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The relationship between members of the canonical NF-kB pathway, tumour microenvironment and cancer specific survival in colorectal cancer patients

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Summary. Background. The aim of this study was to investigate the role of the upstream kinase TAK1 and the canonical NF-κB pathway in colorectal cancer (CRC). Immunohistochemistry was used to assess the expression of TAK1/pTAK1 and canonical NF-κB pathway members in a tissue microarray of 242 patients. The relationship between expression, the tumour microenvironment and cancer-specific survival were examined. Results. All the investigated members of the pathway were expressed in CRC tissue. In addition, cytoplasmic pTAK1 was associated with the tumour microenvironment (P=0.045) and cancer-specific survival (CSS) (P=0.032). When cytoplasmic pTAK1 was stratified by *BRAF* status, cytoplasmic pTAK1 expression association with CSS was strengthened (P=0.014). Cytoplasmic IKKβ was significantly associated with the inflammatory cell infiltrate (P=0.015) as graded by Klintrup Makinen grade, systemic inflammation as assessed by neutrophil-lymphocyte ratio (P=0.03) and CSS (P=0.046). On multivariate analysis cytoplasmic IKKβ was independently associated with CSS (HR 1.75,95%CI 1.05-2.91, P=0.033). Conclusion. Cytoplasmic pTAK1 was significantly associated with CSS and this was enhanced in patients with tumours that expressed wild type *BRAF*. High expression of cytoplasmic IKKβ was significantly associated with decreased CSS and with markers of the tumour microenvironment. These results support the hypothesis that NF-κB pathway members are poor prognostic markers in patients with CRC, but this requires to be validated in a large independent cohort.

Key words Colorectal cancer, Immunohistochemistry, Tumour microenvironment, Inflammation

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Introduction

Colorectal cancer remains the second most common cause of cancer death in Europe. Over the past few decades outcome has improved, but survival still remains poor with a 5-year survival of around 60% (Cancerresearchuk, 2017). Currently TNM-based staging is used in colorectal cancer, however this is not optimal. Identification of factors relating to both the tumour and the microenvironment may help predict prognosis and be used to improve current tools such as TNM. Such factors may also provide an insight into potential novel therapies.

The work of Hanahan and Weinberg (2011) described the importance of tumour associated inflammation in progression of cancers. Recent studies in colorectal cancer have identified a role for the local inflammatory cell infiltrate in determining patient outcome (Roxburgh and McMillan, 2012). On review of the literature the presence of a local inflammatory infiltrate was consistently associated with an increase in survival and a decrease in disease recurrence (Roxburgh

and McMillan, 2012; Mei et al., 2014). Evidence therefore suggests that loss of this inflammatory cell infiltrate may play a role in tumour progression and metastasis (Park et al., 2014a,b).

While a local inflammatory response has been observed to associate with better cancer-specific survival, an elevated systemic inflammatory response has been associated with poorer cancer-specific survival and an increased risk of recurrence in a number of cancers including colorectal cancer (McMillan, 2013). Markers of the inflammatory response, such as acute phase proteins and some components of the white cell population, are measured routinely. These biomarkers can be used in conjunction with TNM-based staging as prognostic indicators (Watt et al., 2015a,b; Park et al., 2016). One potential mechanism linking tumour cell signalling with the local and systemic inflammatory responses is activation of the Nuclear Factor kappa B (NF-κB) pathway. The NF-κB family of transcription factors functions via two main pathways – the canonical and the non-canonical pathways (Perkins and Gilmore, 2006). In the canonical pathway the IKK complex, which is composed of the catalytic subunits IKKα and IKKβ and the regulatory scaffold protein NEMO, is maintained in the cytoplasm. On stimulation of the pathway by cytokines such as TNFα or IL-1, the complex is activated and phosphorylates IκB which leads to the release of p50/RelA which then translocates to the nucleus (Perkins and Gilmore, 2006). This translocation enables transcription of a variety of genes important in inflammatory responses and cell survival and proliferation.

In vitro studies have suggested that transforming growth factor-β-activated kinase 1 (TAK1), an upstream kinase in the pathway, may have a role to play in activation of the pathway. TAK1 has been demonstrated to be an activating kinase for the IKK complex of the canonical NF-κB pathway (Sakurai et al., 1998, 1999). Interestingly TAK1 has also been associated with a possible third NF-κB pathway which is independent of the controls recognised in the other two pathways (Margalef et al., 2012, 2015).

The aim of this study was to investigate the role of components of the canonical NF-κB pathway and the upstream kinase TAK1 in relation to the tumour microenvironment and survival in patients with colorectal cancer. Our hypothesis is that activation of this pathway and expression of these proteins have an important role in the determination of the tumour phenotype and subsequent outcome.

Materials and methods

Patients

In the current study, 242 patients were selected from a cohort of 274 who had undergone elective, potentially curative resection for stage I-III colorectal cancer between 1997 and 2007 in a single surgical unit – Glasgow Royal Infirmary. A tissue microarray (TMA) had previously been constructed. The selection criteria excluded patients who had emergency resection, neoadjuvant therapy or who had died within 30 days of surgery. For this cohort no information was available with regard to treatment post resection. Local ethical approval was obtained from the West of Scotland Research Ethics Committee and tissue for this analysis was obtained from the National Health Service Greater Glasgow and Clyde Tissue Biorepository.

Markers of systemic inflammation were determined in various ways. Serum C-reactive protein (CRP), albumin and differential white cell count were measured within 30 days prior to surgery and recorded prospectively. Patients with CRP≤10mg/L were given a score of 0, patients with CRP>10mg/L and albumin ≥35g/L a score of 1 and patients with CRP>10mg/ml and albumin <35g/L a score of 2. The modified Glasgow Prognostic Score (mGPS) (Park et al., 2015), the neutrophil:lymphocyte ratio (NLR) (Guthrie et al., 2013) and the neutrophil:platelet score (NPS) (Watt et al., 2015a,b) were used to determine the pre-operative inflammatory state. A NLR>5 was considered elevated; patients with a platelet count $<400x10^9$/L and a neutrophil count $<7.5x10^9$/L were given a score of 0, either a neutrophil count $>7.5x10^9$/L or a platelet count $>400x10^9$/L a score of 1 and those with both an elevated neutrophil and platelet count a score of 2.

Assessment of the tumour microenvironment

The tumour stroma percentage (TSP) and the generalised local inflammatory cell infiltrate have been previously characterised for this cohort of patients (Richards et al., 2012,2014) (Park et al., 2014b). If the TSP was ≤50% it was graded low and >50% graded high. Klintrup- Mäkinen (KM) grading was used as a measure of the local inflammatory cell infiltrate. Low grade was deemed if there was no increase or a mild/patchy increase in inflammatory cells at the invasive margin. High grade was assigned if there was a prominent band-like inflammatory reaction or florid cup-like inflammatory reaction at the invasive margin with destruction of cancer cell islands (Richards et al., 2014). In addition to KM, the immunoscore was used as a measure of local inflammatory cell infiltrate and was calculated from CD3 and CD8 counts.

Assessment of the expression of members of the canonical NF-κB pathway

Immunohistochemistry was performed to assess protein levels of TAK1, TAK1 phosphorylated at threonine 187(pTAK1), IKKβ, p65 (RelA) and phosphorylated p65, using a colorectal cancer TMA of 242 patients (Fig. 1). Prior to this, antibody specificity was confirmed using western blotting to identify a single band of the predicted molecular weight. TMA sections (2.5 μm) were dewaxed by immersion in histoclear then

rehydrated using a series of alcohols. Heat induced antigen retrieval was performed in a solution of either citrate buffer pH6 or Tris-EDTA pH 9 after which the sections were incubated in 3% hydrogen peroxide. Non-specific binding was blocked by incubation in 5% normal horse serum before being incubated with the optimum concentration of primary antibody at either room temperature for 2h or overnight at 4°C. Antibody dilutions were prepared in antibody diluent (Agilent, London, UK). The primary antibodies and concentrations used are as follows; TAK1 (Abcam ab111096) at 1:100, pTAK1 (Immunoway YP0424) at 1:200, IKKβ (Abcam ab32135) at 1:500, IKKα (Genway, GWB-662250) at 1:1000, p65 (Santa Cruz sc-8008) at 1:10 and p-p65 (Abcam ab28856) at 1:50. Staining was visualized using EnVision™ (Dako, Agilent) and 3,3'-diaminobenzidine (DAB, Vector Labs). Tissue was counterstained using Harris Haematoxylin before being dehydrated and mounted using DPX.

The stained TMA sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK at x 20 magnification. Visualization was carried out using Slidepath Digital Image Hub, version 4.0.1 (Slidepath, Leica Biosystems, Milton Keynes, UK) at x 400 magnification. The weighted histoscore method was employed to assess protein expression by observer 1 [LB, MK or JQ], with each protein also being scored by a second independent observer [JE] who scored 10% of all tumours to ensure scoring consistency across the study. Inter class correlation coefficients of greater than 0.8 were deemed acceptable and Bland Altman plots carried out to ensure no scoring bias. All observers were blinded to the clinical and pathological characteristics. Histoscores were calculated using the following formula: 0x % negative staining + 1x % of weakly positive staining + 2x % of moderately positive staining +3x % of strongly positive staining giving a range of 0 (minimum) to 300 (maximum). The results were considered discordant if the histoscores differed by more than 50. Cytoplasmic and nuclear expression, if observed, was calculated separately.

Statistical analysis

For the purposes of statistical analysis, the weighed histoscore for each protein was divided into tertiles to allow us to classify protein expression into low, moderate or high (low, moderate, high)(TAK, low<75, moderate 76-115, high>116)(pTAK1, low <63, moderate 64-106, high>107)(cytoplasmic IKK low<33, moderate 34-80, high>81) (p65 low<47, moderate48-84, high>85) (p-p65, low<150, moderate 151-215, high>216). The relationship between clinicopathological characteristics, markers of inflammation and the tumour microenvironment and members of the canonical NF-κB pathway was examined using the Chi-square test for linear trend. The relationship between cytoplasmic pTAK1, cytoplasmic IKKβ and cytoplasmic pTAK1/IKKβ stratified with *BRAF* mutation and cancer-specific survival was examined using Kaplan-Meier log-rank analysis. The relationship between expression of members of the canonical NF-κB pathway, characteristics of the tumour, microenvironment and systemic inflammatory responses and cancer-specific survival was examined using Cox proportional hazards regression; variables with P<0.05 on univariate analysis were entered into a multivariate model using a backwards conditional model to calculate hazard ratios (HR) and 95% confidence intervals (CI). A P value<0.05 was considered statistically significant. SPSS version 22.0 (IBM SPSS) was used for all analysis.

Results

The characteristics of the 242 patients within the cohort are shown in Table 1. Briefly, 59.5% were 65 or

Table 1. Patient characteristics.

	Patient Cohort n=242 (%)
Age	
<65	98(40.5)
>65	144(59.5)
Tumour Location	
Right-sided colon	88(36.4)
Left-sided colon	71(29.3)
Rectal	83(34.3)
TNM-stage	
I	17(7.0)
II	120(49.6)
III	105(43.4)
T-stage	
1	8(3.3)
2	18(7.4)
3	150(62.0)
4	66(27.3)
N-stage	
0	137(56.6)
1	80(33.1)
2	25(10.3)
Differentiation	
Moderate/well	213(88.0)
Poor	29(12.0)
Venous Invasion	
Absent	157(64.9)
Present	85(35.1)
MMR Status	
Competent	193(79.8)
Deficient	35(14.5)
Unknown	14(5.8)
BRAF	
Wildtype	165(68.2)
Mutated	52(21.5)
Unknown	25(10.3)
Survival	
Alive	97(40.1)
Cancer Death	79(32.6)
All causes of Death	145(59.9)

older and 52.5% were male. 7% had pathological confirmation of stage I disease, 49.6% had stage II disease, and 43.4% had stage III disease. 36.4% had right-sided colon cancer, 29.3% had left-sided colon cancer, and 34.3% had rectal cancer. Mismatch repair (MMR) deficiency was identified in 14.5% and 21.5% had *BRAF* mutations. The median follow up was 99 months (range 37.5-130 months) with 79 cancer deaths and 66 non-cancer deaths.

TAK1 was not significantly associated with cancer-specific survival (P=0.757), although patients that had moderate expression exhibited a reduced mean survival to 118 months (98.5-137.5 months).

Univariate analysis demonstrated that expression of cytoplasmic pTAK1 was significantly associated with cancer-specific survival (P=0.032) (Table 2). Moderate expression of cytoplasmic pTAK1 was associated with decreased cancer-specific survival when compared to low and high expression (P=0.032) (Fig. 2a). Mean cancer specific survival for patients expressing low levels of cytoplasmic pTAK1 was 140 months (122.6-158.1 months) versus 150 months (133.5-166.0 months) for patients with high expression.

Moderate expression of cytoplasmic pTAK1 was

Table 2. Univariate and multivariate analysis of members of the canonical NF-κB pathway and cancer-specific survival in patients with colorectal cancer (n=242).

Biomarker	Univariate HR (95% CI)	P Value	Multivariate HR (95% CI)	P Value
All Patients (n=242)				
Cytoplasmic TAK	1.04(0.78-1.39)	0.757		
Cytoplasmic pTAK	0.90(0.67-1.18)	0.032		
Nuclear pTAK	0.94(0.71-1.26)	0.500		
Cytoplasmic IKKβ	1.47(1.08-2.01)	0.046	1.75(1.05-2.91)	0.033
Nuclear IKKβ	1.03(0.76-1.40)	0.648		
Cytoplasmic IKKα	0.96(0.58-1.60)	0.877		
Nuclear IKKα	0.71(0.42-1.19)	0.193		
Cytoplasmic p65	1.10(0.82-1.47)	0.800		
Cytoplasmic pp65	0.94(0.68-1.29)	0.917		
Nuclear pp65	0.81(0.59-1.10)	0.169		

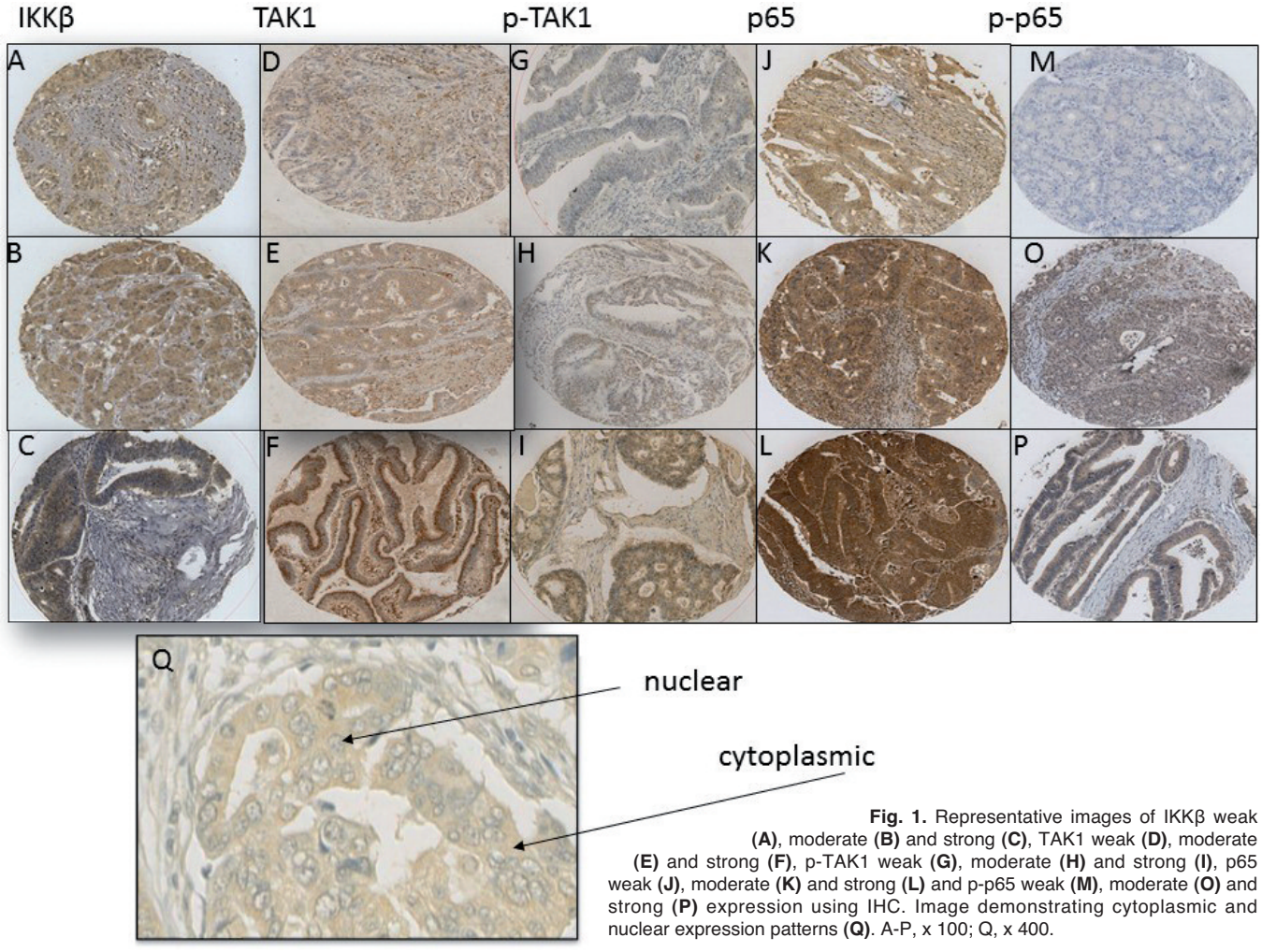

Fig. 1. Representative images of IKKβ weak **(A)**, moderate **(B)** and strong **(C)**, TAK1 weak **(D)**, moderate **(E)** and strong **(F)**, p-TAK1 weak **(G)**, moderate **(H)** and strong **(I)**, p65 weak **(J)**, moderate **(K)** and strong **(L)** and p-p65 weak **(M)**, moderate **(O)** and strong **(P)** expression using IHC. Image demonstrating cytoplasmic and nuclear expression patterns **(Q)**. A-P, x 100; Q, x 400.

associated with decreased cancer-specific survival when compared to low and high expression (P=0.032) (Fig. 2a). When 5 year survival data was considered, 48% of patients with moderate pTAK1 expression survived as compared to 59% and 65% for those with low and high expression.

Cytoplasmic pTAK1 and its relationship with clinicopathological characteristics and MMR status was examined in the 221 patients (Table 3). A direct linear association was observed between cytoplasmic pTAK and age (P=0.030) and an inverse linear association was observed between cytoplasmic pTAK and Glasgow Microenvironment Score (P=0.045) but not with any other characteristics (Table 3).

Association between cytoplasmic pTAK1 expression and cancer-specific survival was then investigated in different subgroups of colorectal cancer patients. Cytoplasmic pTAK1 expression was not significantly associated with cancer-specific survival in any tumour location subgroups (Right sided colon cancer P=0.237, Left sided colon cancer P=0.511, Rectum P=0.338) or in MMR competent or MMR deficient patients (Competent P=0.189, Deficient P=0.460). Interestingly, when stratified by *BRAF* status, pTAK1 expression was significantly associated with cancer specific survival in patients with wild type *BRAF* (P=0.014) (Fig. 3a). When the expression profiles were further analysed, pTAK1 expression showed a significant association between high and moderate expression (P=0.004), but not between high and low expression (P=0.147) or moderate and low expression (P=0.163). Mean cancer specific survival for all patients with wildtype *BRAF* was 137 months (123.5-149.5 months). Moderate expression of cytoplasmic pTAK1 reduced this to 113 months (90.9-136.9 months) as compared to 136 months (113.2-159.7) for low expression and 159 months (141.1-177.4) for patients with high expression. When only stage 2 disease was considered the significant association between CSS and pTAK1 was still observed (n=109, P=0.033). However, when stage 3 disease was considered there was no significant association between CSS and pTAK1 (n=97, P=0.2423).

Table 3. Relationship between cytoplasmic phosphorylated TAK1 and clinicopathological characteristics in patients with colorectal cancer (n=221).

	Low (%)	Mod (%)	High (%)	P-value
Host and tumour characteristics				
Age				
<65	36(16.3)	28(12.7)	22(10.0)	0.030
>65	39(17.6)	46(20.8)	50(22.6)	
Sex				
Female	34(15.4)	36(16.3)	30(13.6)	0.698
Male	41(18.6)	38(17.2)	42(19.0)	
Tumour Location				
Right colon	25(11.3)	28(12.7)	28(12.7)	0.235
Left colon	22(10.0)	17(7.7)	29(13.1)	
Rectum	28(12.7)	27(12.2)	18(8.1)	
TNM-stage				
I	7(3.2)	5(2.3)	3(1.4)	0.775
II	34(15.4)	37(16.7)	38(17.2)	
III	34(15.4)	38(17.2)	31(14.0)	
T-stage				
1	3(1.4)	1(0.5)	4(1.8)	0.868
2	7(3.2)	6(2.7)	2(0.9)	
3	44(19.9)	48(21.7)	47(21.3)	
4	21(9.5)	21(9.5)	19(8.6)	
N-stage				
0	41(18.6)	23(10.4)	11(5.0)	0.443
1	23(10.4)	25(11.3)	7(3.2)	
2	11(5.0)	25(11.3)	6(2.7)	
Differentiation				
Moderate/well	65(29.4)	62(28.1)	66(29.9)	0.336
Poor	10(4.5)	12(5.4)	6(2.7)	
Mismatch Repair Status*				
MRC	61(27.6)	61(27.6)	55(24.9)	0.828
MRD	11(5.0)	10(4.5)	11(5.0)	

Table 3. (Continuation).

Tumour Microenvironment				
Klintrup-Makinen grade (KM-grade, 220)				
Weak	45(20.5)	50(22.7)	52(23.6)	0.273
Strong	30(13.6)	23(10.5)	20(9.1)	
Tumour-Stroma percentage (TSP, 199)				
Low	55(27.7)	46(23.1)	47(23.6)	0.289
High	13(6.5)	20(10.1)	18(9.1)	
Glasgow Microenvironment Score (GMS, 203)				
0	30(14.8)	23(11.3)	20(9.9)	0.045
1	31(15.3)	28(13.8)	33(16.3)	
2	7(3.5)	17(8.4)	14(6.9)	
Immunoscore (198)				
0	23(11.6)	29(14.7)	26(13.1)	0.319
1	6(3.0)	13(6.6)	9(4.6)	
2	12(6.1)	16(8.1)	14(7.1)	
3	11(5.6)	4(2.0)	10(5.1)	
4	12(6.1)	5(2.5)	8(4.0)	
Systemic Inflammatory Response				
Serum CRP (221)				
Normal	41(18.6)	43(19.5)	45(21.4)	0.627
High	34(15.4)	31(14.0)	27(12.2)	
Serum Albumin (221)				
Normal	63(28.5)	62(28.1)	62(28.1)	0.910
Low	12(5.4)	12(5.4)	10(4.5)	
Modified Glasgow Prognostic Score (mGPS, 221)				
0	41(18.6)	25(11.3)	9(4.1)	0.371
1	25(11.3)	23(10.4)	8(3.6)	
2	9(4.1)	20(9.1)	(3.2)	
Neutrophil-Lymphocyte Ratio (NLR, 190)				
≤5	48(25.3)	47(24.7)	50(26.3)	0.571
>5	18(9.5)	12(6.3)	15(7.9)	
Neutrophil-Platelet Score (NPS,117)				
0	24(20.5)	17(14.5)	1(0.9)	0.442
1	17(14.5)	12(10.3)	3(2.6)	
2	1(0.9)	10(8.6)	2(1.7)	

*Of the available patients only 209 had information for both pTAK1 expression and MMR status.

In patients with *BRAF* mutations present within the tumour, no overall significant association with cancer-specific survival and expression of pTAK1 was observed (P=0.105) (Fig. 3b). However, when analysis was performed to compare those patients with high and low expression a significant difference in cancer-specific survival was observed. Those with low pTAK1 expression had a mean cancer-specific survival of 163 months (136.7-190), compared to 85 months (59.5-110.5) for those with high expression. However, as only 49 patients were available for analysis, this should be investigated in a larger cohort.

On univariate analysis expression of IKKβ statistically significantly associated with cancer-specific survival (P=0.046) and was independently associated with cancer-specific survival on multivariate analysis (HR 1.75,95%CI 1.05-2.91, P=0.033) (Table 2).

Cytoplasmic IKKβ was associated with cancer-specific survival, high expression was associated with poor outcome when compared to low and moderate expression (P=0.046) (Fig. 2b). Mean cancer-specific survival for patients expressing low levels of IKKβ was 158 months (142.3-173.2 months) and for those expressing moderate levels was 142 months (123.8-159.9 months); this was reduced to 125 months (105.5-145.0 months) for patients with high cytoplasmic IKKβ expression. When 5 year survival data was considered, 51% of patients expressing high levels of cytoplasmic IKKβ, were alive at 5 years, compared to 59% of patients with moderate expression and 71% of patients whose tumours expressed low levels of IKKβ. When this was assessed across tumour stage 2 and stage 3 the result was negated in stage 2 disease (P=0.446, 99 patients) but enhanced in stage 3 disease (P=0.161, 97 patients) although not reaching significance due to being underpowered.

Cytoplasmic IKKβ and its relationship with clinicopathological characteristics and MMR status was examined in 211 of the 242 patients (Table 4). MMR status was not available for 31 of the patients due to insufficient tissue. A likelihood relationship was observed between cytoplasmic IKKβ and Klintrup-Makinen grade (P=0.015) and a direct linear relationship was observed between IKKβ Glasgow Micro-environment Score (P=0.029) and an indirect relationship was observed between IKKβ and Neutrophil-Lymphocyte ratio (P=0.03). There was no significant relationship observed between any of the other characteristics.

Association between cytoplasmic IKKβ expression and cancer-specific survival was then investigated in different subgroups of colorectal cancer patients. Cytoplasmic IKKβ expression was not significantly associated with cancer-specific survival at any tumour location (Right sided colon cancer P=0.075, Left sided colon cancer P=0.687, Rectal cancer P=0.463), in either MMR groups (Competent P=0.134, Deficient P=0.112) or in *BRAF* wild type or mutated tumours (Wild type

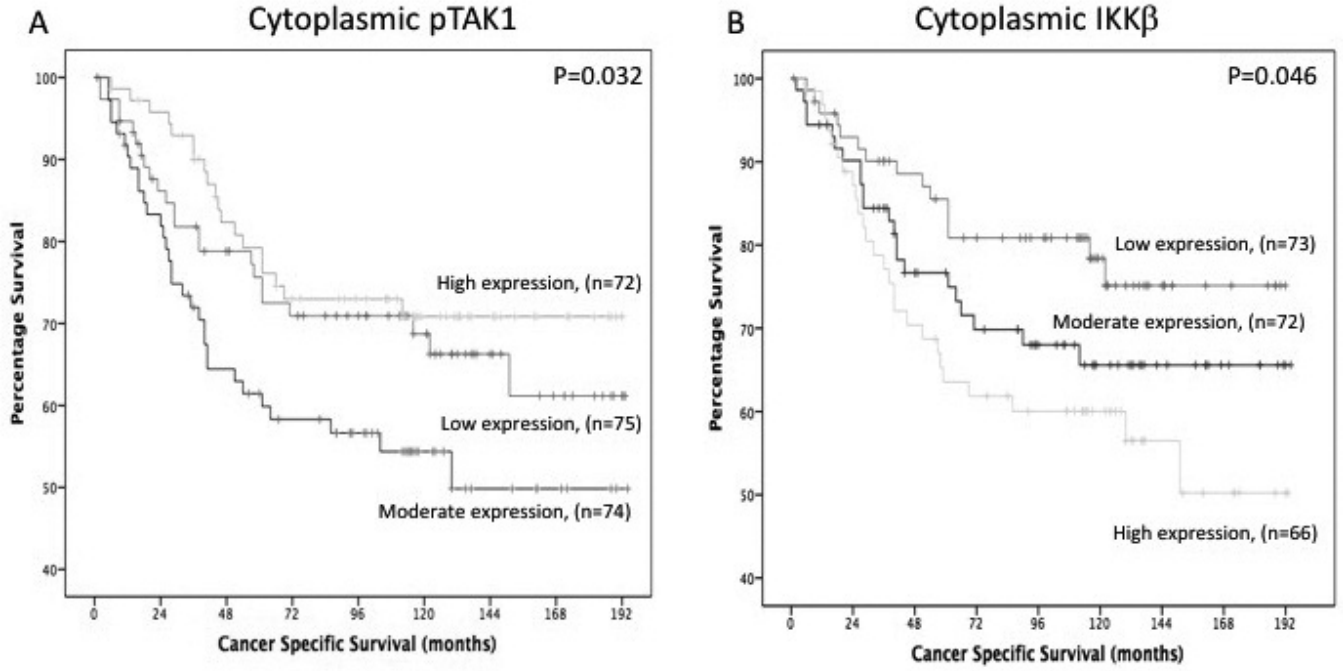

Fig. 2. The relationship between cytoplasmic pTAK1 and cytoplasmic IKKβ expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer: cytoplasmic pTAK1 expression (P=0.032) **(A)** and cytoplasmic IKKβ expression (P=0.046) **(B)**.

P=0.067, Mutated P=0.264).

The relationship between clinicopathological factors, the tumour microenvironment, systemic inflammatory responses, pTAK1, IKKβ and cancer-specific survival was then examined in multivariate analysis (Table 5). Of the factors investigated, those that independently associated with cancer-specific survival were the Glasgow microenvironment score (HR 2.67, 95%CI 1.41-5.03, P=0.002) and cytoplasmic IKKβ (HR 1.75,95%CI 1.05-2.91, P=0.033) (Table 2).

Discussion

In a cohort of 242 patients, cytoplasmic pTAK1 was significantly associated with cancer specific survival and this was enhanced when stratified by *BRAF* status but did not reach significance possibly due to the small number of patients and/or events in the mutated *BRAF* subgroup. In addition, high expression of cytoplasmic IKKβ was significantly associated with decreased survival and with markers of the tumour microenvironment. The present study demonstrates that members of the canonical NF-κB pathway are expressed in tumours of patients with colorectal cancer. Cytoplasmic pTAK1 was associated with cancer-specific survival, which was enhanced by stratification with *BRAF*, but not with microsatellite instability (MSI) status (indicated by MMR protein status). Cytoplasmic IKKβ was associated with decreased cancer-specific survival and with the tumour microenvironment but not with *BRAF* or MSI status. On multivariate analysis only cytoplasmic IKKβ was independently associated with survival.

Surprisingly patients with moderate pTAK1 expression had the poorest survival, with patients who have high or low expression of pTAK having a better prognosis. We hypothesis that this might be due to the fact the TAK1 is normally present and activate in epithelial cells and it is only when the normal expression levels differ i.e. increase or decrease that the normal homeostasis and signalling of the cell is disrupted. An increase or decrease may represent dysregulation of the

Table 4. Relationship between cytoplasmic IKKβ and clinicopathological characteristics in patients with colorectal cancer (n=211).

	Low (%)	Mod (%)	High (%)	P-value
Host and tumour characteristics				
Age				
<65	32(15.2)	27(12.8)	26(12.3)	0.728
>65	41(19.4)	45(21.3)	40(19.0)	
Sex				
Female	37(17.5)	32(15.2)	32(15.2)	0.748
Male	36(17.1)	40(19.0)	34(16.1)	
Tumour Location				
Right colon	30(14.2)	20(9.5)	23(10.9)	0.376
Left colon	20(9.5)	21(10.0)	25(11.9)	
Rectum	23(10.9)	20(9.5)	24(11.4)	
TNM-stage				
I	6(2.8)	4(1.9)	5(2.4)	0.709
II	33(15.6)	38(18.0)	28(13.3)	
III	34(16.1)	30(14.2)	33(15.6)	
T-stage				
1	2(1.0)	4(1.9)	2(1.0)	0.728
2	6(2.8)	4(1.9)	6(2.8)	
3	45(21.3)	48(22.8)	36(17.1)	
4	20(9.5)	16(7.6)	22(10.4)	
N-stage				
0	39(18.5)	28(13.3)	6(2.8)	0.620
1	28(13.3)	21(10.0)	9(4.3)	
2	6(2.8)	26(12.3)	7(3.3)	
Differentiation				
Moderate/well	64(30.3)	66(31.3)	57(27.0)	0.832
Poor	9(4.3)	6(2.8)	9(4.3)	
Mismatch Repair Status*				
MRC	60(28.4)	65(30.8)	56(23.5)	0.461
MRD	12(5.7)	7(3.3)	9(4.3)	
Tumour Microenvironment				
Klintrup-Makinen grade (KM-grade, 210)				
Weak	39(18.6)	52(24.8)	48(22.9)	0.015
Strong	34(16.2)	19(9.1)	18(8.6)	
Tumour-Stroma percentage (TSP, 193)				
Low	51(26.4)	49(25.4)	44(22.8)	0.506
High	15(7.8)	17(8.8)	17(8.8)	
Glasgow Microenvironment Score (GMS, 197)				
0	34(17.3)	19(9.6)	18(9.1)	0.029
1	26(13.2)	32(16.2)	32(16.2)	
2	8(4.1)	17(8.6)	11(5.6)	
Immunoscore (193)				
0	16(8.3)	30(15.5)	28(14.5)	0.069
1	12(6.2)	10(5.2)	8(4.2)	
2	18(9.3)	11(5.7)	10(5.2)	
3	9(4.7)	12(6.2)	3(1.6)	
4	10(5.2)	6(3.1)	10(5.2)	
Systemic Inflammatory Response				
Serum CRP (211)				
Normal	42(19.9)	43(20.4)	41(19.4)	0.583
High	31(14.7)	29(13.7)	25(11.9)	
Serum Albumin (211)				
Normal	65(30.8)	57(27.0)	60(28.4)	0.798
Low	8(3.8)	15(7.1)	6(2.8)	
Modified Glasgow Prognostic Score (mGPS, 211)				
0	42(19.9)	43(20.4)	41(19.4)	0.488
1	24(11.4)	19(9.0)	21(10.0)	
2	7(3.3)	10(4.7)	4(1.9)	
Neutrophil-Lymphocyte Ratio (NLR, 186)				
≤5	46(24.7)	49(26.3)	52(28.0)	0.030
>5	20(10.8)	10(5.4)	9(4.8)	
Neutrophil-Platelet Score (NPS, 117)				
0	26(22.2)	16(13.7)	2(1.7)	0.169
1	16(13.7)	10(8.6)	1(0.9)	
2	2(1.7)	8(6.8)	2(1.7)	

Table 4. (Continuation).

*Of the available patients information for both cytoplasmic IKKβ and MMR status was only available in 209 cases.

pathway from the normal state leading to a worse prognosis. In patients with wild type *BRAF*, moderate expression of cytoplasmic pTAK1 was associated with poor outcome. More detailed analysis revealed a significant association between moderate and high expression but not between low and moderate or low and high. In contrast, in mutated *BRAF*, high expression of cytoplasmic pTAK1 was observed to be related to poor outcome, moderate expression with moderate outcome and low expression with a better outcome. However, although a trend was observed, this did not reach significance possibly due to low patient numbers and events available for analysis in this subgroup. Further analysis of the expression profiles revealed a significant association between low and high expression, but not between moderate expression and low or high

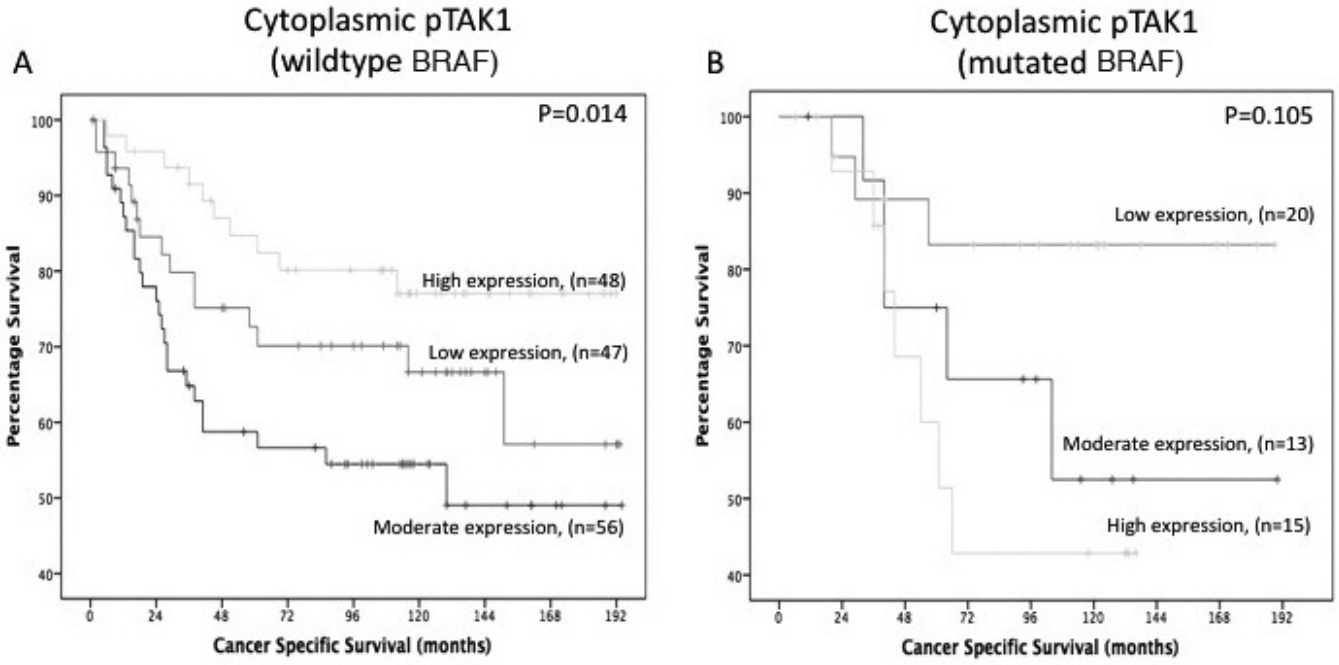

Fig. 3. The relationship between cytoplasmic pTAK1 stratified with *BRAF* expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer: cytoplasmic pTAK1 stratified with wildtype *BRAF* expression (P=0.014) **(A)** and cytoplasmic pTAK1 stratified with mutated *BRAF* expression (P=0.105) **(B)**.

Table 5. Univariate and multivariate analysis of members of NF-κB pathway, characteristics of the tumour, microenvironment, systemic inflammatory responses and cancer specific survival in patients with colorectal cancer (n=242).

Biomarker	Univariate HR (95% CI)	P-value	Multivariate HR (95% CI)	P-value
All Patients (n=242)				
Sex (female/male)	1.18(0.75-1.84)	0.474		
Tumour location (right colon/left colon/rectum)	1.06(0.81-1.38)	0.843		
TNM Stage (I/II/III)	2.26(1.50-3.41)	<0.001		
N-Stage (0/1/2)	1.82(1.35-2.45)	<0.001		
Differentiation (moderate or well/poor)	1.76(0.95-3.25)	0.068		
Mismatch Repair (MRC/MRD)	0.96(0.51-1.83)	0.909		
Klintrup-Makinen Grade (strong/weak)	2.36(1.34-4.14)	0.002		
GMS (0/1/2)	1.95(1.40-2.71)	<0.001	2.67(1.41-5.03)	0.002
Immunoscore (0/1/2/3/4)	0.60(0.48-0.73)	<0.001		
Serum CRP (Normal/High)	1.82(1.17-2.84)	0.007		
Serum Albumin (Normal/Low)	1.94(1.15-3.28)	0.012	0	0.980
mGPS (0/1/2)	1.68(1.25-2.26)	0.001		
Neutrophil count (≤7.5x10^{9}L/>7.5x10^{9}L)	1.84(1.00-3.39)	0.047		
Platelet count (≤400x10^{9}L/>400x10^{9}L)	1.909(0.85-4.20)	0.110		
NPS (0/1/2)	1.98(1.19-3.28)	0.022		

expression. This suggests that pTAK1 expression warrants further investigation in a larger cohort.

TAK1 is a serine/threonine protein kinase of the MAPK kinase family. It is an upstream regulating kinase in the NF-κB pathway and has been recognised as having a role in the activation of the canonical NF-κB pathway (Sakurai et al., 1998, 1999). It has been reported to activate the IκB kinase (IKK) complex which is composed of IKKα, IKKβ and NEMO (Sakurai et al., 1999). It forms a complex with its activator proteins TAK1-binding protein 1 and 2 (TAB1 and TAB2) (Takaesu et al., 2012) and it has been demonstrated that the kinase activity of TAK1 is regulated by modifications to TAK1 and the TABs. Phosphorylation can occur on several serine and threonine residues in the activation loop of TAK1 (Singhirunnusorn et al., 2005; Yu et al., 2008). Phosphorylation at Thr-187 has been associated with activation of TAK1 on stimulation by TNFα (Singhirunnusorn et al., 2005), and this is the main autophosphorylation site.

A study investigating TAK1 in gastric cancer reported that elevated expression of TAK1 was associated with a decreased patient survival and that in cell lines TAK1 selective inhibitors resulted in increased apoptosis (Yang et al., 2017). However, in contrast to the present study only the presence or absence of TAK1 expression was investigated. Multiple studies have investigated TAK1 expression in relation to KRAS status (Singh et al., 2012; McNew et al., 2016); however there is little information on TAK1 expression in relation to *BRAF* status. A limitation of the study is that KRAS status was not available and it would be interesting to investigate further in patients whose KRAS status was known.

In the present study activated TAK1, was assessed by TAK1 phosphorylated at Thr-187 which is recognised as being associated with activation of TAK1 (Singhirunnusorn et al., 2005). Moderate expression of phosphorylated TAK1 at Thr-187, as a measure of activated TAK1, was associated with poor cancer-specific survival. Stratification by *BRAF* status demonstrated that those patients with mutant *BRAF* had decreased cancer-specific survival if cytoplasmic pTAK1 expression was high. KRAS mutations are common in colorectal cancers, and studies employing colorectal cancer cell lines and mouse models have reported that TAK1 is an important regulator of tumour cell viability (Singh et al., 2012). Work carried out in pancreatic cell lines suggests that silencing or inhibition of TAK1 may have a therapeutic effect in relation to chemoresistance (Melisi et al., 2011). However, numbers in our cohort were low and therefore TAK1 expression and activation warrants further investigation in a larger patient cohort.

A role for IKKβ in colitis-associated cancer has previously been reported (Greten et al., 2004). Using mouse models, deletion of IKKβ in intestinal epithelial cells leads to a decrease in tumour size but not a reduction in inflammation (Greten et al., 2004). A role for IKKβ in the initiation of colitis-associated tumours has also been reported (Koliaraki et al., 2015). Vlantis (Vlantis et al., 2011) demonstrated that constitutive activation of IKKβ in mouse intestinal epithelial cells induces tumours. This supports our observation that elevated cytoplasmic IKKβ expression is associated with a reduced cancer-specific survival.

In conclusion the current study investigated the role of components of the canonical NF-κB pathway and the upstream kinase TAK1 in relation to the tumour microenvironment and survival in patients with colorectal cancer. The data from this study suggest that the abundance of pTAK1 and IKKβ in the cytoplasm of CRC cells may have some prognostic value, however this requires validation in a larger independent cohort.

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References

Cancerresearchuk (2017). Bowel cancer statistics.

Greten F.R., Eckmann L., Greten T.F., Park J.M., Li Z.W., Egan L.J., Kagnoff M.F. and Karin M. (2004). IKK beta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118, 285-296.

Guthrie G.J.K., Charles K.A., Roxburgh C.S.D., Horgan P.G., McMillan D.C. and Clarke S.J. (2013). The systemic inflammation-based neutrophil-lymphocyte ratio: Experience in patients with cancer. Crit. Rev. Oncol. Hematol. 88, 218-230.

Hanahan D. and Weinberg R.A. (2011). Hallmarks of cancer: The next generation. Cell 144, 646-674.

Koliaraki V., Pasparakis M. and Kollias G. (2015). IKKbeta in intestinal mesenchymal cells promotes initiation of colitis-associated cancer. J. Exp. Med. 212, 2235-2251.

Margalef P., Fernandez-Majada V., Villanueva A., Garcia-Carbonell R., Iglesias M., Lopez L., Martinez-Iniesta M., Villa-Freixa J., Mulero M.C., Andreu M., Torres F., Mayo M.W., Bigas A. and Espinosa L. (2012). A truncated form of IKKalpha is responsible for specific nuclear IKK activity in colorectal cancer. Cell Rep. 2, 840-854.

Margalef P., Colomer C., Villanueva A., Montagut C., Iglesias M., Bellosillo B., Salazar R., Martinez-Iniesta M., Bigas A. and Espinosa L. (2015). BRAF-induced tumorigenesis is IKK alpha-dependent but NF-kappa B-independent. Sci. Signaling 8.

McMillan D.C. (2013). Cancer and systemic inflammation: Stage the tumour and stage the host. Br. J. Cancer 109, 529-529.

McNew K.L., Whipple W.J., Mehta A.K., Grant T.J., Ray L., Kenny C. and Singh A. (2016). MEK and TAK1 regulate apoptosis in colon cancer cells with KRAS-dependent activation of proinflammatory signaling. Mol. Cancer Res. 14, 1204-1216.

Mei Z., Liu Y., Liu C., Cui A., Liang Z., Wang G., Peng H., Cui L. and Li C. (2014). Tumour-infiltrating inflammation and prognosis in colorectal cancer: Systematic review and meta-analysis. Br. J. Cancer 110, 1595-1605.

Melisi D., Xia Q.H., Paradiso G., Ling J.H., Moccia T., Carbone C., Budillon A., Abbruzzese J.L. and Chiao P.J. (2011). Modulation of pancreatic cancer chemoresistance by inhibition of TAK1. J. Nat.

Cancer Inst. 103, 1190-1204.

Park J.H., Roxburgh C.S.D. and McMillan D.C. (2014a). Comment on 'tumour-infiltrating inflammation and prognosis in colorectal cancer: Systematic review and meta-analysis'. Br. J. Cancer 111, 2372-2372.

Park J.H., Richards C.H., McMillan D.C., Horgan P.G. and Roxburgh C.S.D. (2014b). The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. Ann. Oncol. 25, 644-651.

Park J.H., McMillan D.C., Horgan P.G. and Roxburgh C.S.D. (2015). The clinical utility of a tumour microenvironment-based histopathological score in patients with primary operable colorectal cancer. J. Clin. Oncol. 33.

Park J.H., Watt D.G., Roxburgh C.S.D., Horgan P.G. and McMillan D.C. (2016). Colorectal cancer, systemic inflammation, and outcome staging the tumor and staging the host. Ann. Surg. 263, 326-336.

Perkins N.D. and Gilmore T.D. (2006). Good cop, bad cop: The different faces of NF-kappa B. Cell Death Differ. 13, 759-772.

Richards C.H., Roxburgh C.S.D., Anderson J.H., McKee R.F., Foulis A.K., Horgan P.G. and McMillan D.C. (2012). Prognostic value of tumour necrosis and host inflammatory responses in colorectal cancer. Br. J. Surg. 99, 287-294.

Richards C.H., Roxburgh C.S.D., Powell A.G., Foulis A.K., Horgan P.G. and McMillan D.C. (2014). The clinical utility of the local inflammatory response in colorectal cancer. Eur. J. Cancer 50, 309-319.

Roxburgh C.S.D. and McMillan D.C. (2012). The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. Cancer Treatment Rev. 38, 451-466.

Sakurai H., Shigemori N., Hasegawa K. and Sugita T. (1998). TGF-beta-activated kinase 1 stimulates NF-kappa B activation by an NF-kappa B-inducing kinase-independsnt mechanism. Biochem. Biophys. Res. Commun. 243, 545-549.

Sakurai H., Miyoshi H., Toriumi W. and Sugita T. (1999). Functional interactions of transforming growth factor beta-activated kinase 1 with I kappa B kinases to stimulate NF-kappa B activation. J. Biol. Chem. 274, 10641-10648.

Singh A., Sweeney M.F., Yu M., Burger A., Greninger P., Benes C., Haber D.A. and Settleman J. (2012). TAK1 inhibition promotes apoptosis in KRAS dependent colon cancers. Cell 148, 639-650.

Singhirunnusorn P., Suzuki S., Kawasaki N., Saiki I. and Sakurai H. (2005). Critical roles of threonine 187 phosphorylation in cellular stress-induced rapid and transient activation of transforming growth factor-beta-activated kinase 1 (TAK1) in a signaling complex containing TAK1-binding protein TAB1 and TAB2. J. Biol. Chem. 280, 7359-7368.

Takaesu G., Kobayashi T. and Yoshimura A. (2012). TGF beta-activated kinase 1 (TAK1)-binding proteins (TAB) 2 and 3 negatively regulate autophagy. J. Biochem. 151, 157-166.

Vlantis K., Wullaert A., Sasaki Y., Schmidt-Supprian M., Rajewsky K., Roskams T. and Pasparakis M. (2011). Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice. J. Clin. Invest. 121, 2781-2793.

Watt D.G., Proctor M.J., Park J.H., Horgan P.G. and McMillan D.C. (2015a). The neutrophil-platelet score (NPS) predicts survival in primary operable colorectal cancer and a variety of common cancers. PLoS One 10, e0142159.

Watt D.G., Martin J.C., Park J.H., Horgan P.G. and McMillan D.C. (2015b). Neutrophil count is the most important prognostic component of the differential white cell count in patients undergoing elective surgery for colorectal cancer. Am. J. Surg. 210, 24-30.

Yang Y., Qiu Y.D., Tang M.B., Wu Z.S., Hu W.D. and Chen C.B. (2017). Expression and function of transforming growth factor-beta-activated protein kinase 1 in gastric cancer. Mol. Med. Rep. 16, 3103-3110.

Yu Y., Ge N.L., Xie M., Sun W.J., Burlingame S., Pass A.K., Nuchtern J.G., Zhang D.K., Fu S.B., Schneider M.D., Fan J. and Yang J.H. (2008). Phosphorylation of thr-178 and thr-184 in the TAK1 T-loop is required for interleukin (IL)-1-mediated optimal NF kappa B and AP-1 activation as well as IL-6 gene expression. J. Biol. Chem. 283, 24497-24505.

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