**16**
FAK^{lo}MST1R^{hi}cMET^{hi}	12	20
FAK^{lo}MST1R^{hi}cMET^{lo}	5	24
FAK^{lo}MST1R^{lo}cMET^{hi}	8	25
FAK^{lo}MST1R^{lo}cMET^{lo}	26	45
Total	110	220

	# failed	# censored
Cluster 1	13	49
Cluster 2	81	155
Cluster 3	**16**	**16**

	# failed	# censored
Cluster 1	1	21
Cluster 2	21	28
Cluster 3	**5**	**1**

Fig. 2. Progression-free survival (PFS) and the combined FAK/MST1R/c-MET protein profile. (A) PFS was examined for all eight different combinations of FAK/MST1R/c-MET expression levels, with each marker dichotomized based on its median H-score. Kaplan-Meier curves fall into three distinct clusters 1–3. (B) PFS analysis, based on the combined protein profile clusters 1–3, examining all patients. (C) PFS analysis, based on the combined protein profile clusters 1–3, examining stage II patients only.

our data show that stage II patients with a particular FAK/MST1R/c-MET protein profile characterize an aggressive disease with particularly early progression.

Discussion

Despite the known roles of FAK, MST1R, and c-MET contributing to the malignant behaviour of CRC cells *in vitro*, high expression levels of the three proteins in isolation had little or no prognostic value in our cohort as individual markers. However, the positive correlations seen between the three proteins is in accord with models based on biochemical studies, placing FAK downstream of c-MET and MST1R and attributing this relationship to cancer progression. Interestingly, it was the cluster 3, the combination of high FAK with low c-MET and low MST1R that exhibited the worst PFS, particularly in stage II disease. This observation is in contrast with gastroesophageal adenocarcinoma, where co-expression of MST1R and c-MET was associated with worse survival [19]. Cluster 3 did not stand out from the other clusters with regards to tumor or patient characteristics. One possible explanation for the worse survival of cluster 3 patients is acquisition of FAK autonomy. Upstream signal-independent activation by mutation(s) or other processes may be a possible means by which FAK might gain activity independent of upstream inputs, including c-MET and MST1R (Fig. 4). FAK autonomy would allow cancer cells to avoid anoikis while undergoing epithelial-mesenchymal transition, thereby facilitating invasion and cancer progression. In contrast, in other FAK/MST1R/c-MET combinations, FAK would remain dependent on upstream ligation, even with FAK overexpression, thus acting as a bottleneck in cancer progression. Alternatively, low c-MET and low MST1R may correlate with high expression of another factor upstream of FAK, which, in conjunction with high FAK, would act in synergy with FAK to facilitate disease progression. Future

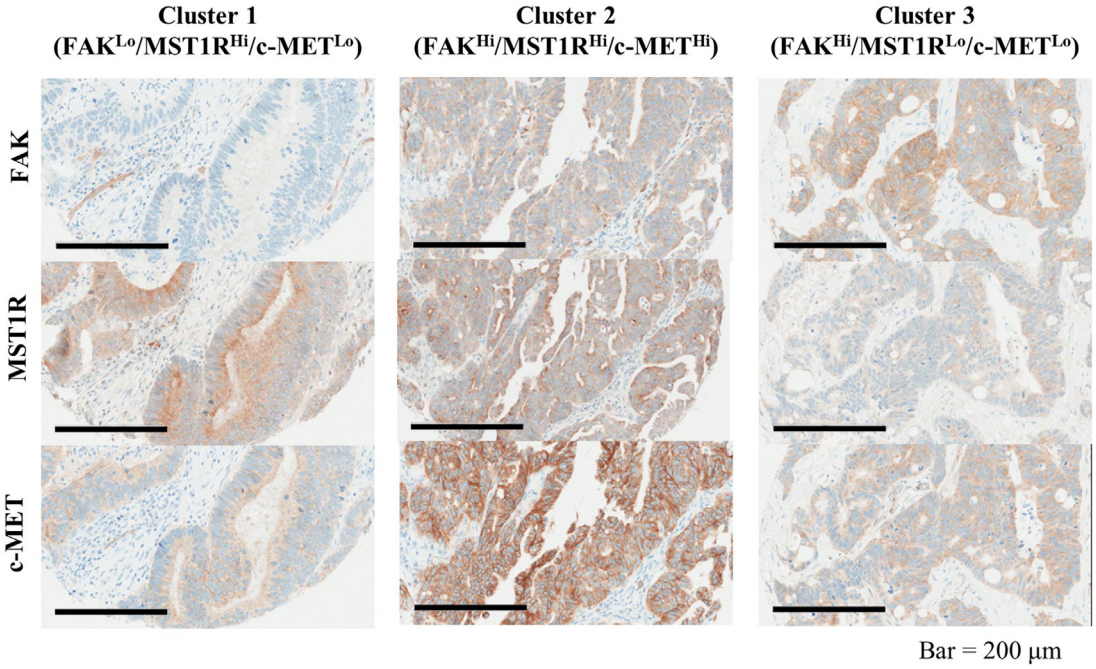


Fig. 3. Representative immunohistochemical stains of CRC tissues illustrating protein expression clusters 1–3, with the indicated combined FAK/MST1R/c-MET protein expression profile.

studies expanding beyond the three proteins will shed light on these possible mechanisms behind the poor prognosis associated with cluster 3.

Table 2
Univariate analyses of FAK/MST1R/c-MET protein expression clusters 1–3 for all patients (MSS, microsatellite stability; MSI, microsatellite instability; NK, not known).

		Cluster 1	Cluster 2	Cluster 3	Log-Rank
Patients		62 (18.8%)	236 (71.5%)	32 (9.7%)	
FAK (H-score)		36.2 ± 2.4	73.1 ± 2.5	95.3 ± 4.4	***p* < 0.0001**
c-MET (H-score)		103.7 ± 3.9	114.1 ± 2.6	74.9 ± 2.7	***p* < 0.0001**
MST1R (H-score)		77.5 ± 3.3	88.0 ± 2.3	56.7 ± 3.6	***p* < 0.0001**
Age (years)		68.3 ± 1.5	65.9 ± 0.8	68.9 ± 2.1	$p = 0.1336$
Gender	F	36 (58.1%)	110 (46.6%)	14 (43.8%)	$p = 0.2341$
	M	26 (41.9%)	126 (53.4%)	18 (56.3%)	
AJCC stage	I	6 (9.7%)	33 (14%)	5 (15.6%)	$p = 0.3873$
	II	22 (35.5%)	49 (20.8%)	6 (18.8%)	
	III	21 (33.9%)	96 (40.7%)	14 (43.8%)	
	IV	13 (21%)	58 (24.6%)	7 (21.9%)	
pT stage	pT1	3 (5.5%)	11 (5%)	2 (6.7%)	$p = 0.2013$
	pT2	6 (10.9%)	38 (17.3%)	2 (6.7%)	
	pT3	38 (69.1%)	114 (51.8%)	19 (63.3%)	
	pT4	8 (14.5%)	57 (25.9%)	7 (23.3%)	
pN stage	pN0	31 (50%)	92 (39.5%)	13 (40.6%)	$p = 0.4210$
	pN1	15 (24.2%)	85 (36.5%)	10 (31.3%)	
	pN2	16 (25.8%)	56 (24%)	9 (28.1%)	
Grade	G1	3 (5.5%)	17 (7.6%)	2 (6.9%)	$p = 0.0615$
	G2	43 (78.2%)	192 (86.1%)	21 (72.4%)	
	G3	9 (16.4%)	14 (6.3%)	6 (20.7%)	
Tumor site	Left	25	113	16	$p = 0.5238$
	Right	37	123	16	
Tumor size (cm)		4.6 ± 0.3	4.6 ± 0.1	4.9 ± 0.4	$p = 0.6172$
Mismatch repair	MSS	48 (77.4%)	200 (84.7%)	29 (90.6%)	$p = 0.0529$
	MSI	11 (17.7%)	21 (8.9%)	1 (3.1%)	
	NK	3 (4.8%)	15 (6.4%)	2 (6.3%)	
Adjuvant therapy	No	40 (64.5%)	130 (55.1%)	18 (56.3%)	$p = 0.4376$
	Yes	21 (33.9%)	100 (42.4%)	13 (40.6%)	
	NK	1 (1.6%)	6 (2.5%)	1 (3.1%)	
Death	No	30 (48.4%)	108 (45.8%)	11 (34.4%)	$p = 0.4002$
	Yes	32 (51.6%)	128 (54.2%)	21 (65.6%)	
Progression	No	49 (79%)	155 (65.7%)	16 (50%)	***p* = 0.0143**
	Yes	13 (21%)	81 (34.3%)	16 (50%)	

p values highlighted in bold denote statistically significant values ($p < 0.05$).

Our study has limitations. While the combined profile was certainly prognostic and robustly so, it is possible that the cluster 3 cohort may only represent a fraction of patients with a meaningful biological FAK/MST1R/c-MET profile. Also, having ultimately focused on stage II patients, the sample size became relatively small, especially for cluster 3 patients, reflected by the wide ranges in the hazard ratio values. The retention of the statistical significance of the prognostic value of the FAK/MST1R/c-MET protein profile in the multivariate analysis, however, reflects the robustness of its prognostic value.

Our results have potential implications for therapy stratification of early stage CRC. While c-MET and MST1R may be interesting therapeutic targets based on *in vitro* work, our data suggests that, clinically, the two proteins would represent poor therapeutic targets, as it is the lower levels of these proteins that are associated with worse PFS. On the other hand, while FAK may be a relevant therapeutic strategy, the group of patients that can benefit from FAK inhibition is likely to be a specific subgroup. Based on our study, such groups would include FAK^{High}/MST1R^{Low}/c-MET^{Low} and FAK^{High}/MST1R^{Low}/c-MET^{High} CRCs, and readily identifying patients with these CRC subtypes would be an important prerequisite in a trial examining the efficacy of FAK inhibitors.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100836.

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Table 3
Multivariate analysis of only stage II CRC patients.

		Hazard ratio	*p*
FAK/MST1R/c-MET Cluster (PFS)	**3 *vs.* 1**	**16.1 (1.6–361)**	**0.0196**
	3 *vs.* 2	2.1 (0.4–8.4)	0.3653
	2 *vs.* 1	**7.6 (1.5–140)**	**0.0107**
pT stage	>pT2	0.32 (0.10–1.10)	0.0692
Tumor grade	>2	0.84 (0.1–4.04)	0.8490
Tumor size (cm)	>5	0.98 (0.33–2.75)	0.9650
Tumor site	Right	0.77 (0.29–1.98)	0.5877
Age at diagnosis (years)	>65	0.72 (0.25–2.08)	0.5430
Gender	Male	0.89 (0.35–2.33)	0.8002

p values highlighted in bold denote statistically significant values ($p < 0.05$).

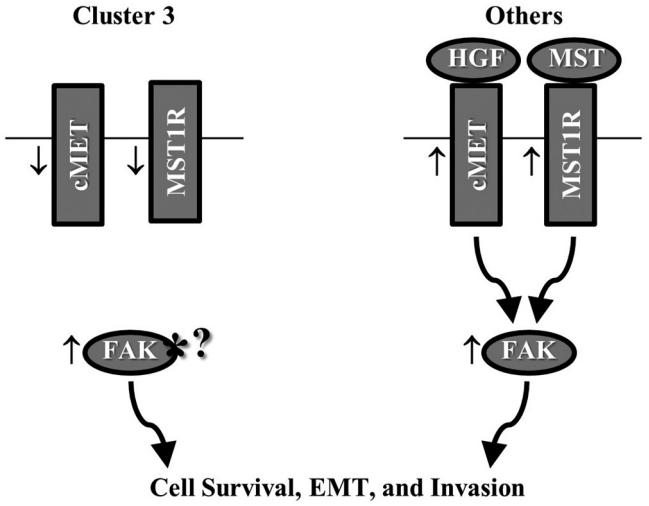

Fig. 4. Schematic model showing the relationship between FAK, MST1R, and c-MET and their potential role in CRC progression. In cluster 3 patients, despite the low c-MET and low MST1R protein levels, activation of FAK (indicated by *) *via* mutation (s) or other processes may allow for autonomous, high activity of FAK, contributing to cell survival, EMT and invasion, ultimately contributing to disease progression. This is contrast with other clusters, where FAK retains its dependence on the upstream factors, including c-MET and MST1R, thereby presenting a potential bottleneck for disease progression. Receptor ligands: HGF, hepatocyte growth factor; MST, macrophage-stimulating protein.

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Author contributions

Ju-Yoon Yoon: Methodology, Validation, Formal Analysis, Data Curation, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Julia Y. Wang:** Formal Analysis, Investigation, Writing – Review & Editing. **Michael H. Roehrl:** Conceptualization, Methodology, Investigation, Supervision, Funding Acquisition, Writing – Original Draft, Writing – Review & Editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: JYY and JYW declare no conflicts of interest related to this study. MHR is member of the Scientific Advisory Boards of Proscia, Trans-Hit, and Universal DX. No other competing interests are declared. None of these companies had any role in support, design, execution, data analysis, or any other aspect of this study.

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